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Review

Seroprevalence of *Coxiella burnetii* in Human and Animal Populations in Türkiye: Meta-Analysis

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ABSTRACT

This study aims to reveal *Coxiella burnetii* by examining the studies reporting Q fever seroprevalence in humans and animals in the last 25 years in Türkiye. In this study, based on PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses), various databases were searched between January 1997 and October. 2022. A literature review was carried out using data analyses performed using the IBM SPSS Version 25.0 statistical package program and Comprehensive Meta-Analysis (CMA) program. Overall prevalence of *C. burnetii* in humans was 22.78% (95% CI: 16.43%-29.12%), overall prevalence in animals was 13.49% (95% CI: 10.04-16.93%) was detected. The mean prevalence of *C. burnetii* in sheep was 19.1%±10.88, 10.46±6.39% in cattle, 15.21±10.01% in studies including cattle and sheep together, 11.17±10.74 in cattle, sheep and goats, and 12.4%±1.15% in sheep and goats. As a result of this study, it was determined that the prevalence of Q fever in humans in Türkiye is high in those dealing with animals, women who had a miscarriage, and infertile individuals. Although it is known that this disease is seen in Türkiye, there are not enough case reports in the literature. Detailed studies on Q fever in humans and animals need to be conducted. Further studies are needed to evaluate Q fever risk factors and prevalence data together within the scope of One Health approach.

Keywords: Cattle, *Coxiella burnetii*, Infertility, Miscarry, Q fever

Introduction

Q fever is a zoonotic disease caused by the intracellular Gram-negative bacterium *C. burnetii* [1]. *C. burnetii* has been accepted as a biological weapon because of its extremely high contagiousness, resistance to harsh environmental conditions and causing severe diseases in humans, and is listed as a Category B biological warfare agent by the Center for Disease Control and Prevention [2]. Although Q fever was first discovered in 1937, this microorganism has come to the fore again in recent years due to the potential of the etiologic agent *C. burnetii* to be used as a bioterrorism weapon and the changes reported in epidemiology in Europe [3].

C. burnetii infects humans and a wide variety of wild and domestic animals. The most common sources of transmission of the agent to humans are farm animals such as sheep, goats and cattle [4]. *C. burnetii* is spread to the environment through infected animals' urine, feces, milk, and birth products [5]. Inhalation of infectious aerosols or contaminated dust-containing bacteria is the leading way of contracting the disease in humans, and it has been stated that a single inhaled microorganism can cause clinical disease [6]. However, consuming raw or unpasteurized milk and dairy products, contact through the skin and mucous membranes, tick bites, blood transfusion, sexual intercourse and transmission through the placenta are the main sources of *C. burnetii* infection [7].

C. burnetii has two different antigenic phases, phase I and phase II, depending on the changes that occur in the organism during *in vitro* culture. In the early stages of infection, antibodies against phase II antigens are formed. However, if the infection continues for a longer period of time, antibodies against phase I antigens predominate in the organism. Although these antibodies are not used in animals, they are used to distinguish acute from chronic infections in humans [1]. The diagnosis of Q fever is made by detecting antibodies to *C. burnetii* using complement fixation, indirect fluorescent antibody (IFA), immunofluorescence, Enzyme Linked Immunosorbent

Assay (ELISA), or agglutination tests. The IFA technique has been recommended as the gold standard method [4].

Q fever is mostly asymptomatic except for some conditions that cause miscarriage, stillbirth, endometritis or infertility [7]. The disease is divided into acute and chronic Q fever in humans. Acute Q fever often causes non-specific liver damage; chronic Q fever causes endocarditis. It has been reported that the mortality rate in patients with acute *C. burnetii* infection generally varies between 1% and 2.4% [8]. This study aims to reveal the seroprevalence of *C. burnetii* by examining the studies reporting Q fever seroprevalence in humans and animals in the last 25 years in Türkiye.

Material and Methods

Literature Search and Research Strategies

This systematic review and meta-analysis were conducted based on the PRISMA guidelines [9]. From January 1997 to October. 2022, a literature review was conducted for studies examining the prevalence of Q fever infection in humans and animals in Türkiye. The study evaluated original scientific studies published in English and Turkish languages in national and international databases (PubMed, Embase, Scopus, Google Scholar, Web of Science and Turkish Medline) between January 1997 and October 2022.

For all English and Turkish population-based studies reporting the prevalence of Q fever in Türkiye, in all electronic databases, "Q fever prevalence in Türkiye", "*Coxiella burnetii* prevalence in Türkiye", "*C. burnetii* prevalence in Türkiye", "*Coxiella burnetii* and Türkiye" and "*C. burnetii* and Türkiye" Various combinations of "key terms" have been used. Three authors did scanning and collection of related articles. Publications for inclusion in the study were evaluated independently, and scientific consensus by the authors agreed upon inconsistencies.

Inclusion and Exclusion Criteria

The inclusion criteria for the study consisted of all original articles with a sample size of more than 30, which reported the prevalence of *C. burnetii* and Q fever in English and Turkish.

Studies with less than 30 samples and not reporting the total number of patients or subjects, studies that do not state positive and/or negative results, studies that do not report the method used for the research, reviews that do not contain original data, theses, case reports/series, letters to the editor, articles whose full text could not be reached, inconsistent data, and congress papers were not included in the study within the framework of exclusion criteria.

The PICOS model was applied for the eligibility criteria [10]. P (Population): "The sample group consists of humans and animals in which Q fever seroprevalence was investigated in Türkiye", I (Intervention): "Prevalence investigation of *C. burnetii* by serological methods"; C (Comparison): "Research articles that study Q fever seroprevalence in the general population or in humans and animals with certain risk factors"; O (Outputs): "A research article should specify the prevalence rate of *C. burnetii* in animals and humans, the characteristics of the sample group, and the serological method studied", S (Study): "This study was planned as a meta-analysis. Research articles published in Turkish or English were included".

Data Collection and Quality Assessment

During the pre-reading process, the titles and abstracts were evaluated, and the full texts of the studies that the authors found appropriate by consensus were reached. Study data; such as the type of study, sample size, clinical characteristics of the sample group, place and time of the study, type of antibody, and seroprevalence of the disease were collected in spreadsheets. Antibodies tested in animal (sheep, goat and cattle) and human studies were grouped as phase I/phase II and IgG/IgM.

Statistical Analysis

The data obtained from the literature review were recorded in Microsoft Excel tables. Mean, standard deviation, frequency etc. The values were analyzed with the help of the IBM SPSS Version 25.0 statistical package program. Meta-analyses were performed using the Comprehensive Meta-Analysis (CMA) program. Effect sizes and heterogeneity (I^2 and Q) of selected studies were

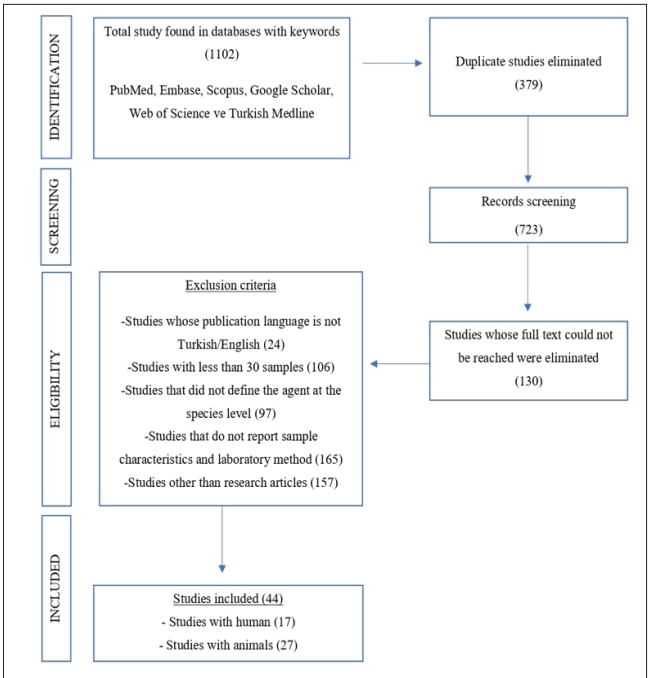

Fig 1. Flowchart of meta-analysis

calculated in CMA, and forest and funnel plots were used to assess publication bias. The heterogeneity in meta-analyses refers to the variation in the results of the selected studies.

Interpretation of I^2 can be misleading as it depends on multiple factors. The values reported in the guidelines for the interpretation of the I^2 value are as follows:

- 0-40%: insignificant,
- 30-60%: moderate heterogeneity,
- 50-90%: may represent substantial heterogeneity,
- 75-100%: considerable heterogeneity.

Results

As a result of the literature review, 1102 studies were found. After 379 repetitive studies were excluded, 723 were reserved for full-text review. The full text of 130 articles has been reached. Among these, studies whose publication language is not Turkish or English (n=24), containing less than 30 samples (n=106), not defining

Table 1. *Characteristics of studies with sample animals included in the meta-analysis [11-34]*

Study	City	Type of Animals	Characteristics of Samples	Phase	Antibody	Number of Sample (n)	Prevalence (%)	Method
Ozgur et al.[11]	Istanbul+Thrace	Cattle	Infertilite			144	9.72	ELISA
Cetinkaya et al.[12]	Multicenter	Cattle + sheep	Normal	Phase II	IgG	827	8.1	IFA
Kalender-1 [13]	Multicenter	Sheep	Normal	-	IgG	227	11.01	IFA
Kalender-2 [13]	Multicenter	Sheep	Abortus	-	IgG	184	38.59	IFA
Seyitoglu et al.-1 [14]	Erzurum	Cattle	Normal	-	-	177	5.65	ELISA
Seyitoglu et al.-2 [14]	Erzurum	Cattle	Abortus	-	-	53	22.64	ELISA
Kirkan et al.[15]	Aydin	Cattle	Normal	-	-	138	4.35	PCR
Ceylan et al.[16]	Multicenter	Cattle + sheep	Normal	Phase II	IgG	184	10.87	ELISA
Karaca et al.[17]	Van	Sheep	Normal	-	-	465	21.08	ELISA
Kilic et al.[18]	Multicenter	Alley Cat	Normal	Phase II	IgG	143	4.9	ELISA
Kennerman et al.[19]	Multicenter	Sheep	Normal	Phase I, Phase II	IgG	743	20.32	ELISA
Arserim et al.[20]	Diyarbakir	Cattle + sheep + cow	Normal	Phase II	IgG	1896	25.63	ELISA
Gazyagci et al.[21]	Konya	Cattle	Normal	Phase II	IgG	322	12.42	IFA
Kucukkalem et al.[22]	Erzurum	Cattle	Abortus	-	-	100	6	PCR
Gunaydin et al.[23]	Multicenter	Cattle + sheep + goat	Normal	-	-	152	7.24	PCR
Parin et al.[24]	Aydin	Cattle + sheep + goat	Normal	Phase I	IgG	600	23.33	ELISA, PCR, IFA
Gulmez et al.[25]	Kars	Cattle + sheep	Normal	-	-	600	26.67	ELISA+PCR
Kilic et al.-1[26]	Multicenter	Sheep	Abortus	-	IgG	350	16	ELISA
Kilic et al.-2[26]	Multicenter	Sheep	Normal	-	IgG	171	7.6	ELISA
Ozkaraca et al.[27]	Multicenter	Cattle	Abortus	-	-	70	1.43	PCR, IHC
Gunaydin and Pekkaya et al.[28]	Afyon	Cattle	Normal	Phase I, Phase II	IgG	92	8.7	ELISA
Karagul et al.[29]	Multicenter	Sheep + goat	Normal	-	-	832	13.22	ELISA
Gulhan et al.[30]	Samsun	Cattle	Normal	Phase I, Phase II	IgG	184	15.76	ELISA
Kilicoglu et al.[31]	Multicenter	Cattle + sheep + goat	Abortus	-	-	270	2.96	PCR
Serifoglu Bagatir et al.[32]	Multicenter	Sheep + goat	Normal	Phase I, Phase II	IgG	1045	11.58	ELISA
Malal et al.[33]	Multicenter	Cattle	Normal	-	-	1114	18.4	ELISA
Ates Kalkan et al.[34]	Multicenter	Cattle	Normal	-	-	200	10	ELISA

-: Unspecified, ELISA: Enzyme-Linked ImmunoSorbent Assay, IFA: Indirect Fluorescent Antibody test, PCR: Polymerase Chain Reaction, IHC: Immunohistochemistry

Table 2. *Characteristics of studies with human samples included in the meta-analysis* [11,14,35-47]

Study	City	Characteristics of Samples	Phases	Antibody	Number of Samples (n)	Prevalence (%)	Male (n)	Female (n)	Age Range (years)	Method
Ozgur et al.[11]	Istanbul+Thrace	Individuals with infertility	-	-	50	22	-	-	-	ELISA
Berberoglu et al.[35]	Multicenter	Normal people	Phase II	IgG	339	7.08	172	167	1-65	ELISA
Sertpolat et al.[36]	Izmir	Farmers, butcher, employee andtradesmen	Phase II	IgG	303	39.27	256	47	18-79	IFA
Eyigor et al.[37]	Aydin	Veterinarians, celebs, butcher	Phase I, Phase II	IgG	92	42.39	85	7	17-63	ELISA, IFA
Seyitoglu et al.[14]	Erzurum	Farmers	-	-	92	19.57	-	-	-	ELISA
Buke et al.[38]	Izmir	Besiciler	Phase II	IgG	96	25	-	-	15-70	IFA
Karabay et al.[39]	Bolu	People living in rural areas	Phase II	IgG	293	20.82	128	165	2-82	IFA
Berktas et al.[40]	Multicenter	Farmers, slaughterhouse workers, butcher	Phase II	IgG	552	36.59	348	204	17-63	ELISA
Arserim et al.[20]	Diyarbakir	Farmers	Phase II	IgG	90	6.67	-	-	18-45	ELISA
Gunal et al.[41]	Tokat	Normal people	Phase II	IgG, IgM	53	35.85	37	16	18-65	IFA
Eyigor et al.[42]	Aydin	Miscarriage women and their husbands	Phase I, Phase II	IgG, IgM	62	40.32	31	31	21-64	ELISA, IFA, PCR
Gunal et al.-1[43]	Multicenter	Normal people	Phase II	IgG, IgM	36	11.11	0	36	-	IFA
Gunal et al.-2[43]	Multicenter	Miscarriage women	Phase II	IgG, IgM	64	15.63	0	64	-	IFA
Cikman et al.[44]	Erzincan	Breeders, normal people	Phase II	IgG	368	8.7	130	238	1-99	ELISA
Erturk et al.[45]	Multicenter	Normal people	Phase I, Phase II	IgG	440	19.09	219	221	8-85	ELISA
Arabaci et al.[46]	Multicenter	Veterinarians, celebs, slaughterhouse butcher, farmers, laboratory workers	Phase I, Phase II	IgG	600	27.17	428	172		ELISA, IFA
Kirecci et al.[47]	Kahramanmaras	Veterinarians, celep andslaughterhouse butcher	Phase II	IgG	40	10	34	6	20-60	ELISA

-: Unspecified, ELISA: Enzyme-Linked ImmunoSorbent Assay, IFA: Indirect Fluorescent Antibody test, PCR: Polymerase Chain Reaction

the agent at the species level (n=97), not reporting the sample characteristics and laboratory method (n=165), and non-research articles (n=157) were eliminated *(Fig. 1)*. Forty-four research papers were included, 27 of which were animal studies and 17 were human studies. The characteristics of the included studies are shown in *Table 1* and *Table 2*.

Findings of Studies with Animal Samples

In the literature review conducted without any date limitation, 27 studies were identified between 1997-2021 that met our inclusion criteria. Of the 27 studies, 55.6% (95% Confidence Interval (CI): 7.14-17.14%) were multicenter, 18.5% (95% CI: 4.14-28.66%) were in the Eastern Anatolia Region and the rest were from other regions as shown in *Table 1* [10-33] made in the provinces. 18.5% of the studies were performed with Phase II, 14.8% with Phase I + Phase II antibodies, and 51.9% with IgG antibodies, and the antibody type studied in 13 studies was not specified. Considering the methods in which antibodies were tested, the ELISA method was used in 59.3% of the studies, the IFA method was used in

14.8% of the studies, and the other methods are listed in *Table 1* [11-34].

The general prevalence of *C. burnetii* in animals was 13.49% (95% CI: 10.04-16.93%), with the most common being 26.67% in Kars and 25.63% in Diyarbakir. Of the animal species, cattle were studied most frequently, with 40.7% (95% CI: 6.16-14.75%) and sheep at 22.2% (95% CI: 7.67-30.52%). The mean prevalence of *C. burnetii* in sheep was 19.1%±10.88, 10.46±6.39% in cattle, 15.21±10.01% in studies including cattle and sheep together, 11.17±10.74 in cattle, sheep and goats, and 12.4%±1.15% in sheep and goats. The prevalence rates of other animal groups are shown in *Table 1* in detail.

Findings of Studies with Human Samples

In the literature review conducted without any date limitation, 17 studies were found between 1997 and 2019 that met our inclusion criteria. Six of the 17 studies were multicenter, four were conducted in the Aegean Region, and the rest were conducted in other provinces as shown in *Table 2* [11,14,35-47]. Of the studies, 11 (64.7%) were performed with Phase II, four (23.5%) with Phase I + Phase II antibodies, 11 (64.7%) with IgG, and four (23.5%) with IgM + IgG antibodies. The ELISA method was used in 47.1% of the studies, and the IFA method was used in 35.3%. The others are shown in *Table 2*.

The general prevalence of *C. burnetii* in humans was found to be 22.78% (95% CI: 16.43-29.12%), and the most common rates of 42.39% and 40.32% were found in Aydın and İzmir provinces. Men constituted 53.51% of the general sample. *C. burnetii* prevalence was the highest, respectively; it was determined that individuals engaged in animal husbandry (30.82%±13.61), women with miscarriage (27.97±17.45%), infertile individuals (22%), normal population (18.28±12.73) and breeders (17.5±10.6). There was no statistically significant difference between prevalence rates and characteristics of individuals (P>0.55).

Meta-Analysis of Included Studies

Random effect (REX) and fixed effect (FEX) models were used to calculate the effect size of the studies. Based on the analysis performed at the 95% confidence interval, studies in animals and humans showed significant heterogeneity (I^2 values 92.25% and 92.85%, respectively; P<0.05). The REX model was used in this study to calculate the effect size of the studies. According to the effect size analysis performed at the 95% CI, the effect size of the animal studies was found to be 0.041, and it was found to be low effective. Since the value was close to zero, the effect size of the generalized *C. burnetii* prevalence in animal studies was found to be negligible. The effect size coefficient of human studies was found to be 0.212, and it was found to be moderately effective.

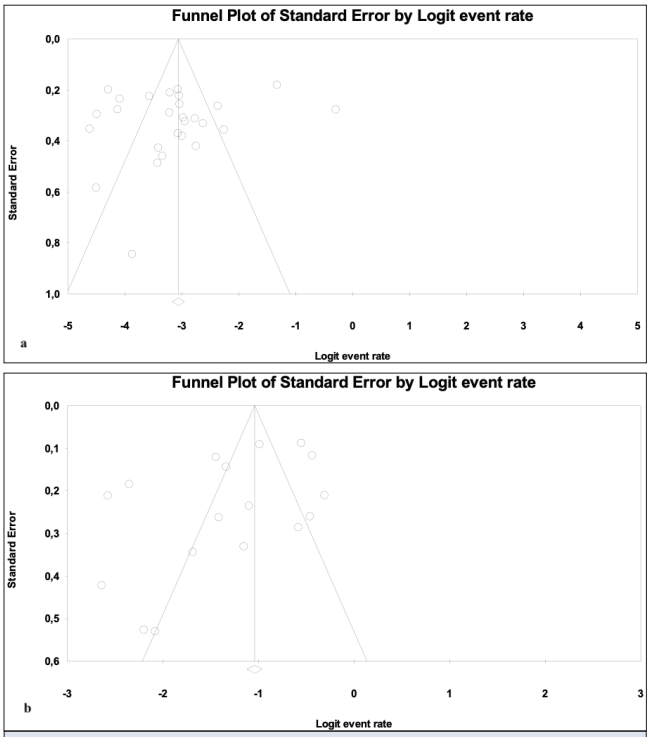

Fig 2. a- Funnel plot of studies involving animals, **b**- Funnel plot of studies that included humans

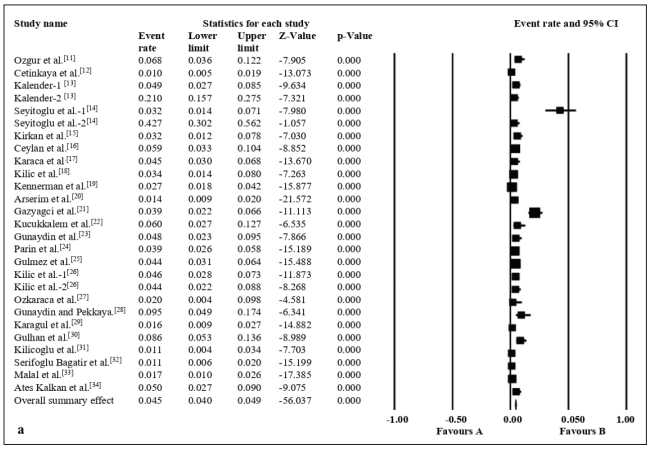

Fig 3. a- Forest plot of studies that included animals [11-34]; **b**- Forest plot of studies that included humans [11,14,35-47]

As can be seen from the funnel plot in *Fig. 2-a*, it was observed that 12 of the studies with animal samples included in the meta-analysis were between the axes, four were on the axis line, and 11 were off the axes. For

this reason, 11 studies in the meta-analysis were not included in calculating the overall effect size, but 16 studies contributed to calculating the overall effect size coefficient. In the funnel plot in *Fig. 2-b*, it is seen that eight studies with human samples included in the meta-analysis were between the axes, and nine were outside the axes. *Fig. 2-a* it can be said that the graphs in *Fig. 2-b* are not asymmetrical; therefore, no bias was detected.

Fig. 3-a and *Fig. 3-b* shows the forest plot of the prevalence rates of *C. burnetii* reported in the studies included in the meta-analysis. It is seen that a study investigating prevalence in animals in *Fig. 3-a* and three studies investigating prevalence in humans in *Fig. 3-b* have P values greater than 0.5 (not statistically significant) and intersect with the 0.5 line.

Discussion

This systematic review and meta-analysis study reports the seroprevalence of Q fever among humans and animals in Türkiye. The results of our study showed that the overall prevalence of *C. burnetii* in Türkiye was 22.78% (95% CI: 16.43-29.12%) in humans. In seroprevalence studies with people included in this meta-analysis, the lowest rate was 6%, and the highest rate was 42.39%. El-Mahallawy et al.[5] reported the rate of Q fever seropositivity as 10% in China in their systematic reviewbetween 1989 and 2013. In a systematic review study conducted in Kenya, the seroprevalence of *C. burnetii* was found to vary between 3% and 35.8% in humans [48]. The prevalence of *C. burnetii* obtained in our study was found to be higher than in other studies in the literature. The highest prevalence rate, respectively, is expected to be detected in livestock workers, women who have had a miscarriage, and infertile individuals. However, the rates between countries may vary depending on the differences in environmental, social, cultural and economic conditions, the exposure of people living in each region to animals and the differences in the infection levels of these animals.

The findings of this study showed that the average prevalence of *C. burnetii* across all studies was 30.45% in butchers, 26.51% in farmers, 14.43% in livestock breeders and 6.5% in veterinarians.In a study conducted in South Korea in 2022, the seroprevalence of *C. burnetii* was found to be 7.9% in people working in veterinary services [49]. Ricco et al.[50] reported an average pooled seroprevalence of 44% in workers, most of whom were agricultural workers, in their meta-analysis study. Subgroup estimates found an average of 2.8% for forest rangers, 49.2% for animal breeders, and 73.7% and 75.9% for slaughterhouse workers and veterinarians, respectively. Woldeyohannes et al.[51] reported the *C. burnetii* seroprevalence rate in slaughterhouse and slaughterhouse workers between 4.7% and 91.7% in their meta-analysis of 19 studies. The findings of this meta-analysis show similar results to other reviews and original studies in the literature.

Of the studies included in this meta-analysis, 11 (64.7%) were performed with Phase II, four (23.5%) with Phase I + Phase II antibodies. In a meta analysis study by Mobarez et al.[7] in Iran, the prevalence of *C. burnetii* IgG phase I and II antibodies in humans was reported to be 19.80% and 32.86%, respectively. In a study conducted to detect *C. burnetii* antibodies among slaughterhouse workers and veterinarians in Canada, antibodies against Phase II *C. burnetii* were detected in 49.0% of veterinarians and 35.0% of slaughterhouse workers. Antibodies against Phase I *C. burnetii* antigens were detected in 30.0% of veterinarians and 14.5% of slaughterhouse workers [52]. In a study by Ali et al.[53] in Pakistan, 25 serum samples (8.4%) were found to be seropositive for Q fever, 17 were positive for Phase I, and 21 of them were positive for phase II antibodies. As in the findings of our study and other studies compared in the literature, Phase II antibodies were mostly used for diagnosis. This is because the antibody titer against Phase II antigens in acute Q fever is higher than that against Phase I antigens.

ELISA was used in 11 of the human studies included in this meta-analysis, IFA was used in nine, and PCR was used in one. It was found that ELISA was used in 18 of the animal studies, PCR was used in seven and IFA tests were used in five. Ricco et al.[50] reported that three of the studies they included in the meta-analysis used IFA, three used ELISA, and one used the complement fixation test (CFT). Woldeyohannes et al.[51] stated that in their meta-analysis of studies measuring the prevalence of C. burnetii in slaughterhouses and slaughterhouse workers, seven of the studies used the CFT method, five used the ELISA method, and two used the IFA method. It has been observed that the most common methods used in seroprevalence studies are ELISA and IFA tests. In particular, the IFA technique is accepted as the reference method in the diagnosis of Q fever by many centers. IFA is a guide in the diagnosis of both acute and chronic Q fever. It is known that the IFA test is the gold standard in the diagnosis of *C. burnetii*. Advantages of this method: it requires a very small amount of antigen and can detect IgG, IgM and IgA antibodies against Phase I and Phase II *C. burnetii* [54]. However, they also have disadvantages such as the need for experienced personnel, lack of standardization between laboratories, not being suitable for large-scale seroprevalence research, and not being able to be automated [55].

As a result of this study, it was determined that the general prevalence of *C. burnetii* in animals in Türkiye was 13.49% (95% CI: 10.04-16.93%). The mean prevalence of *C. burnetii* in sheep was 19.1±10.88, 10.46±6.39% in cattle, 15.21±10.01% in studies including cattle and sheep,

11.17±10.74 in cattle, sheep and goats, and 12.4%±1.15% in sheep and goats. Mobarez et al.[7] found an average of 31.97% seroprevalence of *C. burnetii* in goats in their meta-analysis study in Iran. Q fever seropositivity in goats; was reported to vary between 12% in Africa, between 20% and 46% in a systematic review in Kenya, and between 0.8% and 60.6% in a systematic review made in China [5,39,56]. In this meta-analysis, *C. burnetii* seroprevalence rates in goats were found to be similar to the data in the literature. Guatteo et al.[57] made a review of studies conducted worldwide on the prevalence of *C. burnetii* in domestic ruminants. The review found that the seroprevalence of *C. burnetii* infection at the individual and herd level was 15-20% (prevalence rates depending on the individual and herd level are 20-37.7%, respectively)in many countries, regardless of species, and the prevalence in cattle was found to be higher than that in sheep (15-25% prevalence depending on individual and herd level in sheep and goats, respectively). In the meta-analysis of studies conducted by Rabaza et al.[58] reporting the herd-level prevalence of *C. burnetii* in cattle, the pooled prevalence rate was reported as 37.0% (min. 25.2%-max. 49.5%) in America, Europe, and Asia countries. Nokhodian et al.[59] found the cumulative seroprevalence of Q fever in animals to be 27% in their systematic review including 27 studies. They reported that this prevalence rate was 33% in goats, 27% in sheep and 17% in cattle. In this meta-analysis, *C. burnetii* seroprevalence rates in sheep, goats and cattle were found to be similar to the data of other meta-analysis and systematic review studies around the world. Differences in prevalence rates; it may be caused by different climatic conditions, geographical location, sample size of the study and the time period in which it was conducted, animal species for prevalence screening, serological methods and cut-off values of laboratory tests.

Conclusion

As a result of this study, it was determined that the prevalence of Q fever in humans in Türkiye is high in those dealing with animals, women who had a miscarriage, and infertile individuals. *C. burnetii* is known to cause abortion in animals. When the data obtained are evaluated, it can be concluded that *C. burnetii* may also be associated with miscarriage in humans. The seroprevalence findings in animals reveal that Q fever is common among sheep, goats and cattle and that a surveillance strategy should be applied for this zoonosis. Although it is known that this disease is seen in Türkiye, there are very few case notifications in the literature. In addition to seroprevalence findings, there is a lack of data on the pathogenesis and molecular biology of the disease, and further studies are needed. It is important to carry out detailed studies on Q fever risk factors in humans and animals and to evaluate these factors together within the scope of the One Health approach. Effective vaccination programs should be applied to individuals and animal herds, especially in the risk group dealing with herds of animals.

Highlight Keypoints

o It was determined that the prevalence of Q fever in humans in Türkiye is high in those dealing with animals, women who had a miscarriage, and infertile individuals.

o The seroprevalence findings in animals reveal that Q fever is common among sheep, goats and cattle and that a surveillance strategy should be applied for this zoonosis.

o Effective vaccination programs should be applied to individuals and animal herds, especially in the risk group dealing with herds of the animal.

o More studies are needed to carry out detailed studies of Q fever in humans and animals and to evaluate risk factors and prevalence data together within the scope of the One Health approach.

Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author (E.P. Kahraman Kilbas) upon reasonable request.

Financial Support

No financial support was received for this study.

Conflict of interest

The authors declare that they have no conflict of interest.

Author Contributions

IHC and EPKK designed the study. IK performed the literature review and wrote the article. IHC reviewed and revised the manuscript. All authors have read and accepted the final version of the manuscript.

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Review

Identification and Recognition of Animals from Biometric Markers Using Computer Vision Approaches: A Review

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ABSTRACT

Although classic methods (such as ear tagging, marking, etc.) are generally used for animal identification and recognition, biometric methods have gained popularity in recent years due to the advantages they offer. Systems utilizing biometric markers have been developed for various purposes in animal management, including more effective and accurate tracking of animals, vaccination, disease management, and prevention of theft and fraud. Animals' irises, retinas, faces, muzzle, and body patterns contain unique biometric markers. The use of these markers in computer vision approaches for animal identification and tracking systems has become a highly effective and promising research area in recent years. This review aims to provide a general overview of the latest developments in image processing approaches for animal identification and recognition applications. In this review, we examined in detail all relevant studies we could access from different electronic databases for each biometric method. Afterward, the opportunities and challenges of classical and biometric methods were compared. We anticipate that this study, which conducts a literature review on animal identification and recognition based on computer vision approaches, will shed light on future research towards developing automated systems with biometric methods.

Keywords: Animal, Biometric markers, Computer vision, Identification, Recognition

Introduction

The worldwide purpose of animal identification is to identify and register animals, ensure effective control of animal diseases and movements, maintain records related to livestock support, health, breeding, and statistics. Tracking, monitoring, and individually identifying farm animals hold significant socio-economic importance. Additionally, the increasing consumer demands for food safety have underscored the necessity of secure traceability systems for the origin and production stages of animals and animal products [1,2]. Many international organizations, such as the World Health Organization (WHO) and Food Safety Authorities, actively support the development of identification and traceability systems, recognizing their importance in ensuring food safety and animal health.

Permanent and reliable identification is the primary goal of animal tracking systems. Traditional identification methods (fleece marks, tattoos, ear notches, plastic or metal ear tags) are inconvenient for sheep-goat-type animals and increase cost, especially in large herds [3]. The reasons for the ineffectiveness of these methods are; losses, deletion, short reading distances, reading errors, adversely affecting the welfare of animals, and being vulnerable to cheating [1]. For this reason, the necessity of reliable methods that can be an alternative to classical identification methods emerges. With epidemics, it was understood that animal identification methods were insufficient in monitoring animals, and new traceability tools were needed [4]. Thus, the process of utilizing biometric technologies as well as electronic identification systems in the traceability of animals and animal products has begun.

The World Organization for Animal Health (WOAH) attaches great importance to the individual identification and tracking of animals, especially farm animals. In addition, consumers all over the world want to have information about the source and production stages of the products they consume, worrying about animal health and the safety of animal products [5]. The transmission

of some diseases such as mad cow, anthrax, alum, tuberculosis, brucellosis, and rinderpest to humans by crossing the species barrier in animals brings animal food safety to the fore. From the point of view of the country's economy, the identification and tracking of animals are of great importance. As a matter of fact, in the first mad cow case in England in 1996, meat consumption fell by 40%, resulting in serious economic losses. Beef and beef exports were stopped in 53 countries in 2003 due to the mad cow case in a single cow. This situation caused an economic loss of 3.2-4.7 billion dollars [6]. With an effective animal identification and tracking system, the disease outbreak can be tracked, slowed, and isolated quickly [6]. For these reasons, monitoring livestock has developed rapidly all over the world and continues to evolve. Major exporters such as Australia and Canada have mandatory cattle identification systems. South Korea introduced a comprehensive beef monitoring system in December 2008. In the US, a total expenditure cost of $75.87 million was incurred in 2009 for the identification of cattle alone [6]. In our country (Türkiye), the Ministry of Food, Agriculture, and Livestock started the tagging and registration procedures for bovine animals in 2002.

Animal welfare is an extremely important issue, and some regulations have been made in developed countries, especially in the European Union (EU) countries, with laws and regulations to raise animal welfare standards in animal production and to make animals happier [7]. Animal welfare positively affects the quantity and quality of products obtained from animals. Ear tag application is one of the most used identification methods in our country and all over the world. Ear tagging can not only cause pain and stress in animals but also cause tissue reactions and infections. While ear wounds lead to earring loss, re-identification of the animal will cause pain once again and cause an extra cost. In addition, it is known that pain and stress negatively affect hereditary characteristics such as growth rate, resistance to diseases, milk production, and reproductive ability in farm animals [8]. Also, the ear tag can be easily copied or removed and discarded after the animal is stolen. In this case, there is nothing to be done. Because with other systems other than biometric systems, device tracking is done, not animal tracking. Biometric methods have become one of the popular topics of recent times, as they do not have the above-mentioned disadvantages of classical methods [9-11].

The use of artificial intelligence methods in animal husbandry is becoming increasingly widespread day by day [12-15]. In parallel with this, in recent years, the topic of animal identification and automatic animal recognition through computer vision approaches has also gained popularity [16]. With this system, a database is created from images obtained from animal biometrics. In almost all scientific researches, biometric data collected from animals are not publicly published for researchers to use. To our knowledge, there are several publicly accessible databases that can be used for animal identification and recognition. These databases consist of bovine iris images [17], cattle body patterns [18], and cattle muzzle images [19]. Several biometric features are extracted from the biometric data taken from each living thing, and individual identification and recognition are performed based on this feature set.

The iris patterns, retinal vascular patterns, face, muzzle, and animal body pattern biometric data of animals are analogous to human fingerprints and contain unique biometric features for every living thing. Thanks to these biometric markers, identification, and recognition in animals are successfully performed with the computer vision approach.

This review aims to examine in detail the recognition and identification studies with the computer vision approach and biometric methods in the field of animal husbandry and draw a roadmap for researchers who will work in this field. In addition, the comparative analysis of classical identification methods and biometric identification methods is among the objectives of the study. Considering the contribution of agriculture and animal husbandry to the national economy, the importance of this study can be understood more clearly. In this sense, the importance of this review, which will shed light on the studies to be carried out in the field of agriculture and animal husbandry, becomes more evident.

Animal Identification and Recognition Methods

Classical identification methods leave their place for modern methods due to disadvantages such as animal welfare, losses, deformations, and fraud [20]. Since biometric methods are modern methods and cannot be copied, frauds can be prevented, and there is no such thing as loss or deformation as long as the animal lives. With biometric methods, the animals are not stressed, and animal welfare is protected as an important advantage. Animal welfare is at the center of all these. Animal welfare is a concept related to meeting the needs of the animal in its natural life. In animal breeding, natural lifestyles, shelter conditions suitable for their unique behaviors, feeding the animal without disturbing its physiological, biological, and psychological integrity, and carrying out production activities in a way that does not impair the health of the animal and not restrict its movements are the basic elements of animal welfare [21].

Animal identification systems have been an essential component used in traceability for centuries and are divided into four categories: permanent, temporary,

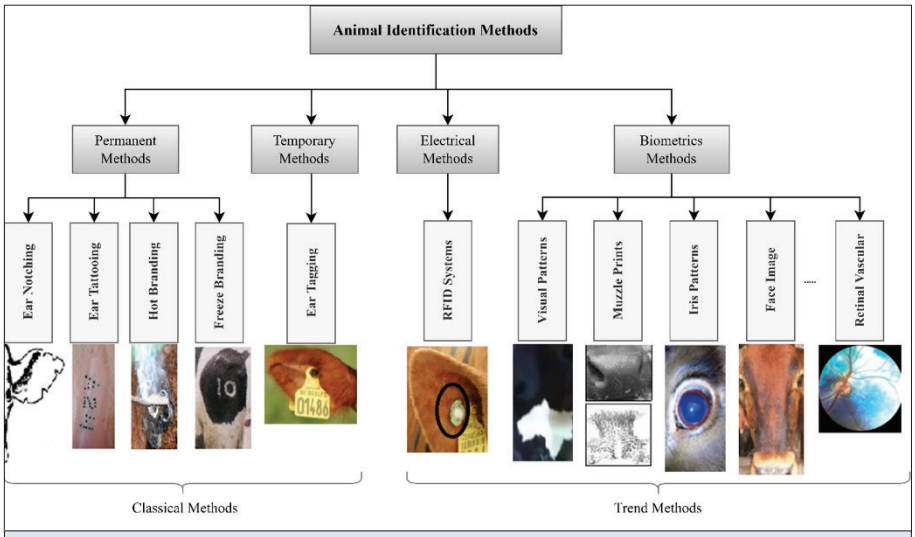

Fig 1. Classical and trend methods in animal identification

electrical, and biometric [20]. These identification methods are presented in *Fig. 1*.

In recent years, identification methods using biometric markers in animals are called trend identification methods. Retinal vessel patterns in animals, muzzle prints, iris, face, and patterns in various parts of their body are biometric markers. Biometric markers are a good alternative to classical methods as they are unique, cannot be changed by others, do not require additional costs, and most importantly do not adversely affect animal welfare. Opportunities and challenges of these classical and trend methods used for identification are presented in *Table 1*.

Traditional methods are still used for identification in many countries. As seen in *Table 1*, traditional identification methods have several disadvantages. RFID microchip technology, one of the trending methods, has been widely employed in recent years, especially in pet identification. However, this method also has a series of disadvantages similar to traditional identification methods. For instance, microchip implantation requires a medical procedure, must be administered by a specialist, carries a risk of infection, microchips can migrate within the body, they can malfunction, and can only be read with specialized scanners. While RFID is considered a trending approach, it structurally differs from other trending identification methods, such as biometric-based identification, as shown in *Table 1*. As observed, biometric-based identification methods offer numerous advantages, making them a popular research topic in recent years [22].

Biometric Methods in Animal Identification a Recognition

Biometrics is an automated system that measures an individual's physical or behavioral uniqueness and performs identification by comparing it to existing records. Biometric identification methods do not cause pain and do not change the appearance of the animal. Biometric features are unique, non-replicable, reusable, measurable, robust, and have distinctive physical features. There is no confusion in biometric systems compared to traditional methods [23].

Biometric identifiers include retinal vascular patterns,

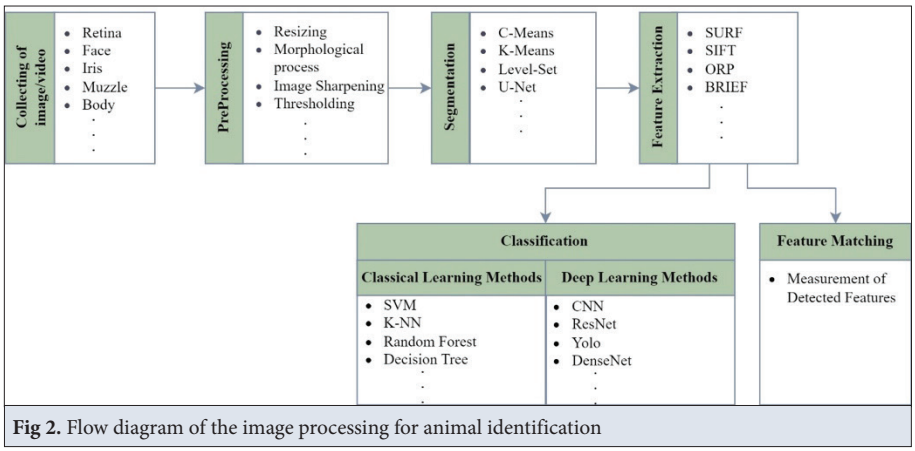

Fig 2. Flow diagram of the image processing for animal identification

Table 1. *Opportunities and challenges of identification methods*

Parameters	Method	Opportunities	Challenges
Trend Identification Methods	RFID	● wide range of applications ● can store information (i.e., owner, the farm, diseases, and the animal›s vaccination status) ● integrated with mobile computing ● easily managed remotely	● painful ● costly ● needs a professional person ● low reliability ● minimum recognition rate ● object tracking, not animal
	Retinal Vascular Pattern	● non-painful ● time-immutable ● applied across a wide variety of animals ● less prone to error and fraud ● useful in monitoring and tracking ● injury to the cornea does not interfere with the retina image ● considerable identification accuracy	● processing time ● the difficulty of capturing a retinal image due to eye diseases
	Muzzle Print	● non-painful ● time-immutable ● less prone to error and fraud ● cannot be forged or altered ● useful in monitoring and tracking	● capturing accurate images ● difficult image processing
	Face	● non-painful ● time-immutable ● less prone to error and fraud ● cannot be forged or altered ● useful in monitoring and tracking	● capturing accurate images ● pose, expression, illumination, aging, and disguise
	Iris Pattern	● non-painful ● less prone to error and fraud ● cannot be forged or altered ● useful in monitoring and tracking	● time-mutable iris texture (with age, disease, and medication) ● difficult to capture iris images (blurred images and images occluded by eyelids or eyelashes)
	Body Pattern	● non-painful ● time-immutable ● less prone to error and fraud ● cannot be forged or altered ● useful in monitoring and tracking	● applicable to animals with the pattern ● capturing accurate images
Traditional Identification Methods	Ear notching	● Permanent ● relatively quick and simple ● highly visible ● low cost	● painful ● laborious operation /An exhausting ● time-consuming ● limited scalability (not suitable for large-sized farms) ● unbounded cost ● susceptible to theft, fraud, and duplication ● not useful in monitoring and tracking
	Ear tattooing	● less painful ● permanent ● low cost ● equipment portable	● limited scalability ● time-consuming ● laborious operation ● time-mutable ● less useful dark animals ● susceptible to theft, fraud, and duplication ● not useful in monitoring and tracking
	Hot iron branding	● easy and simple	● painful ● manually identified ● time-mutable ● susceptible to theft, fraud, and duplication ● not useful in monitoring and tracking ● causes inflammation
	Freeze branding	● faster application time	● painful ● manually identified ● susceptible to theft, fraud, and duplication ● not useful in monitoring and tracking
	Ear tagging	● low cost ● most widely used ● relatively atraumatic ● quick and easy to perform	● painful ● time-mutable ● susceptible to damages, duplications, losses, unreadability, and fraud ● do not perform well as a long-term identification ● can cause infection ● unbounded cost (tags can fall out) ● not useful in monitoring and tracking

muzzle print images, face images, animal body patterns, and iris patterns. Thanks to image processing techniques, animal identification is successfully carried out with these biometric identifiers. *Fig. 2.* illustrates the image processing steps commonly employed in past and present studies of animal identification.

The image processing steps provided in the flowchart may vary depending on the requirements of the specific study; therefore, additional steps can be added to these procedures, or certain steps may not be implemented.

This article reviews the evolution of animal recognition and tracking from traditional methods to animal biometrics. It also reports on traditional and trend animal identification methods, their advantages, and their disadvantages. Also, this article explains the use of biometric identifiers to recognize, identify and trace animals effectively. This review presents the latest research findings in animal biometrics with a strong focus on biometric descriptors such as muzzle prints, animal body patterns, iris patterns, and retinal vascular patterns. A discussion of the current challenges in biometric-based identification systems is included in the results that may guide future research directions. In the following titles, the types of biometric methods and the studies carried out in these fields are examined separately.

Recognition and Identification Based on Retinal Vascular Pattern

The retinal vascular pattern at the back of the eye is used as a biometric marker in humans and animals. Although this biometric descriptor has been extensively studied in the literature for humans, limited studies have been conducted on animals. Due to the static nature of retinal vasculature throughout the animals' lifespan, retinal imaging remains impervious to alterations. In stark contrast, items like ear tags are susceptible to replacement, removal, or misplacement [24]. The animal retina has similar features to the human retina, and it is known that it is a biometric feature that does not change throughout its life [25].

Image processing techniques are increasingly being utilized in the analysis of retinal vascular patterns in animals within the field. However, it is evident from the current literature that such studies are limited in number. In these studies, identification is typically carried out using vascular patterns in the retinas of animals such as lambs/sheep, cattle, dogs and goats. Nevertheless, in most of these studies, embedded software-based devices are preferred over image processing methods. These devices provide a matching score that determines whether the data is present in the database when brought close to the animal's eye. However, this approach has several disadvantages. Firstly, the processing capabilities of these software devices are limited and cannot perform complex image processing tasks. Additionally, updating and customizing such software is often challenging and costly. The use of image processing techniques may offer greater flexibility and customization possibilities and has the potential to cover a broader animal population. Details of studies conducted based on retinal vascular patterns in

Table 2. *Overview of studies based on retinal vascular patterns*

Year	Country	Animal	#Animal /Images	Method(s)	Best Method	Comparison Metric(s)	Accuracy	Ref.
2006	Colorado, USA	Dog	18/18	Technology Driven Products GNU Gimp	Technology Driven Products GNU Gimp	Multivariate ANOVA	Age 5 94.00±6.00%	[26]
2008	North Ireland	Cattle	869/1738	Optibrand Software	Optibrand Software	Optibrand Matching Engine	98.30%	[27]
2008	Ireland	Sheep	64/128	Statistical Methods, Image Matching	Statistical Methods, Image Matching	ROC, Matching Score	93.10%	[28]
2008	Ireland	Lamb	19/38	Regression-based Random Effect	Regression-based Random Effect	Matching Score	Age: 1 week 86.00% Age: 8 weeks 96.00%	[25]
2011	Ireland	Sheep	160/320	Optibrand Software	Optibrand Software	Matching Score	Age<2 96.16% Age>2 96.89%	[29]
2012	Barcelona, Spain	Lamb	143/2534	CATMOD ML	CATMOD ML	Accuracy	94.80±0.60%	[30]
2019	Türkiye	Sheep	60/360	Matching Scores, Pearson Correlation Coefficient	Matching Scores, Pearson Correlation Coefficient	Matching Score	Right eyes 75.46% 78.93% 79.97% Left eyes 89.28% 89.10% 89.74%	[31]
2021	West Bengal, India	Goat	12/200	Template Matching, Hamming Distance, CLAHE	Template Matching, Hamming Distance, CLAHEv	Accuracy, Recall, Precision	99.00%	[32]

Table 3. *Overview of studies based on muzzle print*

Year	Country	Animal	#Animal /Images	Method(s)	Best Method	Comparison Metric(s)	Accuracy	Ref.
2023	-	Cattle	20/600	SIFT, BRISK, ORB, KAZE, AKAZE	KAZE	Matching score	76.18%	[35]
2022	India	Cattle	186/930	Shi-Tomasi, SURF, SIFT, MLP, DT, RF	Shi-Tomasi, SURF, SIFT +RF	Accuracy, TPR, FPR, AUC	83.35%	[36]
2022	USA	Cattle	268/4923	59 deep learning models	VGG	Accuracy, Processing speed, CI	98.70%	[19]
2021	-	Dog	302/2561	DNNet	DNNet	ROC, FAR	98.99%	[37]
2021	Nigeria	Cow	400/4000	CNN, DBN	DBN	Accuracy	98.99%	[38]
2020	Nigeria	Cow	400/4000	CNN, SDAE, DBN	DBN	Accuracy	98.99%	[39]
2020	-	Arabian horse	50/300	SVM, SVM-GWO	SVM-GWO	Accuracy	99.60%	[40]
2020	Korea	Dog	11/1045	SIFT, SURF, BRISK, ORB	ORB	EER	65.00%	[41]
2020	Indonesia	Cattle	60/460	SIFT- RANSAC	SIFT- RANSAC	Accuracy	93.05%	[42]
2018	India	Cattle	500/5000	SDAE, CNN, DBN	DBN	Accuracy	98.99%	[43]
2016	-	Cattle	31/217	AdaBoost, k-NN, Fk-NN	AdaBoost	Accuracy, Sensitivity, Specificity, AUC, Error	99.50%	[44]
2015	-	Cattle	52/1040	MSVMs	MSVMs	Accuracy	96.20%	[45]
2014	-	Cattle	31/217	NN, NB, SVM, k-NN	SVM	Accuracy	99.50%	[46]
2013	Indonesia	Cattle	48/1440	SIFT	SIFT	EER	99.70%	[33]

the literature are presented in *Table 2*.

The implementation of identification from retinal images has some challenges. For instance, the devices used to obtain retinal images are costly. Obtaining retinal images from animals can be challenging, and external conditions (such as lighting, flash, etc.) can affect the quality of retinal images. Additionally, since obtaining retinal images requires close contact, it can be dangerous to capture images from predatory animals.

Recognition and Identification Based on Muzzle Print

Muzzle prints in animals contain some distinctive features similar to fingerprints in humans. These features are unique to the living thing, do not change over time, and cannot be changed. The use of the muzzle mark as a recognition tool dates back to 1921 [33]. The first image-processing studies were made from the prints of the ink applied to the animal's muzzle and taken on paper. Although the accuracy of this identification method has been proven, it has been accepted as a disadvantage that the printing process takes time, and the ink print images are not of sufficient quality to be used in the computer environment [34].

Collecting muzzle print images has been a subject of extensive research in this field due to its relative ease compared to other biometric features, and ongoing efforts are being made in this regard. In the literature, machine learning methods are commonly employed in studies in this field, and in recent years, identity verification and recognition using deep learning techniques have gained momentum. When examining studies based on muzzle prints, it is generally observed that muzzle prints from various animals such as dogs, cows, horses, and cattle are utilized. Information pertaining to these studies is presented in *Table 3*.

The uniqueness of each animal's muzzle print, as with other biometric identifiers, is advantageous, making muzzle prints a viable authentication tool. Furthermore, the ease and low cost of collecting muzzle print data from animals in a manner that minimizes stress is considered an advantage. However, in implementation, environmental factors such as dirt, humidity, or lighting conditions may affect the quality of muzzle prints, and collecting muzzle prints from certain animals, especially larger and more dangerous species, can be challenging. Additionally, disparities may arise when comparing muzzle print images acquired using different camera types or under different conditions.

Table 4. Overview of face image-based image processing studies

Year	Country	Animal	#Animal /Images	Method(s)	Best Method	Comparison Metric(s)	Accuracy	Ref.
2023	-	Horse	-/1103	YOLOv7	YOLOv7	Precision	99.50%, 99.70%	[49]
2022	-	Sheep	81/5265	CNNs	ResNet50V2-ArcFace	Average Precision, Recall, F1-Score	97.00%	[50]
2022	China	Sheep	67/6526	GGFace, AlexNet, ResNet50, YOLOv3, YOLOv4	YOLOv4-CBAM-TL	mAP	91.58%, 90.61%	[51]
2021	-	Sheep	420/1680	Resnet50, VGG16	VGG16	Precision, Recall, F1-score, Support, Accuracy, Macro Average, Weighted average	94.00%	[52]
2020	Brazil	Cattle	51/27849	DenseNet, ResNet50, InceptionResnetV2	DenseNet	Precision, Recall, F1-score, Accuracy	99.85%	[53]
2020	China	Pig	30/1800	CNN	CNN	Accuracy	83.00%	[54]
2019	-	Sheep	52/52000	CNN, Cosine, AlexNet	CNN	Accuracy	98.00%	[48]
2019	West Africa	Chimpanzee	23/10 million	CNN	CNN	Accuracy	92.50%	[55]
2019	Türkiye	Cattle	5/1579	Faster R-CNN	Faster R-CNN	Accuracy	98.44%	[56]
2018	-	Dog	500/5000	SVM, FLPP, PCA, LDA, ICA, LBP, SURF	SVM-FLPP	Accuracy	96.87%	[57]
2018	Scotland	Pig	10/1553	CNN, Fisher face, VGG-Face + SVM	CNN	Accuracy	96.70%	[58]
2015	Varanasi	Cattle	120/1200	PCA, LDA, ICA SURF, LBP	SURF- LBP	Accuracy	92.50%	[59]
2007	-	Sheep	50/200	Cosine distance	Cosine distance	Accuracy	96.00%	[47]

Recognition and Identification Based on Face Images

Another biometric-based method using image processing technology is animal face recognition. Like the face recognition system in humans, animal recognition and identification can be performed using facial images of animals. Animal facial biometrics includes important features that can be used in recognition, such as eyes, muscles, mouth, and many hidden features [47,48].

In the literature, it has been seen those different types of animals, such as cattle, horses, pigs, sheep, dogs, and non-human primates, can be successfully recognized and identified from facial images. Various summary information about the identification, classification, and recognition studies based on facial biometrics are presented in *Table 4*.

Identification from facial images also has some practical challenges. Animals can often be restless, and ensuring they stay still to capture facial images can be difficult. External conditions can affect the quality of facial images. Particularly, lighting conditions can influence image quality. The facial structures of some animals can complicate the recognition process. Obtaining facial images may require close contact with animals, which can be dangerous in some cases. Differences between facial images obtained with different camera types or under different conditions can impact recognition accuracy.

Recognition and Identification Based on Animal Body Pattern

Some animals exhibit patterns or spots on their bodies, representing a biometric approach utilized in animal identification or recognition studies. Research based on such patterns has a limited overall impact in the literature when compared to more common biometric features like facial recognition or retinal scans. This limitation stems from several key factors, including the limited population of animals with these patterns, the highly individualistic nature of these patterns, and the restricted scope of applications. For example, in the literature, studies have been conducted on identification using patterns on the bodies of cattle and cows, speckle patterns on the skin of Atlantic salmon, patterns on the shells of sea turtles, black stripes on the body of Sumatra barbs, and feather patterns of Saimaa Ringed Seals. Detailed information on these studies is provided in *Table 5*.

Table 5. *Overview of pattern-based image processing studies*

Year	Country	Animal	#Animal /Images	Method(s)	Best Method	Comparison Metric(s)	Accuracy	Ref.
2022	US	Cattle	268/4923	59 DL models	VGG16_BN	Accuracy CI	98.70%	[19]
2022	-	Cattle	48/12000	SVM	SVM	Precision, Recall, Average precision, F1-score	98.67%	[60]
2021	-	Cattle	46/4736	CNN	CNN	Accuracy	93.80%	[61]
2021		Cattle	18/10402	CNN, GMM	GMM	Accuracy	76.90%	[18]
2021	Norway	Atlantic salmon	328/1312	CNN	CNN	Accuracy	96.70%	[62]
2021	Lundu, Sarawak	Sea turtles	16/70	SIFT, SURF, ORB, HOG	HOG	Accuracy	65.00%	[63]
2021		Sumatra barb	43/215	HOG, LBP, HP and body shape	LBP	Accuracy	93.00%	[64]
2021	-	Cow	4/2500	YOLO	YOLO	Precision, F1-score, Accuracy	90.00%	[65]
2019	-	Cattle	17/147	YOLO v2, LRCN	LRCN	Accuracy	94.40%	[66]
2019	-	Cattle	66/528	FAST, SIFT, FLANN, ORB, Brute Force	FAST + SIFT + FLANN	Accuracy	96.72%	[67]
2017	China	Cattle	10/1965	QDA, SVM	QDA	Precision, F1-score, Accuracy	99.70%	[68]
2015	Varanasi	Ringed seal	46/2000	PAT, ROT, TOP, k-NN	PAT + ROT + TOP +	Accuracy	88.60%	[69]

Pattern-based identification studies face several challenges, including the following: Some animals' body patterns may change over time or differ as the animals grow. This can make it difficult to record animal body patterns in a standardized manner. The visibility and quality of patterns can be influenced by environmental factors. Especially in open areas or natural habitats, environmental conditions (such as seasonal changes or their living environment) can affect the perception of body patterns. Developing suitable technologies to record and recognize body patterns may be necessary. The cost and complexity of these technologies can pose challenges to implementation.

Recognition and Identification Based on Iris Patterns

Animal iris, like human iris, is a unique biometric marker. The use of iris images for biometric identification is common. Recognition systems have been developed using iris images of animals such as tarentola, cattle, Atlantic salmon, goats, cow, horse, owl, and tiger, as given in *Table 6*.

When examining recognition studies conducted using iris images, it is observed that a wide range of animal species, from fish to tigers, have been utilized in these studies. Among biometric markers, iris is considered more reliable compared to other methods such as facial recognition, pattern recognition, and muzzle prints. Additionally, the collection of iris images is easy and cost-effective when compared to retina scans, making it a more practical choice in biometric recognition systems. Iris-based recognition is seen as a potentially valuable tool for the identification and tracking of animals.

Identification from iris images also has challenges in real-world applications, including the following: The mobility of animals can make iris scanning challenging. Ensuring that the animal remains still, as the eye must stay fixed, can be a challenging process. The structure and characteristics of irises can vary among different animal species.

Conclusion and Recommendations

Animal identification is essential to prevent epidemics, administer vaccines, monitor animal health, and prevent theft and fraud. Many methods have been used to identify animals from the past to the present. Traditional methods of identification, which adversely affect animal welfare,

Table 6. Overview of iris-based recognition studies

Year	Country	Animal	#Animal /Images	Method(s)	Best Method	Comparison Metric(s)	Accuracy	Ref.
2023	India	Black Bengal Goats	49/ 5880	Resnet152V2	Resnet152V2	Accuracy	82.49%, 92.68%, 77.17%, 87.76%	[70]
2023	-	Angus	11/80	15 DNN models	U-Net + VGG16	Dice, Accuracy, Precision	99.50%	[71]
2020	Norway	Atlantic Salmon	14/41	VeriEye	VeriEye	Matching Score	98.00%	[72]
2020	India	Black Bengal Goats	5/25	Hamming Distance	Hamming Distance	Matching Score	59.00%	[73]
2019	-	Cattle	11/80	HSV, Watershed Segmentation	HSV + Watershed Segmentation	Precision, Recall, F1-score, Intersection over union	96.25%	[74]
2017	-	Horse	28/2000	DCNN	DCNN	EER	90.50%	[75]
2017	-	Horse	145/1015	Circular Hough Transform, Canny Edge Detection, K-means clustering	Circular Hough Transform + Canny Edge Detection + K-means clustering	Jaccard similarity	95.00%	[76]
2015	-	Owl	Different images of Owl	Hamming Distance	Hamming Distance	Accuracy	94.00%	[77]
2015	-	Cow	8/48	SURF	SURF	Accuracy	91.67%	[17]
2014	-	Cow	6/60	Hamming Distance	Hamming Distance	Matching Score	98.33%	[78]
2013	-	Lizard (Tarentola geckos)	54/924	I^3S	I^3S	Accuracy	95.00%	[79]
2012	-	Tiger	Different images of Tigers	Hamming Distance	Hamming Distance	Matching Score	not specified	[80]

have begun to give way to modern methods with the development of technology. Ear notching, ear tattooing, hot iron branding, and freeze branding, which are among the traditional methods, are almost no longer used, and the ear tagging method is used more widely. While classical identification methods negatively affect animal welfare, problems such as loss, repetition, fraud, and security cannot be avoided.

Unlike traditional methods, biometric identification using retinal vascular patterns, iris patterns, muzzle prints, facial features, and body patterns offers numerous advantages over conventional approaches. These applications are painless, ensuring animal welfare, and are highly resistant to fraud or loss. Technological advancements and increased interdisciplinary studies have driven a growing shift towards biometric methods for animal identification. This review aims to provide a comprehensive examination of studies employing biometric techniques for identification and recognition, presenting both their strengths and weaknesses to researchers. In this review, studies that utilized retinal vascular patterns, iris patterns, muzzle prints, facial recognition, and body pattern biomarkers were scrutinized meticulously, and their advantages and disadvantages were assessed individually. Additionally, the challenges that may be encountered in the practical implementation of these biomarkers for identification were presented. As a result of this review, it is evident that using retinal vascular patterns for animal identification stands out as the most advantageous method, ensuring both animal welfare and economic benefits at a national level.

This review article presents the following recommendations as a result:

· The widespread adoption of biometric animal identification methods, especially those based on facial, iris, and retinal

vein patterns, is essential.

· The use of biometric methods in international trade transactions should be encouraged as it can support both animal welfare and economic growth.

· To develop successful biometric-based identification systems, attention must be paid to the accuracy and precision of data collection processes, ensuring high-quality images are captured.

· Making animal biometric data accessible to researchers can facilitate advancements in the field of animal science by increasing the quantity and quality of interdisciplinary studies.

The review emphasizes the significance of utilizing biometric markers in the development of animal identification and recognition systems. Considering the undeniable role of animal husbandry in national economies, it is anticipated that the use of the mentioned biometric markers will become a necessity in the near future.

Abbreviations

ANN: Artificial Neural Networks

ARI: Adjusted Rand Index

AUC: Area Under Curve

BRISK: Binary Robust Invariant Scaling Keypoints

CI: Comprehensive Index

CLAHE: Contrast Limited Adaptive Histogram Equalization

CNN: Convolutional Neural Network

CWT: Complex Wavelet Transform

DBN: Deep Belief Network

DCNN: Deep Convolutional Neural Networks

DL: Deep Learning

DNNet: Dog Nose Network

EER: Equal Error Rate

EU: European Union

FAR: False Acceptance Rate

FAST: Features from Accelerated Segment Test

Faster R-CNN: Faster Regional-Convolutional Neural Networks

Fk-NN: Fuzzy K-Nearest Neighbor

FLPP: Fisher Linear Projection and Preservation

FPR: False Positive Rate

GMM: Gaussian Mixture Model

GWO: Gray Wolf Optimization

HOG: Histogram of Directed Gradients

HP: Horizontal Density Profiles

HSV: Hue, Saturation, Value

I^3S: Interactive Individual Identification System

ICA: Independent Component Analysis

IoU: Intersection over Union

KLT: Kanade-Lucas-Tomasi

k-NN: k-Nearest Neighbor

LBP: Local Binary Pattern

LDA: Linear Discriminant Analysis

LRCN: Long-term Recurrent Convolutional Network

ML: Maximum Likelihood

MSVMs: Multiclass Support Vector Machines

NB: Naive Bayes

NN: Nearest Neighbor

ORB: Oriented FAST and Rotated BRIEF

PCA: Principal Component Analysis

QDA: Second-order Discriminant Analysis

RANSAC: Random Sample Consensus

RFID: Radio Frequency Identification

ROC: Receiver Operating Characteristic

ROI: Region of Interest

SDAE: Stacked Denoising Auto Encoder

SIFT: Scale Invariant Feature Transform,

SURF: Speeded Up Robust Feature

SVM: Support Vector Machine

TPR: True Positive Rate

WLD: Weber's Local Descriptor

WOAH: The World Organization for Animal Health

YOLO: You Only Look Once

YOLOv4-CBAM-TL: Convolutional Block Attention Module- Transfer Learning

Availability of Data and Materials

Not applicable.

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PC: Conceptualization, designed, planned, methodology, investigation, writing original draft. AS, NEO and MA: Conceptualization, methodology, writing and editing. All authors read and approved the final manuscript.

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Review

Distribution, Infection, Diagnosis, and Control of *Avibacterium paragallinarum* in Poultry

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ABSTRACT

Avibacterium paragallinarum is widely distributed all over the world in poultry farms. The purpose of this review was to describe IC disease in chickens caused by *A. paragallinarum* in terms of incidence, pathogenicity, diagnosis, and management. The disease is characterized by upper respiratory affection that is represented by conjunctivitis, sinusitis, facial and wattle edema, growth retardation, a marked drop in egg production, and a high morbidity rate. Complications with other bacterial and viral infections and environmental stressors increase the severity of the clinical signs, lesions, and mortality rate. For serological evaluation of the bacterium, there were two schemes. Page scheme classified the bacterium into serovars A, B, and C, whereas Kume scheme divided it into serogroups I, II, and III. Page serovars were further classified and associated with the Kume serogroup. There are 9 *A. paragallinarum* serovars of Kume scheme represented as A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3, and C-4. Laboratory diagnosis of *A. paragallinarum* is based on conventional methods of isolation and identification as well as serotyping and molecular detection. Strict biosecurity measures are important for the prevention of such infections. However, inactivated polyvalent bacterins are widely used to prevent the possibility of infection. The lack of cross-protection among serovars is the major challenge in the vaccination program.

Keywords: Clinical signs, Incidence, Infectious coryza, Treatment, Vaccination

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Introduction

Respiratory affections result in severe negative economic impacts on poultry production [1]. Snot or infectious coryza (IC) is a widely distributed cosmopolitan bacterial [2] and highly contagious acute upper respiratory disease of chickens caused by a bacterium of the Pasteurellaceae family, *Avibacterium paragallinarum* commonly known as *Haemophilus paragallinarum* [3]. As a result of phenotypic and genotypic characterization, taxonomic differences showed the designation of the bacterium as *A. paragallinarum* [4].

Chicken is the most susceptible host for *A. paragallinarum* and can acquire the infection mainly via aerosol droplets or direct contact with carrier birds [5]. The clinical syndrome of IC has been recognized since the 1930s [6]. Growth retardation and increased culling rate of broilers, drop in egg production of layers and breeders (10-40%), mortality (2-10%), and increased medication costs are the most common economic losses caused by IC [7]. Infection with *A. paragallinarum* is characterized by conjunctivitis, nasal discharge, facial edema, drop in egg production in layers, and high morbidity with a low mortality rate [8]. The epidemiology of the disease is complicated. However, outbreaks of IC are most common in multiple age farms. Severe cases of the disease were also recorded in intensive poultry production systems, especially in developing countries, where poor management conditions and the existence of multiple infections are common [9,10].

Page and Kume schemes subtyped *A. paragallinarum* into 3 serogroups (A, B, and C) and 9 serovars (A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3, and C-4) based on hemagglutination inhibition (HI) test [11]. Diagnosis of IC infection relies on conventional isolation and identification of the causative agent and serotyping [12]. However, recent molecular techniques are used for the rapid and accurate identification of *A. paragallinarum* [13]. Prevention and control of IC can be achieved through the application of strict biosecurity measures, vaccination, antimicrobial agents, and probiotic supplementation [14,15]. Inactivated autogenous water or oil-based bacterins, including *A. paragallinarum* of serovars A, B, and C, are commercially available for breeders and laying hens [16]. The applied vaccines should be crossly ponded with the

predominant local serovars. Moreover, an antibiotic sensitivity test is a must to overcome such an infection. Despite the presence of several antimicrobial agents that are effective in eliminating *A. paragallinarum* infection, the development of resistance is a common issue [17,18].

Therefore, this review article was designed to discuss IC disease caused by *A. paragallinarum* in poultry with emphasis on the disease incidence, pathogenicity, clinical and pathological signs, diagnosis, and control.

History

In Holland, De Blieck [19] described a disease in chickens termed as "contagious or infectious catarrh, roup, or cold" caused by *Bacillus haemoglobinophilus coryza gallinarum*. However, Elliot and Lewis [20] and Delaplane et al.[21] proposed the name *H. gallinarum* as a causative agent of IC based on bacteriological characterization and binomial nomenclature system. Both X (hemin) and V (nicotinamide adenine dinucleotide, NAD) factors were discovered as essentials for the cultivation of the bacterium in media [22-25]. As a result of discovering X-factor-independent isolates of the bacterium [26,27], *H. paragallinarum* became the name of the causative microorganisms of IC [28]. Therefore, *H. paragallinarum* was V-factor-dependent, but X-factor-independent although V-factor-independent strains have been recently identified.

Incidence

Table 1 presents different reports on the incidence of IC in birds such as poultry, quail, and emu in different countries.

Etiology

Avibacterium paragallinarum (formerly *H. paragallinarum)* is a fastidious, Gram-negative, polar staining, non-motile, and non-spore former coccobacillus of the family Pasteurellaceae [55]. There are 2 schemes for the serological classification of *A. paragallinarum*. Page scheme classifies the bacterium into 3 major serovars of A, B, and C using the plate agglutination test [27], while Kume scheme divides it into 3 major serogroups as I, II, and III using HI test [56]. Page serovars are further classified by the HI test associated with the Kume serogroup. Accordingly, Page serovars A, B, and C represent the modified Kume serogroups I, II, and III, respectively. The 9 *A. paragallinarum* serovars of Kume scheme are then classified into A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3, and C-4 [56,57]. Serotyping of *A. paragallinarum* strains is performed using specific antisera in HI test as described by Kume serotyping scheme [11]. Countries, such as the United States, Germany, Mexico, China, South Africa, Thailand, and Taiwan, reported the presence of serovars A, B, and C of *A. paragallinarum*. However, Japan and Australia reported only serovars A and C [39,58].

In 1989, new isolates of *A. paragallinarum* were identified in South Africa where they did not require nicotinamide adenine dinucleotide (NAD) for growth [49]. These isolated strains of the bacterium were regarded as NAD-independent. Most of them are Page serovar A [59] and some strains are serovar C [34]. The NAD-independent *A. paragallinarum* strains become more common than classic strains and they are incriminated in the production of airsacculitis and vaccination failure than NAD-dependent strains [34].

Pathogenicity

The pathogenicity and virulence of *A. paragallinarum* depend on the presence of hemagglutinin protein (HMTp210) which is important for the hemagglutination (HA) process [60]. The HMTp210 deficient mutants cause no HA and accordingly do not induce HI antibodies in vaccinated chickens. Besides, they indicate a decrease in their adherence to tissue cultures and biofilm production. Therefore, these mutants have less virulence than their wild-type strains.

The other essential virulence factor of *A. paragallinarum* is the capsule which plays an important role in the adhesion, colonization, and multiplication of the organism to the nasal mucosa of infected chickens [61]. The encapsulated bacterium is virulent and produces pathological signs, while the non-encapsulated one is regarded as non-virulent [62]. Thus, the somatic antigen of non-encapsulated strains of *A. paragallinarum* is unable to adhere to the host cells [63]. The presence of the capsule may help an increase in the resistance of the bacterium against the bactericidal activity of the host.

In the same context, the similarity of the outer-membrane proteins of *A. paragallinarum* to iron regulation mechanisms of other bacterial pathogens as *Pasteurellae* is demonstrated [64].

Susceptibility of Avian Species

All types of chickens in multiage flocks could be infected with *A. paragallinarum* [65]. Although chicken is the most common host of IC, some reports have confirmed the susceptibility of other avian species, such as ornamental birds [30], Japanese quail [11,66], emu [32], and pheasant in any age [42]. The disease has not been reported in turkeys [6]. Indigenous domestic local fowls are also liable to IC [51,67,68]. Intensive layer chicken farms after 20 weeks, especially on large-scale egg production complexes, and breeding farms are more vulnerable to IC infection than younger ages [5]. Moreover, the spread of IC to successive age groups usually happens within 1-6 weeks after moving chickens from brooder houses to growing batteries close to older groups of infected birds.

Table 1. *Incidence of Avibacterium paragallinarum infection in different countries all over the world from 1991 to 2022*

Country	Reference	Animal Species	Detection and Prevalence	Antibiotic Sensitivity
	Rajurkar et al.[29]	Layer	Six isolates of *A. paragallinarum* were characterized from 109 samples of adult chickens	All the isolates were sensitive to enrofloxacin, ampicillin, and kanamycin, and 100% resistant to tetracycline and streptomycin. Two isolates were sensitive to cotrimoxazole (33%)
	Priya et al.[30]	Ornamental birds	The *A. paragallinarum* isolates were morphologically and biochemically identified from ornamental birds	The bacterium was sensitive to gentamicin, ceftriaxone, tobramycin, chloramphenicol, and nitrofurantoin, but it was resistant to neomycin, sulfadiazine, tetracycline, enrofloxacin, metronidazole, and ciprofloxacin.
	Thenmozhi and Malmarugan [31]	Japanese quail	The cultural and molecular identifications of *A. paragallinarum* isolates from 53 samples of Japanese quail revealed the presence of 8 strains with an amplicon size of 500 bp.	All strains showed 100% resistance to ampicillin, neomycin, pefloxacin, cotrimoxazole, furazolidone, streptomycin, cephalexin, and amikacin. 90% resistance to gentamycin and 70% to oxytetracycline
	Nabeel Mohammad and Sreedevi [32]	Emu	The presence of *A. paragallinarum* was confirmed by PCR. The prevalcne was 30-72% among collected samples.	
China	Guo et al.[33]	White leghorn chicken	Forty strains of *A. paragallinarum* were isolated and identified from diseased chickens during 2019 to 2020. The HI test results revealed presence of 11 isolates with serovar A, 10 with serovar B, and 19 with serovar C	High sensitivity to sulfamethoxine and oxytetracycline was detected. Out of 40 A. *paragallinarum* isolates, sulfamethoxine with concentrations of 30%, 10%, and 15% had minimum inhibitory concentration values of 64 µg/mL, 128 µg/mL, and 256 µg/mL, respectively. However, 85% of strains showed minimum inhibitory concentration values of 64 µg/mL or more for oxytetracycline. The minimum inhibitory concentration values for β-lactamase (amoxicillin, ampicillin, and ceftiofur) were low, with 77.5%, 70%, and 92.5% of strains showed minimum inhibitory concentration values of ≤1 µg/mL, respectively.
	Chen et al.[34]	Layer, broiler, and breeder	The PCR detected 14/14 of the infected chickens in a challenge trial as compared with 13/14 for culture. In addition, PCR yielded 15/39 birds and 6/8 commercial farms positive as compared with 8/39 birds and 4/8 farms positive by culture. All positive farms by PCR had chickens showing the typical signs of IC, indicating that culture failed to confirm coryza on 2 farms that had the typical signs of the disease	
	Mei et al.[10]	Layer	The existence of *A. paragallinarum* Page serovar A was confirmed from chicken cases using isolation and a species-specific PCR test. Moreover, fowl adenovirus-4 was molecularly identified from these chickens as a concurrent infection	

Table 1. *Incidence of Avibacterium paragallinarum infection in different countries all over the world from 1991 to 2022 (continued)*

Country	Reference	Animal Species	Detection and Prevalence	Antibiotic Sensitivity
Indonesia	Poernomo et al.[35]	Layer	*A. paragallinarum* were found in 24 out of 30 samples (80%) from vaccinated layers. The isolates showed tiny, circular, transparent, dewdrop-like Gram-negative coccobacilli colonies based on Gram staining. The isolates were non-motile, negative in urease, catalase, indole, and oxidase tests and were able to ferment sorbitol, lactose, mannitol, and maltose	24 *A. paragallinarum* isolates were sensitive to ampicillin and amoxicillin (100%), 91.6% of isolates were sensitive to chloramphenicol, 79.2% sensitive to enrofloxacin, 75% to Fosfomycin, and 54.2% to ciprofloxacin
	Wahyuni et al.[11]	Quail	Five out of 9 strains (55.5%) from quails were identified as NAD-independent *A. paragallinarum* using traditional isolation methods. Three out of the isolated strains were serovar B	All strains were susceptible to amoxicillin and ampicillin, but resistant to amikacin, erythromycin, gentamycin, and tetracycline. In addition, 80% of strains were resistant to kanamycin and trimethoprim, 60% to chloramphenicol, and 20% to enrofloxacin
	Tangkonda et al.[36]	Layer	Four strains of *A. paragallinarum* were isolated from 12-layer chicken using conventional identification techniques. Serotyping of strains using plate HA method revealed that 2 were serotype B and the others were serotype C	
	Fauziah et al.[37]	Layer	Out of the total 30 samples from layer chickens, 24 (80%) were biochemically identified as *A. paragallinarum*	The isolated strains were sensitive to ampicillin and amoxicillin (100%), and chloramphenicol (91.6%), but resistant to erythromycin (100%), tetracycline (87.5%), streptomycin (83.3%), doxycycline and kanamycin (70.8%), and trimethoprim (62.5%)
Thailand	Akter et al.[38]	Layer	From 21 sinus and tracheal swabs of layer chickens, 3 only were positive for *A. paragallinarum* after cultural, staining, morphological, motility, and biochemical characterizations of the bacterium	Strains were sensitive to ciprofloxacin, chloramphenicol, and gentamicin, but resistant to ampicillin, amoxicillin, oxytetracycline, erythromycin, and sulphamethoxazole
	Chukiatsiri et al.[39]	Layer	Eighteen isolates of *A. paragallinarum* were confirmed by PCR. However, 10, 5, and 3 isolates were serovar A, B, and C, respectively	All isolates were sensitive to amoxicillin-clavulanic acid, but there was a high level of resistance to lincomycin, erythromycin, cloxacillin, and neomycin. The challenge test in 4-week-old layers showed that all isolates induced typical signs of IC and could be re-isolated at 7 days post-challenge

Table 1. *Incidence of Avibacterium paragallinarum infection in different countries all over the world from 1991 to 2022 (continued)*

Country	Reference	Animal Species	Detection and Prevalence	Antibiotic Sensitivity
Korea	Han et al.[40]	Layer	In the period from 2009 to 2012, *A. paragallinarum* was detected in 7 chicken farms using PCR and they were serotyped as serovar A by multiplex PCR. The serological surveys using the HI test showed high positivity for serovar A in rates of 86.4% in 2009, 78.9% in 2010, 70.0% in 2011, and 69.6% in 2012	Isolated strains showed susceptibility to erythromycin, gentamicin, lincomycin, neomycin, oxytetracycline, spectinomycin, and tylosin.
	Jeong et al.[41]	Layer and broiler	Twenty strains of *A. paragallinarum* were identified in chickens using HPG-2 PCR assay and biochemical tests. Sixteen out of 20 strains required NAD and an enriched CO_2 for growth, while one isolate needed increased levels of NAD and serum for good growth. Three isolates showed NAD-independent growth on blood agar under aerobic conditions. Three biochemical biovars were demonstrated. The 16 typical NAD-dependent strains were serovar A, however both NAD-independent strains and that with increased NAD dependency (variants) were untypeable	All strains were sensitive to amoxicillin-clavulanic acid, ceftiofur, gentamicin, and spectinomycin, but resistant to lincomycin, cloxacillin, and erythromycin. Variants were more resistant to antibiotics than the typical NAD-dependent strains
United States of America	Crispo et al.[42]	Broiler	Both *A. paragallinarum* and infectious bronchitis virus were detected in the respiratory tract and brain of 29-day-old broiler chickens	
	Crispo et al.[43]	Broiler, layer, and backyard chickens	Fifty-four samples represented as broilers (n = 40), layers (n = 11), and backyard chickens (n = 3) were examined. *A. paragallinarum* was identified by PCR from the respiratory tract and from extra-respiratory organs. Concomitant infections with infectious bronchitis virus and infectious bursal disease virus as well as *Mycoplasma* species, *Escherichia coli*, *Ornithobacterium rhinotracheale*, and *Gallibacterium anatis* biovar *haemolytica* were reported. Thirteen A. *paragallinarum* strains were serovar C2. Isolates of *A. paragallinarum* shared a unique enterobacterial repetitive intergenic consensus PCR	Isolates showed high minimum inhibitory concentration values for tetracycline
USA (Pennsylvania)	Kuchipudi et al.[13]	Broiler, layer and pullet	Real-time PCR was applied on 419 samples from broilers, layer pullets, and laying hens. Positive *A. paragallinarum* was detected in 94 samples based on culture isolation. Moreover, the recN PCR assay with HPG-2 based real-time PCR assay showed a PCR efficiency of 79%	
Bulgaria	Giurov [44]	Layer poults	Based on culturing and biochemical reactions, *A. paragallinarum* strains were detected. Strains were pathogenic for 8-week-old birds and poults and also induced death of 4-7-day-old chick embryos within 48 h of inoculation	The disk-diffusion method showed sensitivity of isolates for streptomycin, tetracycline, chloramphenicol, gentamycin, erythromycin, spectinomycin, furazolidon, imekil, cosumix, trimetoprim, and sulfadoxin

Table 1. *Incidence of Avibacterium paragallinarum infection in different countries all over the world from 1991 to 2022 (continued)*

Country	Reference	Animal Species	Detection and Prevalence	Antibiotic Sensitivity
Argentina	Sandoval et al.[45]	Broiler and layer	Seventeen complicated outbreaks of IC in layer, broiler-breeder, and broiler chicken's flocks were detected. Layer flocks were suffered from up to 35% drop in egg production, while broilers showed mortality 2-5%. *H. paragallinarum* was isolated in all of the outbreaks either as a pure or mixed infections from the liver, kidney, tarsal joints, and ocular globes. Serovars A, B, C, and untypable serovars were isolated in 8, 6, 1, and 2 outbreaks. The severity of these outbreaks was increased by concomitant salmonellosis, pasteurellosis, and mycoplasmosis. Under certain circumstances, *H. paragallinarum* was able to cause septicemia. Moreover, 10 of the farms were vaccinated against IC before the outbreaks	
Mexico	García et al.[46]	Layer	Two strains of *H. paragallinarum* were detected in layer chickens using PCR and conventional identification methods. They were NAD-independent, serovars B-1 and C-2, and pathogenic for susceptible chickens The strains were associated with drop in egg production up to 20% over a 3 weeks and mortality ranged from 0.1% to 0.2%	
Germany	Heuvelink et al.[18]	Dutch layer	Almost all 44 field strains of *A. paragallinarum* from 25 outbreaks showed sensitivity to antimicrobial agents that are used for the treatment of IC. However, a quarter of strains with high minimal inhibitory concentration of tetracycline showed *tet* resistance genes. Of most agents, low minimum inhibitory concentration results were determined for the 9 serovars reference strains, and negative PCR results for resistance genes	
Netherlands	Feberwee et al.[3]	Layer	Eighteen NAD-independent *A. paragallinarum* isolates were identified from outbreaks of IC in layer flocks based on isolation, biochemical identification, PCR tests, and serotyping. Molecular typing by ERIC-PCR and sequence analysis of the partial HPG2 region of *A. paragallinarum* were applied. All isolates were detected by the species-specific conventional PCR, but 33% of the isolates were missed by the species-specific real-time PCR. Sequence analysis showed a probe mismatch as a result of a single nucleotide polymorphism. Sequence analysis of the partial HPG2 region was in concordance with ERIC-PCR indicating presence of 2 major genotypes. Serotyping revealed existence of serovars A-1, A-2, and B-1. The pathogenicity test of one strain of the most prevalent genotype of serovar A-1 in layer hens induced typical disease of IC.	
United Kingdom	Welchman Dde et al.[47]	Layer	Strains of *A. paragallinarum* were identified from outbreaks in mixed-age layer flock. Coinfection with *Ornithobacterium rhinotrachale* and infectious bronchitis virus was identified by real time PCR	

Table 1. *Incidence of Avibacterium paragallinarum infection in different countries all over the world from 1991 to 2022 (continued)*				
Country	**Reference**	**Animal Species**	**Detection and Prevalence**	**Antibiotic Sensitivity**
Peru	Morales-Erasto et al.[48]	Pathogen-free chickens	Severe coinfection outbreaks of *A. paragallinarum* and *Ornithobacterium rhinotracheale* were detected through isolation, PCR, and sequencing of the 16S rRNA gene	Isolated strains were sensitive to amoxicillin-clavulanic acid and florfenicol. They were resistant to oxacillin and sulfamethoxazole-trimethoprim. Chickens inoculated with both *A. paragallinarum* and *Ornithobacterium rhinotracheale* showed severe clinical manifestations compared with that inoculated with *A. paragallinarum* alone
South Africa	Horner et al.[49]	Layer, broiler, and pullet	NAD-independent *H. paragallinarum* and *H. avium* were isolated from chickens in an overall age range of 14 days to 64 weeks. The whole cell protein profiles of NAD-independent *H. paragallinarum* isolates were identified from 5 different flocks but they were differed from that of a typical isolate	
Iran	Nouri et al.[50]	Backyard chickens	From 18 collected choanal swab samples, four (22%) isolates of *Av. Paragallinarum* were detected by culture methods and confirmed by the biochemical reaction of Catalase and Oxidase tests. PCR (HPG-2) indicated 12of 18 (66%) of sampled birds were infected with *Av. Paragallinarum*. (66%) positive reactions were detected by observing expected 500 bpb and using PCR (HPG-2) on swab samples.	The isolates were resistant to amoxicillin, oxytetracycline, streptomycin, trimethoprim/ sulfamethoxazole (up to 75%) and sensitive to cefalexin, ceftriaxone, enrofloxacin, florfenicol, gentamycin, linco-spectin, neomycin, doxycycline (50%), danofloxacin (75%), flumequine (50%), ofloxacin (75%)
	Beiranvand et al.[51]	Backyard chickens	*A. paragallinarum* were isolated from 7 out of 10 samples with typical IC clinical signs. Most isolates (4/7) showed the typical requirement for nicotinamide adenine dinucleotide (NAD) and an enriched CO2 atmosphere for growth. Three of the seven strains were obtained to be novel NAD-independent under anaerobic conditions.	All isolates were sensitive to gentamicin and spectinomycin. There was greater antibiotic resistance in the three NAD-independent isolates than in normal NAD-dependent bacteria.
Iraq	Rashid and Poeiecha [52]	Layer	An outbreak of IC in a poultry farm. The morbility was 80%.	
Egypt	Ibrahim et al.[53]	Layer	*A. paragallinarium* was isolated from 162 layers and 205 broiler chicken flocks in the Upper Egypt (33% Prevalence). Serovars A, B, and C were detected	
	Badr et al.[54]	Layer, broiler	From 41 different samples, only four (9.7%) were positive. Three positive samples (7.3%) were confirmed by PCR using HPG-2. Multiplex PCR indicated that all strains were of type B. All positive samples belonged to layer chickens.	

Epidemiology

Infection of *A. paragallinarum* usually occurs via the inhalation of infectious droplets and ingestion of contaminated feed and drinking water with infective nasal exudates [27]. Horizontal transmission of the disease commonly occurs through aerosol and direct contact. Recovered chronic carriers or sub-clinically infected chickens are important sources of IC transmission [8]. *Fig. 1* shows the infection and transmission of IC in poultry farms.

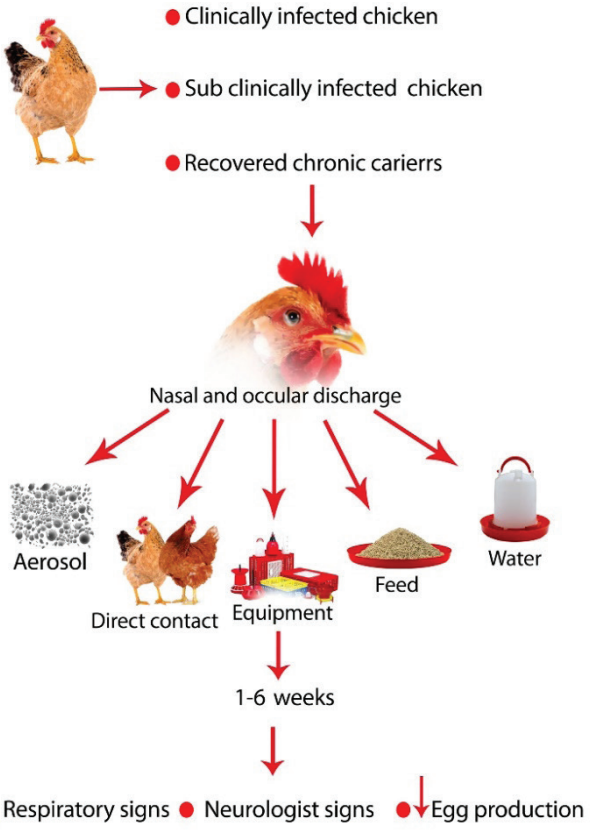

Fig 1. Transmission and infection of *Avibacterium paragallinarum* in poultry farms (The figure is designed by the corresponding author)

Clinical Signs and Pathology

The severity of clinical signs of IC depends on some factors, such as age, breed, feeding, management, parasitism, and mixed infections [69]. Besides, the clinical signs of the disease are independent of the infective serotype [39]. Infectious coryza is associated with acute respiratory distress and a decrease in egg production up to 40% in layer chickens [4]. The clinical findings are limited to the upper respiratory tract and appear as sneezing, nasal and ocular discharge, conjunctivitis, swelling of the infraorbital sinuses and wattles, and facial edema. Young chickens show decreased feed intake, reduced body weight, and diarrhea. A drop in egg production of layers and breeders may reach 10-15% and last for 6 weeks. Severe neurologic signs were also reported in chickens in California where *A. paragallinarum* infection was concomitant with the infectious bronchitis virus [42]. Subclinical form of IC infection is usually without signs but infected chickens are carriers and show intermittent shedding of the bacterium through the respiratory tract. The disease is associated with high morbidity of up to 60-80% and mortality ranges of 1-15% according to the complications with either concomitant infection or environmental stressors [70]. Infectious coriza is characterized as a rapid spread disease with a short incubation that does not last for more than 3 weeks although the duration of the infection can be prolonged and may reach 7 weeks in case of complications. The severity, duration, and mortality rate of IC may increase as a result of infections with bacteria, including *Pasteurella multocida* [45], *Ornithobacterium rhinotracheale* [48], *Gallibacterium anatis* [71], *Staphylococcus aureus* with avian influenza virus [72], *Escherichia coli* with *Proteus* [73], *Salmonellae enterica* [74], viruses, such as infectious bronchitis virus [42], infectious laryngotracheitis virus, and fowlpox virus [16,47], as well as bad environmental conditions [40].

Experimental infection of *A. paragallinarum* is usually associated with the appearance of typical upper respiratory disease signs but without mortality [71]. The bacterium adheres and colonizes the upper respiratory mucosa by both HA antigen and capsule, and then it proliferates and produces some toxic substances to induce the clinical signs. However, some studies have indicated the absence of clinical signs in the inoculated chickens which may be due to an increased level of lipid peroxidation by the epithelial surface and leucocytes in the systemic circulation [75]. The early response strategy against *A. paragallinarum* has been demonstrated in chickens through the anti-oxidant mechanism [76].

The post-mortem lesions of IC are restricted to the upper respiratory tract and reflect themselves as catarrhal to serous rhinitis, conjunctivitis, and sinusitis. However, complicated conditions result in chronic respiratory diseases, swollen head-like syndrome, airsacculitis, and septicemia, especially in broilers [16].

The microscopic observations include sloughing, disintegration, hyperplasia of mucosal and glandular epithelium, and hyperemia with infiltration of heterophil in lamina propria of the mucous membranes. Severe and complicated cases are indicative of severe subacute to chronic pyogranulomatous pneumonia, airsacculitis, pericarditis, perihepatitis, synovitis, and myositis [77]. Complicated immunosuppressant infections such as infectious bursal disease result in severe lymphoid

depletion of the bursa of Fabricius and prepare conditions for co-infections [78].

Laboratory Diagnosis

Conventional Phenotypic Characterization

Laboratory diagnosis of IC is based on conventional methods of isolation and identification of the causative agent. Swabs from nostrils, infraorbital sinuses, or trachea should be taken for the isolation process of *A. paragallinarum*. However, for the first time, Abd El-Ghany [79], and Odor et al [80] demonstrated the isolation of the bacterium from the non-respiratory organs, such as liver, kidney, and tarsus of septicemic cases. *Avibacterium paragallinarum* should be isolated during the acute stage (1-7 days) of infection to prevent complications that counteract the isolation process [81]. The bacterium grows well in brain heart infusion broth or on blood or chocolate agar containing supportive growth factors, such as 0.25% NAD or feeder organism of *Staphylococcus aureus* (V factor), hemin (X factor), and 1% chicken serum [41]. The organism grows at 37°C under microaerophilic or anaerobic conditions for 24-48 hours. Some isolates of *A. paragallinarum* are NAD-dependent, but others are NAD-independent. On blood agar plates, NAD-dependent *A. paragallinarum* isolates produce tiny dewdrop, circular, convex, smooth, non-pigmented, and non-hemolytic colonies that only grow near the feeder strain. Besides, NAD-dependent strains produce satellite growth after 24 or 48 h of inoculation, while NAD-independent strains produce no satellite growth [82]. Cultures from acute pathogenic strains of *A. paragallinarum* appear as big mucoid colonies, while those of non-pathogenic strains are much smaller colonies [83].

Avibacterium paragallinarum is a Gram-negative, non-motile, and non-spore former pleomorphic coccobacilli that appear as 1-3 µm in length and 0.4-0.8 µm in width with filament formulation. After 48-60 hours of incubation, the bacterium shows degeneration with the formation of fragments and indefinite shapes [11,41,84,85].

The biochemical identification of *A. paragallinarum* isolates has revealed negative reactions to catalase, oxidase, urease, indole, methyl-red, hydrogen sulfide, Voges-Proskauer, and gelatin liquefaction tests. The bacterium shows positive fermentation of lactose, maltose, sucrose, mannitol, glucose, fructose, and sorbitol with the production of acid, but does not ferment galactose or trehalose [36].

Traditional characterization of *A. paragallinarum* has some limitations. These limitations include isolation of the bacterium in the acute stage of infection, fastidious and slow growth nature of the bacterium, and the presence of a usual mixed overgrowth of other bacteria and faster-growing commensals [13]. The presence of NAD-independent *A. paragallinarum*, *Ornithobacterium rhinotracheale*, and NAD-independent strains of *Pasteurella* species increases the complexity of phenotypic detection of the bacterium [82].

Serological Tests

Hemagglutination Assay Test: The plate HA test using chicken antisera was used to classify *A. paragallinarum* into serovars A, B, and C according to the Page classification scheme [27]. Two of the three *A. paragallinarum* groups were subdivided into 3 serotypes each forming a total of 7 serotypes designated as HA-1 to HA-7. Thus, both Page and Kume schemes are mainly used for serotyping of *A. paragallinarum* strains [85].

Hemagglutination Inhibition Test: The HI test has been also recommended for serotyping of *A. paragallinarum* strains by Page scheme [57]. Kume scheme depended on the characterization of the isolated *A. paragallinarum* strains with specific rabbit's antisera using HI tests [56]. Yamaguchi et al.[86] demonstrated the importance of the HI test for the detection of the relation between the titer of infection and the protection level against IC. There are 3 types of HI tests. The simple HI test using whole bacterial cells of *A. paragallinarum* Page serovar A and chicken erythrocytes can detect antibodies only to serovar A [6]. The eracted HI test is based on using sonicated cells of *A. paragallinarum* and glutaraldehyde-fixed chicken erythrocytes and can detect only Page serovar C [36]. Most of the infected chickens with serovar C show a seronegative reaction [86]. Finally, the treated HI test that is based on using hyaluronidase-treated whole bacterial cells of *A. paragallinarum* and formaldehyde-fixed chicken erythrocytes is employed to detect Page serovars A, B, and C in vaccinated chickens [87]. Accordingly, the simple HI test is suitable for detecting infections or vaccinations associated with serovar A, the extracted HI test is used for vaccination associated with serovar C, while the treated HI test is good for infections and vaccination associated with all serovars. In an Egyptian study, Ibrahim et al.[53] reported 15 out of 22 *A. paragallinarum* isolates of layer chicken flocks, which showed HA against chicken erythrocytes with the presence of serotypes A, B, and C using the HI test. In Thailand, *A. paragallinarum* serovar B was detected in a layer farm using HI [85]. It could be concluded that the presence of antibodies against IC is not likely to be induced by *A. paragallinarum* infection, thus, HI may not be a reliable tool for the diagnosis of infection [88]. Page or Kume serogroups delineate three different immunovars [70]. No cross-protection among serovars has been found and the cross-protection within Page serovar B is not common [89]. There is generally good cross-protection among serotypes A1-A4. However, some of the serotypes (C1-C4) showed partial cross-protection [90]. Serovar B-1 is common in the Americas, Ecuador, Mexico, and Panama [91].

Enzyme-Linked Immuno-Sorbent Assay: Despite Enzyme-Linked Immuno-Sorbent Assay (ELISA) being specific and sensitive, it can detect antibodies only against *A. paragallinarum* Page serovars A and C. Accordingly, the monoclonal antibody-based ELISA has shown the potential for the diagnosis of IC, particularly based on Page serovar B [70].

Multiplex molecular-based serotyping is used for molecular serotyping of *A. paragallinarum* [92]. According to a recent study by Tan et al.[12], serovar A-2 contains a chimeric haemagglutinating HMTp210 gene caused by the recombination of serovar A-1 and serovar C-1 and this gene is enough to distinguish serogroups A, B, and C.

Molecular Techniques

Polymerase Chain Reaction: Polymerase Chain Reaction (PCR) techniques are a more rapid diagnostic means of *A. paragallinarum* infection, compared to conventional methods [3,46,51]. In comparison with the traditional methods, PCR is regarded as an accurate, sensitive, easy, highly sensitive, efficient, and reliable diagnostic tool for the detection of *A. paragallinarum* field isolates from any clinical samples [93]. Species-specific (HPG-2 PCR), DNA restriction endonuclease analysis, ribotyping, ERIC-PCR, real-time PCR, and 16S ribosomal RNA (rRNA) sequencing have been implemented in the rapid diagnosis of IC [90,94-96]. For example, a rapid HPG-2 PCR test is used after isolation to replace the biochemical tests and reduce the complexity and costs of other diagnostic techniques [97]. The HMTp210 gene, which encodes *A. paragallinarum* HA antigen, could be also detected using PCR [98].

A multiplex PCR is used to amplify 0.8, 1.1, and 1.6 kbp fragments for A, B, and C serovars, respectively [88]. Moreover, multiplex PCR can be used for serotyping of *A. paragallinarum* targeted HMTp210 gene [92]. This technique is employed for molecular identification of the bacterium using 16S ribosomal RNA (rRNA) sequencing [99]. Corney et al.[95] detected *A. paragallinarum* in the presence of other bacteria using a 5' Taq nuclease assay. Real time PCR is considered as the most sensitive and specific technique for the detection of the DNA repair protein gene of *A. paragallinarum* [13].

Therefore, molecular diagnostic methods can replace traditional cultural characterization methods for the epidemiological studies of IC. Furthermore, they would be much valuable for the quick and correct prevention and control measures against IC infection [99].

Prevention and Control

Biosecurity Measures

One-day-old chicks should be chosen from vaccinated breeder flocks and they should be kept away from the old flock during rearing. Depopulation of the infected or recovered flock which are reservoirs of infection is important. It has been documented that *A. paragallinarum* can survive in exudates at low temperatures for many days [84]. Accordingly, the crucial preventive measures include strict husbandry and management procedures, good cleaning and disinfection of the houses and equipment, and keeping houses vacant for 2-3 weeks before restocking [6]. Isolation of age groups of chickens on an all-in-all-out basis should be taken into consideration. According to Crispo et al.[43], high biosecurity standards and proper immunization of susceptible, multi-age flocks should always be implemented and adjusted as needed.

Vaccination

Infected chickens with Avibacterium *paragallinarum* during the growing period were protected against a drop in egg production in the laying period [100]. It has been found that IC bacterin produced in broth culture was more protective than that produced in the chicken embryo [101]. Inactivated IC bacterin is effective based on the relief of the clinical signs and the decrease in the bacterial re-isolation rate [40]. The ability of the IC vaccine to elucidate protective immunity relies on the stimulation of innate immune organs via recognition of immunostimulatory components, such as adjuvants and other intrinsic pathogen-associated molecular patterns (bacterial cell wall). The HA antigen of the bacterium [63] and the polysaccharide capsule are regarded as antigens of *A. paragallinarum* and they can induce protective immunity [102,103]. Most IC bacterins contain only a single serovar which provided complete protection against the homologous serovar or partial protection against heterologous serovar. Therefore, bacterins provided only serovar-specific immunity [104].

A bivalent bacterin containing *A. paragallinarum* Page serovars A and C [105], a trivalent bacterin containing serovars A, B, and C [105], and a tetravalent bacterin containing serovars A, B, C, and B variant [106] have been used to prevent IC infection in chicken flocks. There has been a cross-protection between *A. paragallinarum* serovars A and C. The bivalent bacterin depends on the concept that Page serovar B was not a true serovar, while serovars A and C-based bacterins provide cross-protection. Nevertheless, Page serovar B become distinct and commercial trivalent and tetravalent bacterins are available [107]. Serovars A-1, B-1, C-1, or C-2 are also used in IC bacterins [48]. Page serovars could be distinguished from each other as the antibodies for each serovar cannot protect chickens from the other serovars, but can protect against serovars of the same group. For instance, a bivalent vaccine that contains serovar A and C cannot provide protection against serovar B-1 infected chickens, while it protects chickens against serovars A-1, A-2, A-3, A-4, C-1, C-2, C-3, and C-4 infection [58].

The challenge study is still the best method to evaluate the protective efficacy of IC vaccines [108]. Aluminum hydroxide adjuvanted IC bacterin could be used in double doses with 3 weeks interval for increasing the immunity that ends 30-40 weeks post-vaccination of chickens. In another study, aluminum hydroxide gel and montanide ISA71 adjuvanted bacterins containing *Salmonella* Typhimurium and *Salmonella enteritidis* combined with *A. paragallinarum* serovars A, B, and C were prepared in Egypt [53]. Both vaccines were tested in 6-week-old layer chickens by inoculation of double doses of each vaccine 3 weeks apart. The results indicated that both bacterins were efficient in terms of induction of better immune response and higher protection rates in vaccinated chickens, as compared with non-vaccinated ones. A similar study was conducted by Akeila et al.[109] who reported that a combined bacterin against *A. paragallinarum* and *Salmonella enteritidis* could protect chickens against both bacteria and elucidated maximum antibody titers levels at week 6 post-vaccination. Furthermore, inactivated multivalent bacterins that compromise *A. paragallinarum*, infectious bronchitis virus, egg-drop syndrome' 76 viruses, and Newcastle disease virus have been developed to reduce the time and costs of vaccination [110,111].

A virulent or live attenuated strain of *A. paragallinarum* was used for the production of live IC vaccines. Although living IC vaccines showed better cross-serovar protection when compared with inactivated vaccines, genetic transmutation of live *A. paragallinarum* strains into more pathogenic serovars is possible [112]. Thus, inactivated vaccines are still widely used around the world.

The protective efficacy of the IC vaccines decreases over time due to the continuous changes in the antigenic structure of *A. paragallinarum* and the development of new biovariants. The absence of local pathogenic serotypes of the bacterium in vaccines and the presence of multiple serovars with lacking cross-protection among them results in ineffective vaccination protocols [96,106]. Ideal vaccines against IC should induce protective immunity and decrease the possibility of infection and bacterial shedding. According to Conde et al.[113], 56 chicks were subcutaneously vaccinated at the hatchery with 0.1 mL of the Hepa Inmuno NC vaccine including bacterial antigens of *A. paragallinarum* (serogroups A, B, variant B, and C). On day 31 of the mentioned study, the broilers were challenged with three serogroups A, B, or C of *A. paragallinarum*. On days 2 and 5 post-challenge, the clinical signs were recorded and the infraorbital sinuses were sampled for the presence *of A. paragallinarum*, respectively. The vaccine could reduce the number of broiler chickens with clinical signs after a challenge with serogroup B, and significantly fewer vaccinated broilers were positive for the presence of *A. paragallinarum* after challenge with serogroup C. On the other hand, no significant protection was observed when broiler chickens were challenged with *A. paragallinarum* from serogroup A necessitating the need for further cross-protection studies on vaccines to include all *A. paragallinarum* strains in a vaccine, especially a serogroup A.

Treatment

Despite the application of strict hygienic measures and using of prophylactic vaccines, outbreaks of IC are still worldwide. Vaccines provide incomplete protection against the disease. Accordingly, using antimicrobials either in feed or drinking water is a must to overcome such infection. Many antibiotics can alleviate the severity and course of IC, however, no antibiotics have a bactericidal effect on *A. paragallinarum*. The development of bacterium resistance since the presence of multidrug-resistant plasmid has been reported [84,114]. For instance, more than 75% of *A. paragallinarum* isolates were found resistant to some antibiotics in Taiwan [115]. In addition, discontinued treatment results in relapses and the development of persistent carrier birds [116]. Thus, the choice of appropriate antimicrobial against *A. paragallinarum* infection should be taken into consideration as the treatment can only reduce the severity of the clinical signs but not completely cure and eradicated the disease [14]. According to Wodegebriel et al.[117], the medicinal plant families including Solanaceae, Rutacceae, and Maliaceae have been more frequently used by farmers to prevent and treat infectious diseases of poultry, such as IC.

Effective Antibiotics

An early study by Rimler [118] demonstrated that isolates of *H. paragallinarum* were susceptible to erythromycin, chloramphenicol, gentamicin, nalidixic acid, furoxone, novobiocin, neomycin, spectinomycin, and tetracycline. However, in Taiwan, Lu et al.[119] found that *A. paragallinarum* isolates were sensitive to oxytetracycline, erythromycin, sulfamonomethoxine, sulfadimethoxine, ormetoprim, tylosin, and streptomycin. Sensitivity to tetracycline, chloramphenicol, streptomycin, gentamicin, erythromycin, and spectinomycin was also reported for 10 *A. paragallinarum* Bulgarian isolates [120], while susceptibility to erythromycin, neomycin, ampicillin, penicillin, streptomycin, and tetracycline was detected in 73% of 75 isolates [114]. Regarding the effectiveness of quinolone derivative against *A. paragallinarum* infection, isolates were sensitive to enrofloxacin [121], ofloxacin [122], and ciprofloxacin and pefloxacin [123]. There were also reports on the sensitivity of *A. paragallinarum* to a combination of sulfachloropyridazine/trimethoprim [124], and sulfamethoxazol/trimethoprim [122,125]. It should be mentioned that sulfa drug compounds may cause a decrease in egg production in layers and their overdoses may be

toxic. Streptomycin, erythromycin, sulfodimethoxine, tylosin tartrate, and spectinomycin were also used successfully [5]. Isolated Indian strains of *A. paragallinarum* showed that 28 NAD-dependent isolates were sensitive to gentamicin (50%) and enrofloxacin (40.91%), while 6 NAD-independent isolates revealed high susceptibility to gentamicin (66.67%) [126]. Rajurkar et al.[29] demonstrated that all Indian *A. paragallinarum* strains were 100% sensitive to chloramphenicol, enrofloxacin, kanamycin, and ampicillin. The antimicrobial susceptibility test of 24 *A. paragallinarum* Indonesian isolates revealed that all isolates were sensitive to ampicillin and amoxicillin (100%), and 91.6% of isolates were sensitive to chloramphenicol. The isolates were 79.2% sensitive to enrofloxacin, 75% to Fosfomycin, and 54.2% to ciprofloxacin [35]. Recently, 100% of *A. paragallinarum* isolates were susceptible to ampicillin and amoxicillin, while 91.6%, 79.2%, and 54.2% were sensitive to chloramphenicol, enrofloxacin, and ciprofloxacin, respectively [37].

On the contrary, some strains of *A. paragallinarum* showed resistance to cloxacillin, erythromycin, ampicillin, and lincomycin [127] as well as neomycin, cotrimoxazol, amikacin, and cephalexin [128].

As a result of poor vaccine protective efficacy and antibiotic resistance, stimulating the production of antimicrobial peptides is an innovative antimicrobial strategy for the prevention of IC [129]. Some pro-inflammatory cytokines such as Il1β are produced at a high level and induced β-defensins to remove *A. paragallinarum*.

Conclusion

It could be concluded that IC is an important disease of economic importance in the poultry production system. Accordingly, there is a need to conduct more studies addressing the disease epidemiology, diagnosis, prevention, and control.

Competing Interests

The authors declared that there are no competing interests.

Authors' Contributions

WAA suggested the subject of study and wrote the draft of the manuscript. DB contributed to gathering the data, designed the original image of the study, and revised the draft of the manuscript. All authors revised and approved the final version of the manuscript.

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RESEARCH ARTICLE

Development of Multiple Real-time Fluorescent PCR for Detection of *Porcine parvovirus* (PPV), *Porcine circovirus* Type 2 (PCV2) and *Haemophilus parasuis* (HPS)

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ABSTRACT

Porcine Parvovirus (PPV) and *porcine circovirus type 2* (PCV2) often have mixed infection in the process of clinical breeding, and PCV2 infection will cause immunosuppression in pigs, which is easy to stimulate or complicated with other infectious pathogens. *Haemophilus parasuis* (HPS) is a typical "opportunistic" pathogen, which often leads to mixed infection with PCV2 as a secondary pathogen. In order to establish a rapid and simultaneous detection of three pathogens of PPV, PCV2 and HPS, referring to the relevant genome sequence of GenBank, specific primers were designed according to the conserved region of *VP2* gene of PPV, *Cap* gene of PCV2 and *infB* gene of HPS, and the amplified fragments were cloned into the vector to construct plasmid standard. Using standard samples with different dilutions as templates, adjusting primer concentration, annealing temperature and other conditions, a real-time fluorescence quantitative PCR method for PPV, PCV2 and HPS triple SYBR Green I was established. Three specific Tm peaks could be generated on the same melting curve without cross-reaction with other pathogens. The minimum detection limits of this method were 153 copies/μL, 128 copies/μL and 91 copies/μL, with good specificity and repeatability, which provided technical support for rapid diagnosis of these three diseases and could be used for clinical tissue material detection.

Keywords: *Differential diagnosis, Haemophilus parasuis, Multiple RT-PCR, Porcine circovirus type 2, Porcine parvovirus*

INTRODUCTION

Porcine parvovirus (PPV) is a member of parvovirus genus of *Parvoviridae* [1]. It is the smallest single stranded linear autonomous replication virus in animal DNA virus. It is a disease causing reproductive disorder of sows and causing huge losses to the global pig industry [2]. PPV is mainly harmful to sows. The common clinical symptoms of the disease are abortion, stillbirth, mummification and other phenomena in pregnant sows, but the sows themselves have no obvious symptoms [3]. PPV is also one of the main causes of reproductive disorders in sows and post weaning multisystem failure syndrome in piglets infected with *porcine circovirus type 2* (PCV2) [4]. Once the pig farm is infected with PPV, it is difficult to eliminate it. Therefore, the main measure to control the epidemic of the disease is vaccination.

Porcine circovirus is a single negative strand circular DNA virus without capsule, belonging to *circovirus* genus of *circovirus* family, which is the smallest known animal virus [5]. *Porcine circovirus* is classified into three serotypes, PCV1, PCV2, and PCV3. The diseases caused by PCV2 infection are collectively known as *porcine circovirus* associated paraplegics. Clinical manifestations include weight loss, dyspnea, pale skin, emaciation, and jaundice [6]. In addition, PCV2 infection can lead to immunosuppression in pigs, and a variety of concurrent or secondary diseases, resulting in a large number of deaths in pigs and huge economic losses [7].

Haemophilus parasuis (HPS) belongs to *haemophilus* pasteurelaceae, which is a gram-negative *Bacillus* with capsule structure, no spores and flagella. HPS infection can cause polycellulose serositis, arthritis and meningitis

in pigs, which is also known as porcine Glasser's disease [8]. HPS can achieve a dynamic equilibrium symbiosis with the immune system in the upper respiratory tract of pigs [9]. However, when the body is stimulated by stress or mixed infection, especially when secondary infection or mixed infection occurs with immunosuppressive pathogens, it has a high morbidity and mortality [10]. With the development of intensive pig farming, the prevalence of HPS has become increasingly serious, causing huge economic losses to the pig industry [11].

Under the conditions of high density and intensive breeding, the phenomenon of mixed infection or secondary infection of multiple pathogens often occurs in pigs, which increases the difficulty of prevention and control and causes serious losses. PPV and PCV2 are common reproductive disorders in the breeding process. PCV2 infection will lead to immunosuppression, which is easy to stimulate or complicated with other infectious diseases, and HPS is often a secondary pathogen infection. It is difficult to determine which pathogen is caused by the disease only by clinical symptoms [12]. Therefore, the establishment of rapid and specific diagnostic methods is of great practical significance for the control and epidemiological investigation of these three diseases.

In clinical detection methods, the differential diagnosis of multiple viruses or bacteria is more common, but the differential diagnosis of mixed infection of viruses and bacteria is less, and has not received due attention [13]. At present, the existing diagnostic methods mainly include pathogen isolation and identification, serological detection, molecular biology detection and so on. Multiplex real-time quantitative PCR technology is a detection method that can simultaneously amplify multiple target nucleic acid fragments in the same PCR reaction system using multiple specific primers. In this study, SYBR Green dye was used to establish multiplex real-time fluorescent quantitative PCR technology that could make some specific diagnosis of these three pathogens [14].

Material and Methods

Ethical Statement

Experimentation with animals was approved by the Experimental Animal Management Methods of Xinxiang Medical University (Approval number: 201206078) and followed Henan Authority's Experimental Animal Regulations. Written informed consent was obtained from all the participants prior for the publication of this study.

Strains

PK-15 cells, PPV, PCV2, *Classical swine fever virus, Pseudorabies virus, Porcine epidemic diarrhea virus, Swine Japanese encephalitis virus, Actinobacillus pleuropneumonia, Streptococcus, Pasteurella multocida,* HPS, *Porcine reproductive* and *Respiratory syndrome virus* were kept by Henan institute of science and technology laboratory. Disease materials from Henan province animal prevention and control center gift.

Primers

According to the *NS1* gene sequence of PPV (M38367.1), *Cap* gene sequence of PCV2 (JX912914.1) and *infB* gene sequence of HPS (CP001321.1) logged in GenBank, Primer 5.0 biological software was used to design specific primers for the conserved regions of these three pathogens, and the modified primers were synthesized in Shanghai Bioengineering Co., LTD.

Standard Recombinant Plasmid Construction

According to the instructions of the DNA extraction kit, the viral and bacterial genomes were extracted respectively, and the target genes were amplified by PCR. The amplification conditions were as follows: A total of 40 cycles were carried out, including pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. Agarose gel electrophoresis was used to retrieve the target fragment, and the target gene fragment was linked with PMD-19T vector and transformed into DH5a escherichia coli susceptible cells. After enzymatic digestion, the positive plasmid was sent to Wuhan GeneCreate Biological Engineering Co., LTD for sequencing. The copy number of extracted plasmid was calculated according to the formula: copies/μL = (6.02 x 10^{23}) x (plasmid concentration ng/μL x 10^{-9})/(DNA length x 660). The plasmid concentration was measured by NanoDrop 2000 spectrophotometer.

Simplex SYBR Green I Fluorescence Quantitative PCR Assay

The positive plasmids correctly sequenced were named PMD-PPV, PMD-PCV2 and PMD-HPS, respectively, and were diluted by 10 times ratio. The standard sample of the diluted plasmids was used as the template for fluorescence quantitative PCR amplification test with a total volume of 10 μL. The specific reaction system was SYBR PreMix enzyme with 5 μL. Primer and primer 0.5 μL, template 1 μL, DEPC water 3 μL; The setup procedure of fluorescence quantitative PCR was as follows: 95°C for 5 min, 95°C for 30 s, and 72°C for 60 s, with a total of 40 cycles. Meanwhile, DEPC hydraulic negative control without template was set, and standard curves of PPV, PCV2 and HPS were drawn according to the results.

Duplex SYBR Green I Fluorescence Real-time PCR Assay

Gradient diluted standard plasmids of PPV, PCV2 and HPS were used as templates to conduct fluorescence

quantitative PCR amplification test of the 20 μL reaction system. Two standard plasmids of pathogens were used as templates in each test. The specific reaction system was as follows: SYBR PreMix enzyme was 10 μL, the upper and lower primers of the two pathogens were 0.5 μL each, the standard plasmid template of the two pathogens was 1 μL each, and DEPC water was 6 μL. The setup procedure of fluorescence quantitative PCR was as follows: 95°C for 5 min, 95°C for 30 s, and 72°C for 60 s, with a total of 40 cycles. Meanwhile, DEPC water without template was set as negative control [15].

Multiple SYBR Green I Fluorescence Real-time PCR Assay

The following ingredients were added into the 20 μL reaction system: SYBR PreMix enzyme 10 μL, PPV DNA template 1 μL, PCV2 DNA template 1 μL, HPS DNA template 1 μL, then PPV, PCV2 and HPS upstream and downstream primers were added to 0.5 μL respectively, supplemented with DEPC water, and then centrifuged and mixed. The procedure of fluorescence quantitative PCR instrument was set as follows: 95°C for 5 min, 95°C for 30 s, and 72°C for 60 s, with a total of 40 cycles, and negative control without template DEPC water was set [16].

Sensitivity, Specificity and Repeatability Analysis

DNA/CDNA 1 μL of PPV, PCV2, PRRSV, CSFV, PRV, PEDV, JEV, HPS, APP, SS and PM positive samples were used as templates, and DEPC water negative control was set up for amplification according to triple fluorescence quantitative PCR reaction system. Verify the specificity of the method.

Three standard plasmids with the same concentration gradient of PPV, PCV2 and HPS were randomly selected as templates, and the established triple SYBR Green I real-time fluorescence quantitative PCR method was used for continuous anti-proliferation for 3 times. The stability of the method was verified by analyzing CT value, Tm value and other data.

Gradient diluted PPV, PCV2 and HPS standard plasmids were used as templates for PCR amplification using the established method. The dilution concentration and CT value of fluorescence signal could not be detected by fluorescence quantitative PCR instrument, and the lower limit of sensitivity detected by the method was calculated.

Clinical Sample Testing

Thirty samples of suspected PPV, PCV2 and HPS mixed infection tissues were detected by the established triple SYBR Green I fluorescent quantitative PCR method and conventional PCR method. The detection rates of the two methods were compared and their clinical practicability was evaluated.

Results

Construction and Identification of Standard Recombinant Plasmid

The constructed standard plasmids of PPV, PCV2 and HPS were amplified by conventional PCR. The size of PPV was 352 bp, PCV2 was 168 bp and HPS was 183 bp. From *Fig. 1* that the amplified product fragment was consistent with the expected size. After enzyme digestion identification, the product was consistent with the expected band.

Establishment of Standard Curve of Simplex Fluorescence Quantitative PCR

From *Fig. 2-A*, *Fig. 3-A*, and *Fig. 4-A*, the kinetic amplification curves of the plasmids with different concentrations of the three pathogens were smooth and evenly separated, and the CT values of repeated samples at each dilution gradient were the same. With the increase of the dilution degree of the standard substance, the CT values gradually increased. By observing the Tm values of PPV, PCV2 and HPS plasmid standards with different concentration gradients when amplified, from *Fig. 2-B*, *Fig. 3-B*, and *Fig. 4-B* that the melting curves of the three pathogens all have only one melting peak, and the Tm values of PPV plasmid standards with different dilution concentrations are about 77.07°C. The Tm values of PCV2 plasmid standard were 79.83°C, and those of HPS plasmid

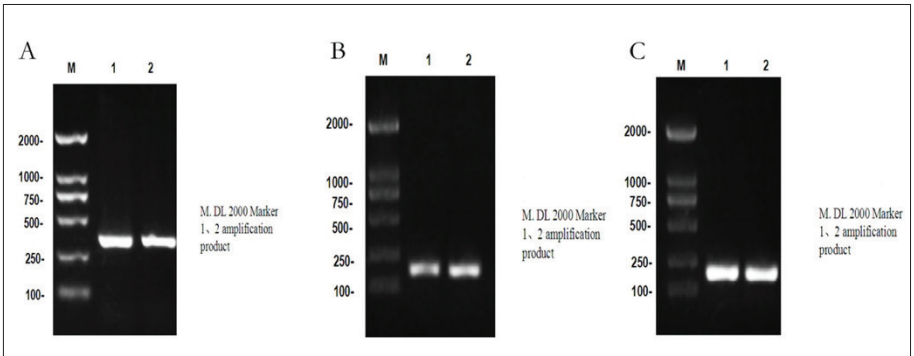

Fig 1. PCR amplification of PPV, PCV2 and HPS plasmid. (A) Amplification product of PPV plasmid; (B) Amplification product of PCV2 plasmid; (C) Amplification product of HPS plasmid

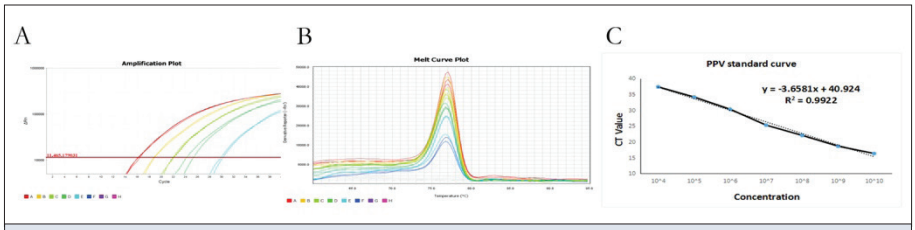

Fig 2. Simplex real time PCR assay of PPV. (A) The amplification curves of different plasmid concentrations of PPV; (B) The melting curves of different plasmid concentrations of PPV; (C) The standard curve of PPV

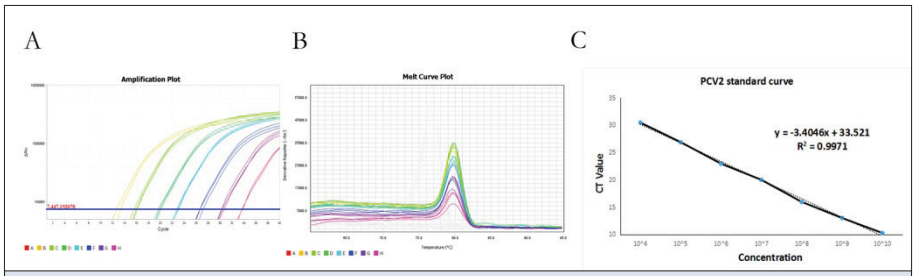

Fig 3. Simplex real time PCR assay of PCV2. (A) The amplification curves of different plasmid concentrations of PCV2; (B) The melting curves of different plasmid concentrations of PCV2; (C) The standard curve of PCV2

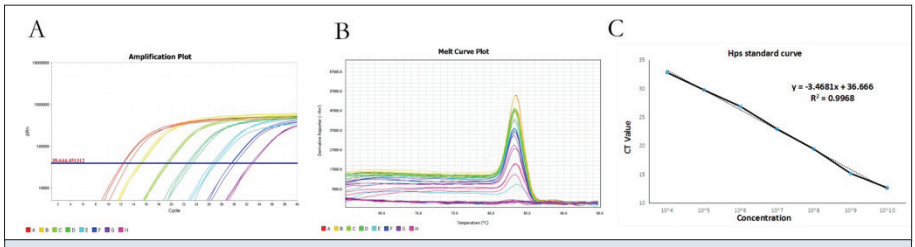

Fig 4. Simplex real time PCR assay of HPS. (A) The amplification curves of different plasmid concentrations of HPS; (B) The melting curves of different plasmid concentrations of HPS; (C) The standard curve of HPS

standard were 83.27°C. According to the correlation diagram of standard plasmid concentration and CT value, three standard curves were obtained. The equation is as follows: PPV standard curve: Y = -3.6581X + 40.924, E = 87.7%, R^2 = 0.9922; PCV2 standard curve: Y = -3.4046X + 33.521, E = 96.7%, R^2 = 0.9971; and HPS standard curve: Y = -3.4681X + 36.666, E = 94.2%, R^2 = 0.9968.

Determination of Dissolution Curve and Tm Value by Double Fluorescence Quantitative PCR

From *Fig. 5-A*, the method established in this study generates two specific peak values on a smooth melting line, namely, Tm values of PPV and PCV2. Tm values of PPV are between 77.03-77.16°C, and Tm values of PCV2 are between 79.80-80.27°C. The results were consistent with the fusion line Tm values of PPV and PCV2 single SYBR Green I real-time fluorescence PCR. From *Fig. 5-B*, two specific peaks are generated on a smooth melting curve, namely, Tm values of PPV and HPS. Tm values of PPV and HPS are between 77.03-77.15°C and 83.27-83.58°C, respectively. It was consistent with the Tm value of the fusion curve in PPV and HPS single SYBR Green I real-time fluorescence PCR detection method. From *Fig. 5-C*, a smooth melting produces two specific peak values on the line, which are respectively the Tm values of PCV2 and HPS. The Tm values of PCV2 are 79.93-80.12°C and the Tm values of HPS are 83.28-83.54°C. The results were consistent with the Tm values of the fusion curves of PCV2 and HPS single SYBR Green L real-time fluorescence PCR.

Establishment of Multiplex Fluorescence Real-time PCR Assay

From *Fig. 6-A*, the method established in this study produces three specific peaks on A smooth melting curve, namely, Tm values of PPV, PCV2 and HPS. Tm values of PPV are between 76.67-76.93°C, and Tm values of PCV2 are between 79.74-80.12°C. The Tm values of HPS ranged from 83.13°C to 83.58°C, which was consistent with the Tm values of the fusion curves of PPV, PCV2 and HPS single SYBR Green I real-time fluorescence PCR.

Specificity, Repeatability, and Sensitivity Analysis

From *Fig. 6-B*, the detection method for fluorescence

quantitative PCR amplification, in a melting curve produced three specific Tm peak, only group a specific amplification, do not cross reaction with other patients, other pathogens are not present amplification curve, judged to be negative, the detection method has good specificity.

From *Fig. 6-C*, *Table 2*, the error between each repeated test is less than one cycle, the fusion curve has a high degree of coincidence, the corresponding Tm value is relatively stable, the standard deviation is less than 0.2, and the coefficient of variation is less than 0.2%, indicating that the method has good repeatability.

The established triple fluorescence quantitative PCR method of PPV, PCV2 and HPS was used to detect the standard plasmid with gradient multiplication dilution. The lower limit of PPV detection was 153 copies/μL, the lower limit of PCV2 detection was 128 copies/μL and the lower limit of HPS detection was 91 copies/μL.

Clinical Sample Testing

Ordinary PCR and triple SYBR Green I real-time fluorescence PCR were respectively used to detect 30 samples of suspected PPV, PCV2 and HPS mixed infection tissue samples. The detection results are shown

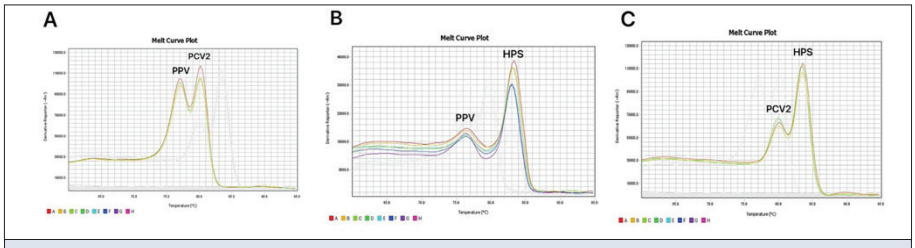

Fig 5. Double real time PCR results. (A) PPV and PCV2 double SYBR Green I real-time fluorescent quantitative PCR results; (B) PPV and HPS double SYBR Green I real-time fluorescent quantitative PCR results; (C) PCV2 and HPS double SYBR Green I real-time fluorescent quantitative PCR results

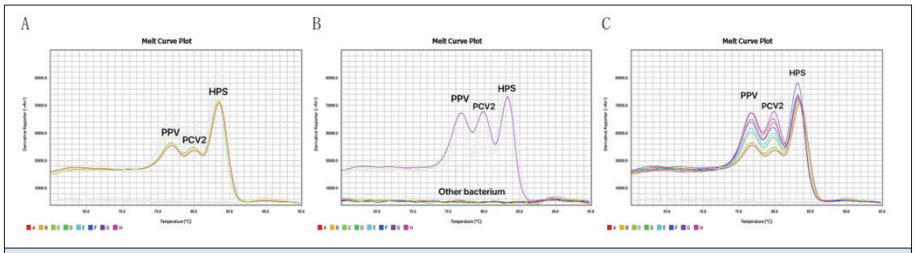

Fig 6. PPV, PCV2 and HPS multiple SYBR Green I real-time fluorescent PCR results. (A) The determination melting curve; (B) The specific test results; N = 8 (C) The repeatability detection results of different concentration gradients

Table 1. Primers used in the FQ-PCR of PPV, PCV2 and HPS gene

Gene	Primer	Sequence (5'-3')	Length/bp
PPV	Forward	GATGGCTCAAACCGGAGGAG	352
	Reverse	TGGAAAGTTCACATTGGCTGC	
PCV2	Forward	TGCCCTAACCTATGACCC	168
	Reverse	TGTAGTTTGTAGTCTCAGCCAG	
HPS	Forward	CACCCTTATCCTTTGTTGCC	183
	Reverse	CACTTTCTGAGATTCACTCCACC	

Table 2. The Tm analysis of repeatability for the multiplex SYRR Green I real time PCR intra-assay

Pathogens	The Tm/°C Values of 3 Tests			Means	S	CV(%)
	1st	2nd	3rd			
PPV	76.80	76.73	76.67	76.73	0.05	0.069
PCV2	80.12	80.05	79.99	80.05	0.05	0.066
HPS	83.58	83.43	83.43	83.48	0.14	0.169

Table 3. *Triple SYBR Green* I *real-time fluorescent PCR detection method in clinical samples*

Method	PPV	PCV2	HPS	Control
Conventional PCR method	7	9	13	0
Real-time PCR	9	17	23	0

in *Table 3*. CT values of all positive samples were between 16.24 and 35.68. No more than 38 cycles, neither method can detect negative samples. When using conventional PCR, the positive rate of PPV, PCV2 and HPS was 23%, 30% and 43% respectively. When using triple real-time fluorescence PCR, the positive detection rate of PPV was 30%, 7% higher than that of conventional PCR; the positive detection rate of PCV2 was 56.7%, 23.7 higher than that of conventional PCR: The positive rate of HPS was 76.7%, 33.7% higher than that of conventional PCR, and the method could detect all the positive samples detected by conventional PCR.

Discussion

In recent years, respiratory and reproductive diseases occur frequently in the process of pig disease prevention and control, which poses a serious threat to the development of pig industry. PPV and PCV2 are common reproductive disorders in the process of breeding. In the process of breeding, these two pathogens often have mixed infection [17]. HPS often appears as a secondary pathogen of porcine parvovirus disease, leading to secondary infection or other infectious diseases, resulting in a large number of deaths of pigs [18]. When respiratory diseases occur in pigs, it is difficult to accurately judge the pathogen only by clinical symptoms. Therefore, it is of great practical significance to establish a rapid and specific diagnosis method for disease control and epidemiological investigation.

In the process of differential diagnosis of swine diseases, the differential diagnosis between viruses or bacteria is more common, especially in the daily detection of swine diseases, the single detection and verification of pathogens is more common. However, the identification of mixed infection or secondary infection between viruses and bacteria has not been paid enough attention and ignored. In the daily detection of these pathogens, it is found that the mixed infection between viruses and bacteria is increasing day by day, especially the mixed infection between viral pathogens such as PPV and PCV2 and bacterial pathogens such as HPS is more frequent [19, 20]. Therefore, rapid and accurate diagnostic detection of these three pathogens is essential for early detection, surveillance and prevention of swine diseases.

Due to the establishment of differential diagnosis methods for viral and bacterial pathogens has not been paid enough attention, so this study established a real-time fluorescence PCR method for simultaneous detection of these three pathogens [21]. The method can not only bind the double-stranded DNA with SYBR Green fluorescent dye, but also accurately quantify the target nucleic acid. Furthermore, multiple PCR reactions for the diagnosis of multiple diseases can be performed simultaneously in the same reaction system, and differential diagnosis can be achieved by monitoring the Tm peak position of the fusion curve of the target product [22].

In this study, the sensitivity, specificity and repeatability analysis of the method showed that the lower limit of PPV detection was 148 copies/μL, the lower limit of PCV2 detection was 153 copies/μL, and the lower limit of HPS detection was 91 copies/μL. There were three specific peaks in the melting curve. The Tm values of 77.07°C, 79.96°C and 83.27°C showed good specificity and repeatability, indicating that the established SYBR Green I real-time fluorescence PCR technology could achieve the purpose of differential diagnosis of these three pathogens, and provide a more convenient and reliable monitoring means for the routine diagnosis of clinical pig mixed infection. It also provides a basis for the revision of immunization procedures for this disease.

In conclusion, through the optimization of test conditions, a triple SYBR Green I real-time fluorescent PCR method was established for simultaneous identification and diagnosis of PPV, PCV2 and Hps without cross-reaction with other pathogens, indicating that a triple real-time quantitative fluorescent PCR method for simultaneous detection of PPV, PCV2 and Hps was successfully established. To provide technical support for rapid diagnosis of mixed infection between these viruses and bacteria.

Ethical Statement

Experimentation with animals was approved by the Experimental Animal Management Methods of Xinxiang Medical University (Approval number: 201206078) and followed Henan Authority's Experimental Animal Regulations. Written informed consent was obtained from all the participants prior for the publication of this study.

Availability of Data and Materials

The data sets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Competing Interests

The authors declare no conflict of interest.

Authors' Contributions

Y. Z. performed experiments and wrote the manuscript. Y. Z., N. Y., L. W. performed experiments, Y. D. and H. X. wrote the article and conceived the experiments.

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RESEARCH ARTICLE

Fattening Performance and Some Carcass Characteristics of Hereford and Angus Steers Fed a High Roughage

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ABSTRACT

This study was carried out to compare the fattening performances, some slaughter and carcass characteristics of 10-month-old Hereford (HER) and Angus (ANG) steers fed a high roughage with the same care and feeding conditions in Türkiye. A total of 40 steers, 20 HER and 20 ANG from the same herd, were selected as homogeneously and randomly divided into two groups. The average body weights of the breeds were determined as 276.85±9.70 and 288.10±9.86 kg, respectively, and the differences between the breeds were insignificant (P>0.050). All animals were fed *ad-libitum* with two different Total Mixed Rations (TMRs) prepared with a concentrate: roughage ratio of 34:66% during the fattening. In the experiment, fattening feed containing 14.12% crude protein (CP) and 2671 kcal/kg metabolic energy (ME) for the first 300 days and fattening finisher feed containing 14.11% CP and 2717 kcal/kg ME for the last 45 days were used. At the end of the study, which lasted for a total of 345 days, the 1 kg live weight costs of the animals were calculated. Differences were insignificant between the two breeds in terms of body weights, final weights (slaughter weights), total weight gains, daily live weight gains, daily dry matter consumption, feed conversion ratios, hot and cold carcass weights, head weight to carcass ratio and longissimus muscle area (LMA) during fattening (P>0.050). Head and skin weights (P=0.000 and P=0.003) and subcutaneous fat thickness (SFT) (P=0.000) were higher in the HER breed. The profit ratio with the cost did not change. It was concluded that both breeds had similar characteristics.

Keywords: Angus, Carcass, Cattle, Fattening performance, Hereford, Roughage

INTRODUCTION

Due to the rapidly growing population of the world, people's food demands have also increased. Countries are developing different approaches and strategies in order to meet the demand for healthy and reliable animal protein, which increases in parallel with the increase in population. For this, both the number of animals and the productivity per animal need to be increased. The inability to meet this demand is due to the inadequacy of the number of animals and breeding meat breeds in Türkiye [1]. As of June 2021, the total number of cattle in Türkiye was 17.875 672 and the total number of sheep/goats was 58.447.555 [2].

Generally, in cattle breeding for fattening in Türkiye; combined productive breeds such as Simmental and Brown Swiss, and male offspring of dairy breeds such as Holstein or hybrids of these breeds are used. Since the amount of meat produced does not meet the demand, breeding and butchery beef cattle or carcass meat are imported from time to time [3]. Primarily, it is aimed to increase the number of breeding animals in both fattening and dairy cattle by importing pregnant heifers. To this end; Limousine, Charolais, Angus, Hereford and Simmental are the most preferred breeds among imported cattle breeds [4,5]. However, it is seen that there is not enough benefit from the breeders brought to Türkiye for fattening and the required breeder production is not at a sufficient level [5].

In order to increase productivity in animal production, housing high-yielding breeds that can transform this genetic potential into products, as well as high genetic capacities, in appropriate environmental conditions is the basis of economic and profitable production [6]. Animal material and feed constitute the most important cost in meat production in Türkiye. Their share in the costs is approximately 90% and varies according to the fattening period and feed efficiency values. The remaining 10%

belongs to other expenses [4]. Livestock production in Türkiye and especially in the Central Anatolian Region is mostly carried out based on high concentrate feeding due to the inefficient and inadequate pasture lands. In the feeding of animals, concentrated feed is used instead of high-quality roughage [5]. In addition, short-term fattening is mostly preferred in fattening steers. In recent years, the increase in feed raw material and concentrate feed prices has led breeders to mainly feed their animals mainly roughage. Various crossbreeding studies have been carried out on cattle breeds imported from abroad to Türkiye in the past [7,8]. Recently, the number of studies on the fattening performance, slaughter and carcass characteristics of imported beef cattle is increasing daily [4,5,9].

In this study, roughage was mainly used in the feeding of animals and the fattening period was long. At the end of the study, it was aimed to comparatively reveal the fattening performances and such as weight gain and feed efficiency values and some slaughter and carcass characteristics in the fattening steers of HER and ANG breeds.

Material and Methods

Ethical Statement

This study and all experimental procedures were carried out in accordance with ethical standards approved by Aksaray University Animal Experiments Local Ethics Committee (Approval no: 2021/8-15).

Animals and Feeds

In the study, a total of 40 male steers, 20 each of HER and ANG breeds, at an average age of 10 months, imported in 2021 to a private enterprise with an altitude of 939 m in Aksaray, were used as animal material. The fattening period of the animals started in mid-March 2021 and ended in early March 2022. The animals were kept in a quarantine barn for 21 days and foot and mouth disease and smallpox vaccines and antiparasitic applications were made. During the fattening period, these preventive vaccination and drug application procedures were repeated periodically. The steers were placed in the paddocks in two groups (20 heads each). In a semi-open free stall barn system, 10.5 m^2 area per animal and 60 cm feeder length were calculated. The steers in each group were fed at the same time, under the same environmental conditions and with the same ration. Fattening was continued for 345 days.

All animals were given two different Total Mixed Rations (TMRs) *ad-libitum*, with a roughage: concentrate ratio of 66:34%. Animals were fed with TMRs containing fattening feed (14.12% CP and 2671 kcal/kg ME) for the first 300 days and fattening finisher feed (14.11% CP and 2717 kcal/kg ME) for the last 45 days. TMR application was made twice a day, morning and evening (07:00-17:00). Crude nutrient matters analyzes of feeds were conducted according to the AOAC [10], cell wall components (ADF; acid detergent fibre and NDF; neutral detergent fibre) were determined in accordance with the detergent analysis system reported by Van Soest [11]. The rations given to the animals were formulated according to the daily nutrient requirements of the cattle [12]. The TMR-1, TMR-2 contents and chemical compositions fed to animals are presented in *Table 1*.

Table 1. *Ingredients and chemical composition of the TMRs*

Ingredients, (% in a DM basis)	**TMR-1**	**TMR-2**
Wheat straw	8	7
Alfalfa hay	8	7
Meadow hay	0	10
Alfalfa silage	7.9	0
Corn silage	17	20
Wheat silage	25	21.5
Barley grain, flaked	15	19
[1]Vitamin-mineral premix	0.1	0.5
[2]Fattening feed	19	0
[3]Fattening finisher feed	0	15
Chemical composition, % DM		
Dry matter	64.91	68.32
Crude protein	13.42	12.30
Ether extract	2.10	1.96
Crude ash	8.09	7.37
Crude cellulose	21.04	22.20
ADF	25.24	25.38
NDF	42.04	44.13
Starch	17.90	19.57
[4]ME, kcal/kg	2671.72	2745.15

[1] *Per kilogram of contains: 1.000.000 IU Vit. A, 875.000 IU Vit. D3, 550 mg Vit. E, 625 mg Vit. K3, 625 mg Vit. B1, 63.000 mg Mn, 38.000 mg Zn, 7.700 mg Fe, 7.500 mg Cu, 71 mg Co, I, 80 mg Se*
[2] *Crude protein; 14.12%, 2671 kcal/kg ME*
[3] *Crude protein; 14.11%, 2717 kcal/kg ME*
[4] *ME, metabolizable energy; was calculated according to NRC, 2001*

Fattening Performance Parameters

The animals were subjected to the process of adaptation to feed for 15 days before fattening. At the end of the adaptation period, the animals were fasted overnight and their initial weights were determined by weighing in the morning. Weighing processes were continued regularly every month during the fattening period and the average daily weight gains (ADG) of the animals were calculated individually. Each weighing were carried out before feeding in the morning.

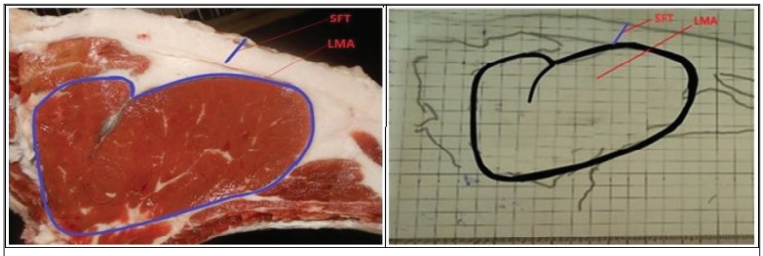

Fig 1. Drawing of LMA and SFT on tracing paper and measurement locations

The rations given to the groups in the group feeding, were mixed daily homogeneously with an uni-feed wagon pulled by a tractor in the form of TMR and poured into the feeders. The feeds given to the groups were recorded daily by weighing them on the scale of the TMR trolley. The amount of TMR consumed by the groups was determined by subtracting the amount of TMR left in front of them the next morning from the amount of TMR given to the animals. This procedure was continued three times in each month (39 replicates in total) during the fattening period. Average dry matter (DM) consumption was calculated by multiplying the consumed TMR amounts and the DM analysis results. The feed conversion ratio (FCR) was calculated by using daily DM consumption and average daily weight gain values.

$$\text{FCR} = \frac{\text{Daily DM consumption (kg)}}{\text{Average daily weight gain (kg)}}$$

At the end of the fattening, the total cost was determined from the cost elements that constituted the cost of animal material, feed, labour, veterinary-health, energy-fuel and other costs (maintenance-repair and general administration expenses). In order to determine the cost of the feed consumed per kg body weight in the groups, the feed prices in that period were taken as a basis. One kg live weight costs of animals were calculated using the ratio of total cost/total end of final weight [13].

Carcass Parameters

The animals that reached the slaughter weight after the fattening period were weighed with the electronic scale in the enterprise after 16 h of fasting, and their live weights were determined before slaughter. The slaughter process of all steers was completed at Aksaray Municipality Meat Integrated Facilities on the same day. After slaughter, some slaughter and carcass characteristics of the breeds were examined. During slaughter, the carcasses were numbered and at the end of the slaughter, the hot weights, skin and head weights of the carcasses were recorded separately. In addition, percentages of skin and head were calculated by dividing skin and head weights by hot carcass weights. Cold carcass weights were determined after the carcasses were rested for 24 h in a cold storage at +4°C. The hot and cold carcass dressing percentages (%) were determined using these data.

$$\text{Hot carcass dressing percentage (\%)} = \frac{\text{Hot carcass weight (kg)}}{\text{Slaughter weight (kg)}} \text{x } 100$$

$$\text{Cold carcass dressing percentage (\%)} = \frac{\text{Cold carcass weight (kg)}}{\text{Slaughter weight (kg)}} \text{x } 100$$

In the shredding process for the determination of some slaughtering and carcass characteristics; forequarter and hindquarter weights were recorded individually. In addition, the longissimus dorsi muscle cross-sectional areas (LMA) and subcutaneous fat thickness (SFT) of all carcasses, 12-13 were drawn on tracing paper from the intervertebral section. Calculations were then made from these drawings using a planimeter and ruler *(Fig. 1)*.

Statistical Analysis

Statistical analyses were performed using SPSS 21.0 for Windows. Fattening duration and initial weights are continuous factors that can affect fattening performance and carcass characteristics except the breed property. Since the fattening duration were equal, the initial weights were considered in the analyses. An independent sample t-test was used to compare the fattening performance, slaughter and carcass characteristics regarding the importance of this factor between breeds. Data were given as mean ± SD and $P<0.050$ was considered statistically significant.

Results

The findings for the fattening performance obtained from the groups at the end of the fattening are presented in *Table 2*. The differences observed among the groups were found to be insignificant in terms of initial and final weights, total weight gain, ADG, FCR, daily TMR, roughage, concentrate feed and total DM consumptions throughout the experiment. No difference also was observed between breeds in terms of feed costs consumed by HERs and ANGs for 1 kg of live weight cost during fattening ($P>0.050$) *(Table 2)*.

The average values of some slaughter and carcass characteristics obtained from the groups at the end of fattening are given in *Table 3*. The differences between pre-slaughter mean body weights, hot and cold carcass weights

Table 2. *The results of the fattening performance and the feed cost obtained from the animals during the experiment*			
Properties	**Hereford (n=20)**	**Angus (n=20)**	**P Values**
Fattening duration (day)	345	345	
Initial age (day)	315.500±7.013	320.900±6.463	0.575
Initial weight (kg)	276.850±9.700	288.100±9.865	0.421
Final (slaughter) weight (kg)	648.400±17.128	653.400±22.042	0.859
Total weight gain (kg)	371.550±13.726	365.300±16.115	0.769
Average daily weight gain (kg)	1.074±0.040	1.056±0.047	0.770
Daily TMR consumption (kg)	19.186±0.433	18.449±0.369	0.199
Daily DM consumption (kg)	12.854±0. 290	12.361±0.247	0.199
Feed conversion rates (FCR)*	11.968±0.270	11.704±0.234	0.462
1 kg live weight cost (₺)	14.039±0.519	13.732±0.605	0.702
** FCR: Daily DM consumption (kg)/Average daily weight gain (kg)*			

Table 3. *Results of slaughter and carcass parameters obtained from the groups at the end of fattening*			
Properties	**Hereford (n=20)**	**Angus (n=20)**	**P Values**
Slaughter age (day)	660.500±7.013	665.900±6.463	0.575
Slaughter weight, kg	648.400±17.128	653.400±22.042	0.859
Hot carcass weight, kg	376.225±11.613	378.700±12.762	0.887
Chilling loss, %	1.361±0.018	1.379±0.030	0.619
Cold carcass weight, kg	371.115±11.469	373.460±12.554	0.891
Hot carcass dressing percentage, %	57.928±0.660	57.996±0.458	0.933
Cold carcass dressing percentage, %	57.139±0.653	57.197±0.448	0.942
Half carcass weight, kg	185.557±5.734	186.730±6.277	0.891
First front quarter, kg	102.763±3.224	104.625±3.502	0.698
First back quarter, kg	82.743±2.585	82.275±2.831	0.904
Second front quarter, kg	105.238±3.229	104.870±3.579	0.940
Second back quarter, kg	80.373±2.517	81.690±2.746	0.726
Head weight, kg	16.225±0.117	15.350±0.171	0.000
Skin weight, kg	54.275±1.549	47.300±1.583	0.003
LMA, cm^2	91.025±1.024	86.375±2.020	0.057
SFT, cm	1.479±0.453	1.197±0.483	0.000
Half carcass, %	49.320±0.009	49.311±0.015	0.613
First front quarter, %	27.676±0.113	28.014±0.116	0.043
First back quarter, %	22.303±0.103	22.034±0.125	0.104
Second front quarter, %	28.354±0.114	28.068±0.129	0.104
Second back quarter, %	21.667±0.128	21.884±0.132	0.245
Head, %	4.384±0.124	4.124±0.115	0.133
Skin, %	14.450±0.095	12.493±0.023	0.000
LMA, %	24.900±0.669	23.355±0.517	0.076
LMA (%): Longissimus muscle area as calculated per 100 kg carcass (LMA / cold carcass weight x 100)			

and dressing percentages, LMAs and mean chilling loss values were insignificant (P>0.050). The mean head and skin weights (P=0.000) and SFT (P=0.000) were found to be higher in the HER breed than in ANG.

Discussion

Selection of breeds suitable for fattening and environmental conditions is very important in order to obtain high fattening performance and quality carcasses. Chambaz et al. [14] reported that the effect of breed is important in fattening, and that there are significant differences in the level of intramuscular adiposity of beef cattle of different breeds under the same conditions. Another important factor affecting the fattening period and fattening performance is the age of the animals at the start of fattening.

The growth rates of animals and feed conversion rates (FCR) differ according to their age [15]. In fact, young animals have low purchasing costs and high feed efficiency. The growth rate remains high until the animal reaches 75-80% of it adult body weight. The growth in this period is mostly in the form of protein and muscle accumulation [16]. As the age of the animal increases, the amount of feed consumed for 1 kg of live weight gain increases and therefore the profitability of the fattening decreases. Therefore, it is recommended to use male bulls in the growth period.

Fattening duration in livestock may vary depending on many factors such as the breed of the animal, age, initial fattening weight, daily live weight gain and market conditions. In our study, the differences in both the initial ages and initial weights were found to be insignificant between the breeds. In some studies using HER and/or ANG breeds to obtain high fattening performance and carcass characteristics, the starting age of fattening has been reported to be approximately 10 months, in line with our study [4,5,17]. On the contrary, there are studies reporting the age of onset of fattening to be approximately 6-9 months [1,18-22].

Fattening duration are generally kept short in the fattening of steers imported to Türkiye. It can be said that this is because the regional pasture conditions are unsuitable for stockbreeding and hence, a high rate of concentrated feed is required in the ration. In studies, there are reports that the average fattening period for HER and/or ANG steers was 5-6 months [1,5,23] or 7-10 months [4,21]. Contrary to these stated times, in this study, fattening was continued for 345 days in order to evaluate the results of traditional fattening carried out under variable market conditions.

While there are studies in which the initial fattening weights of the animals used were similar [4,17,18,24], lower [20,22] and higher [1,5] weights are also noteworthy.

Differences in ADG, FCR and carcass quality of livestock are of enormous economic importance for cattle breeders and the meat industry. Santin et al.[25], reported ADG in pasture-fed ANGs was reported (1.06 kg/day) in consistent with our study. However, there are also studies were lower [26] or higher [1,27,28] ADG values in ANG and/or HER steers were reported, largely depending on the roughage: concentrated feed ratio.

The results obtained in this study regarding feed consumption and DM intake are inconsistent with the results of some studies. According to Fidancı et al.[23] these differences are related to the amount of DM in the diet and the amount of concentrate used. Kazhgaliyev et al. [29] emphasised that feed consumption of Hereford and Angus heifers can be assessed based on their behavioural properties relation to their adaptation capabilities to environmental conditions. While the average feed consumption and DM intake were found to be similar for HER and ANG steers in some studies [27], it was observed that the DM intake obtained in our study was higher [5,28,30]. According to some studies, the lower ADG and FCRs obtained in this study can be explained by the feeding method of the animals, the ration content, the amount of concentrated feed used in the rations, and the length of time the animals remained in fattening.

The slaughter weight of the breeds that were the research material and accordingly the hot and cold carcass weights were higher than some studies with the same breeds [9,24,31] (*Table 3*). Bartoň et al.[17] emphasised that high carcass weights were associated with initial body weight, and the time that had elapsed before and during the fattening period. The carcass weights obtained in our study were consistent with the results reported by Pesonen and Huuskonen [32], and slaughter weights reported by Bureš and Bartoň [21].

Şenyüz et al.[1], Bartoň et al.[17] and Holló et al.[28] showed similar results to the present study for carcass dressing percentage. Although Kayar and İnal [9] reported similar carcass yields for ANGs, they calculated lower yields for HERs. In some studies, lower values for ANGs [22,24,31] and HERs [26,31] have been reported. Duru and Sak [4] reported higher carcass dressing percentage for HERs and ANGs (58.9% vs 58.5%). When the studies were evaluated in general, the differences between hot and cold carcass weights, hot and cold carcass dressing percentages and chilling loss values were found to be insignificant in our study, which can be explained by the similar slaughter weights of the breeds.

Kayar and İnal [9], in accordance with our study, reported that HER and ANGs are morphologically similar to each other and these similarities are reflected in most of the carcass characteristics. In addition, head and skin weight

and skin ratios were higher in HERs in our study. While these values were consistent with those reported by Pesonen and Huuskonen [32], they were higher than those reported by Duru and Sak [4]. Mazzucco et al.[31] reported lower LMA values for HER and ANGs. In our study, LMA values calculated in ANG carcasses were found to be higher than those reported by Albertí et al.[19], Holló et al.[28], Santin et al.[25], Jiu et al.[27] but close to those of Barker-Neef et al.[33] and Retallick et al.[20]. Pesonen and Huuskonen [32] also emphasised that the SFT is higher in HERs than in ANGs. Butler et al.[34] and Kayar and İnal [9] found that cold carcass weights and LMA values were similar in both breeds. When SFT values were analysed, Kayar and İnal [9] obtained higher SFT values in HE carcasses. These findings were compatible with our study in this respect. In contrast to our study, Butler et al.[34] reported that there was no difference in SFT values as well as similarity in cold carcass weights between breeds. The reasons why the LMA and SFT values obtained in our study were not compatible with those reported in previous studies may be explained by the differences in the breed characteristics, slaughter weights and carcass weights of the steers.

As a result, between applying a long-term fattening programme based on roughage in HERs and ANGs in the study and applying a short-term fattening programme based on concentrate feed reported in the previous literature; there were no significant differences in daily body weight gains, FCR and most carcass traits. Additionally, it was observed that HER and ANG breeds did not have any superiority to each other. HER and ANG steers are normally early-developing breeds that achieve weight gain in a short time. With this study, it can be said that the fattening period can be extended to a longer term by increasing the roughage ratio in the ration. Thus, breeders can keep their feed costs at an optimum level for 1 kg of live weight by feeding their animals based on roughage for a longer time, instead of feeding their animals for a shorter time with concentrated feed, and they can make more profit periodically. Furthermore, especially in times of economic crisis and uncertainty, breeders can maintain their activities and production at an optimum level in accordance with meat/feed parity. On the other hand, since the animal costs are high in livestock breeding, it is important to carry out similar studies with livestock of different breeds in terms of efficient use of resources.

Availability of Data and Materials

All data generated or analysed during this study are included in this manuscript. The data are original and available from the corresponding author (T. Kayar) on reasonable request.

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Conflict of Interest

The authors declared that there are no conflicts of interest.

Ethical Statement

This study and all experimental procedures were carried out in accordance with ethical standards approved by Aksaray University Animal Experiments Local Ethics Committee (Approval no: 2021/8-15).

Author Contributions

All authors contributed to design of the study. TK and DB conducted all experiments. TK and DB collected, analysed and interpreted the data. TK wrote the first draft of the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

Blood Feeding Preferences of Laboratory-Reared *Aedes albopictus* for Human Blood Groups and Its Effect on Their Fertility

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ABSTRACT

Female mosquitoes require both sugar and blood for feeding. They show distinct host preferences depending on behavioral, ecological, and physiological factors. Knowledge of the feeding behavior of *Aedes albopictus,* one of the primary vectors of Dengue, is critical in disease prevention measures. This study was aimed to determine the preference of *Ae. albopictus* on human blood groups (ABO) and their effects on female fecundity. Laboratory colonies of these mosquitoes were offered O, A, B, and AB blood via artificial membrane feeders, and blood meal preferences were identified using multiplex allele-specific PCR. Fertility was assessed by the mean number of eggs laid. Results showed that *Ae. albopictus* species significantly preferred the O blood group compared to others and blood type choice did not affect the fertility of the mosquitoes. To our knowledge, this is the first study assessing blood feeding choice of *Ae. albopictus* mosquitoes directly using human blood, hereby reducing the effects of factors such as odor when using human participants. The results of this study provide a new perspective on the still partially unknown ABO blood group host selection of mosquitoes, promoting the personal protection of individuals in at-risk populations.

Keywords: ABO Blood Groups, *Aedes albopictus*, Feeding preference, Multiplex allele-specific PCR

INTRODUCTION

Mosquitoes are a significant threat to public health as a result of their inclination to bite people for blood, and their role in the transmission of diseases. *Aedes*, *Anopheles,* and *Culex* genera contain the most important species that vector viral and protozoal pathogens of several human and animal diseases, like Dengue, Yellow fever, Zika, Chikungunya, and Malaria [1-3]. Such widespread infections have been global health problems for years; this is directly associated with upsurges in international trade and travel, the distribution of vector mosquitoes, and ecological changes affecting these organisms [4,5]. *Aedes albopictus* (Asian tiger mosquito), in particular, is found in many sections of the world such as Southeast Asia, Africa the United States, and Europe [6-8], where it transmits Dengue, Chikungunya, and Zika, which are viral infections that affect nearly half a billion people annually [9-11]. In Türkiye *Ae. albopictus* is present in Thrace, Aegean, Central Anatolia, Marmara, and Mediterranean Regions [12-15]. This invasive species has the potential to further spread to other regions of Türkiye; this might be a huge public health problem as Türkiye receives many immigrants from war-torn areas and the spread of *Aedes*-transmitted diseases will significantly affect more unprotected people [15]. Therefore, countries infested by these mosquitoes should initiate vector and pathogen surveillance and control to hinder any potential epidemics [16,17].

Female mosquitos need blood for oogenesis [18], hence understanding blood meal preferences of harmful species is important in assessing disease risk. Quality and quantity of a blood meal can impact oviposition and consequently affect vector competence and possible population dynamics [19]. For instance, more eggs are oviposited by *Ae. albopictus* after feeding from human blood than on animal blood [20]. Elucidating mosquito attraction to human hosts might help in ascertaining the role of individual mosquito species in diseases epidemics and provide information vital to mosquito surveillance and control [21]. Female mosquitoes locate host using physical, visual, and chemical cues [22] hence they have different levels of attraction to different humans [23] which some studies have linked to genetic factors such as odor, ABO blood group system, and onsite host availability [21].

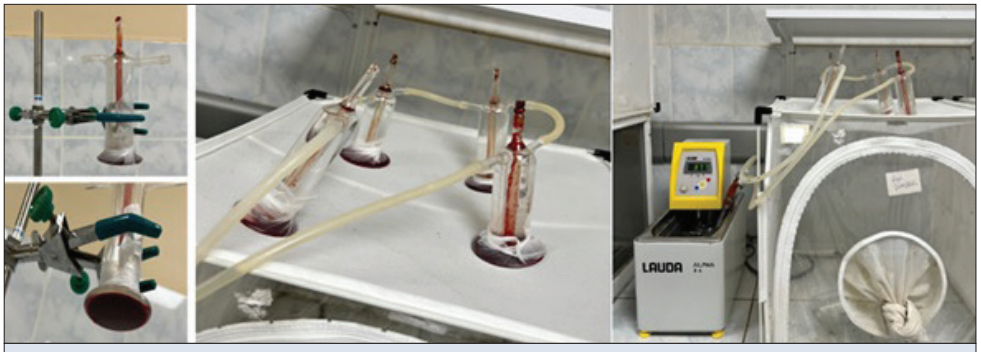

Fig 1. Experimental setup

Studies have been conducted in many parts of the world in regards to mosquito blood preference at feeding time as well as the relationship between different mosquito species and the forms of pathogen transmission [24,25]. Awareness on blood group choice and their effect on fertility is fundamental in developing personal protection and vector control in Türkiye. So, the current study determined which human blood type was preferred by *Ae. albopictus* females and the impact of the blood type on their fertility. Elucidating the effects of different human blood groups on such insects enables better recognition of the behavior of hematophagous arthropods that transmit important vector-borne diseases.

Material and Methods

Ethical Statement

This study was approved by the Aydin Adnan Menderes University Human Ethical Committee (Approval no: 2015-714).

Maintenance of Mosquito Species

Aedes albopictus larvae were collected from Aydın and reared to adult stage in the Vector Control Laboratory, Aydin Adnan Menderes University, under laboratory conditions of 25±2°C temperature and 70±10% relative humidity; adults were obtained from these larvae and reared in the insectarium. Females were fed through a membrane feeding system containing human blood and allowed to lay eggs. Mosquitoes laid eggs in plastic containers filled with water and sides lined with filter papers after about 5 days after blood feeding. The eggs were hatched in plastic trays (size 33-24-8 cm) with distilled water. Groundfish food (Tetramin) was added to each tray as feed for larvae. Pupae were transferred to 50 mL disposable plastic containers filled with distilled water and allow developing to adults. Hundred mosquitoes (50 females: 50 males) were separately removed to six insect cages (50 x 50 x 50 cm) using a mouth aspirator and were given cotton balls drenched with 10% sucrose which were removed from the cages for 12 h prior to blood feeding.

Blood Feeding Choice of Mosquitoes

Fifty newly emerged female mosquitoes (5-7 days old) were transferred to new insect cages (50 x 50 x 50 cm) and starved for 12 h [26]. Commercial human blood in EDTA tubes was obtained from the Blood Bank, Aydin Adnan Menderes University Hospital. Approximately 50 mL of blood was taken under appropriate ethical conditions, transported appropriately, and stored in the refrigerator until use in the experiment and used in the experiment within 2 days.

The females were provided with four blood feeding systems which have blood from different groups in the same time. Feeding experiments using these blood types was conducted with an artificial mosquito membrane feeding system [27,28]. The glass feeding system is made up of an inner chamber which had blood and an outer chamber that had circulating water from a water bath system at 37°C. Parafilm was stretched over the undersides of four feeders and filled with 10 mL of human blood (types O, A, B, and AB) for blood-feeding. The blood in the feeders were warmed up for 30 min before the experiments and mosquitoes were allowed to feed for an hour. All artificial blood feeding procedures were performed in replicates of six on different days *(Fig. 1)*. After the feeding, blood-fed female mosquitoes were collected from the cages with an aspirator, labeled, frozen 4°C and kept until molecular analysis.

Determination of Blood Feed Type

Genomic DNA of 245 blood-fed mosquitoes was isolated to determine the ABO blood groups in mosquito abdomen using the Invitrogen PureLink Genomic DNA isolation kit by following manufacturer's instructions. Unfed *Ae. albopictus* female was used as negative control and identified human ABO blood groups served as positive controls. The amount of isolated DNAs (ng/µL) was measured in the Nanodrop (Thermo Scientific™ NanoDrop™ 2000/2000c Spectrophotometers). Total DNAs obtained from the samples were stored at -20°C until PCR was performed. All extracted DNA was checked to verify human DNA by PCR amplification with human-specific

Table 1. *Primers used in this study to detect blood type in mosquito abdomen*

Target Gene	PCR Reaction	Primer Names and Sequences	Fragment Size (bp)	Allele/Genus/Species Specificity
ABO	1	261G: 5′-CAGTAGGAAGGATGTCCTCGTGtTG-3′ int6: 5′-AGACCTCAATGTCCACAGTCACTCG-3′	205	A101, A102, B101, cis-AB01
		467C: 5′-CCACTACTATGTCTTCACCGACCAtCC-3′ 803G: 5′-CACCGACCCCCCGAAGAtCC-3′	385	A101, O01, O02
	2	297A: 5′-CCATTGTCTGGGAGGGCcCA-3′ int6: 5′-AGACCTCAATGTCCACAGTCACTCG-3′	164	A101, A102, O01, cis-AB01
		467C: 5′-CCACTACTATGTCTTCACCGACCAtCC-3′ 803C: 5′-CACCGACCCCCCGAAGAtCG-3′	381	B101
	3	261A: 5′-GCAGTAGGAAGGATGTCCTCGTGtTA-3′ int6: 5′-AGACCTCAATGTCCACAGTCACTCG-3′	205	001, 002
		467T: 5′-CCACTACTATGTCTTCACCGACCAtCT-3′ 803G: 5′-CACCGACCCCCCGAAGAtCC-3′	381	A102
	4	297G: 5′-CCATTGTCTGGGAGGGCcCG-3′ int6: 5′-AGACCTCAATGTCCACAGTCACTCG-3′	164	B101,002
		467T: 5′-CCACTACTATGTCTTCACCGACCAtCT-3′ 803C: 5′-CACCGACCCCCCGAAGAtCG-3′	381	cis- AB01

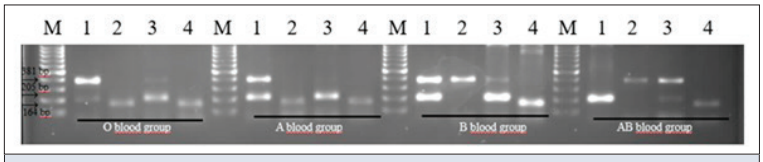

Fig 2. Agarose gel image of selected ABO blood groups amplified by multiplex ASPCR. Lane M, size marker (100-1000 bp ladder); lane 1-4 PCR reaction

primers: Human 741F, UNREV 1025 [29]. A 334 bp PCR fragment indicated human blood. Then the genomic DNA was verified for ABO blood groups by multiplex allele-specific PCR (ASPCR) [30]. Ten different primers were used in a four-reaction multiplex ASPCR genotyping assay to determine the differences in the specific nucleotide sequence between the ABO alleles - A101, A102, B101, 001, 002, and cis- AB01 *(Table 1)*. PCR mixture had 2× Taq DNA Polymerase Master mix, 20 pmol of primers, and 15 ng of template DNA in 25 µL and PCR settings are as follows: denaturation for 5 min at 95°C, 35 cycles of 1 min at 95°C, annealing for 1 min at 58°C, elongation for 1 min at 72°C and a final extension of 7 min at 72°C. The products were run on a 1% agarose gel and visualized under UV light inVilbert lourmat transilluminator.

Effects of Blood Groups on *Aedes albopictus* Female Fecundity

Newly emerged adult mosquitoes were transferred to four cages. Each cage housed 30 males and 30 females. Four artificial membrane feeding systems as described above were used and each was filled with different blood types. Mosquitoes in the cages were allowed to feed on blood for one hour. Afterward, fully engorged females were moved to paper cups (one female/cup) which held water and had filter papers on the sides for oviposition. The total number of eggs laid per individual was collected seven days post-blood-feeding and counted under a stereomicroscope (Leica GZ4 Stereo Zoom Microscope). Feeding success was ascertained by recording the numbers of fully engorged female mosquitoes and calculating the percentage of fed mosquitoes. The study was replicated three times.

Statistical Analysis

Analysis of data was in Minitab 14 and comparison of means of the number of blood fed females and the number of eggs laid by each female mosquitoes was carried out using ANOVA with Tukey's test ($P<0.05$).

Results

Preferences of *Aedes albopictus* from ABO Blood Sources

Out of the 300 mosquitoes used in the experiment, it was determined that 245 females blood-fed according to PCR amplification of human DNA. Agarose gel images of some selected samples are given *(Fig. 2)*.

In the ABO genotyping PCR, the number of mosquitoes that preferred the blood groups are O (n=93), A (n=51), B (n=66), and AB (n=35). The most fed on blood group was O (37.9%). Statistical analysis showed a significant preference in the selection of the blood groups ($F=18.385$; $df=3.15$ $P<0.01$) *(Table 2)*.

Table 2. *Number of blood-fed mosquitoes detected by PCR analysis and mean number of eggs laid per female*

Blood Group	Number of Blood-fed Mosquitoes	Mean (%) no. of Blood-fed Mosquitoes	Mean no. of Eggs Laid/Female
O	93	37.96	55.72±1.08
A	51	26.94	55.01±0.95
B	66	20.82	55.27±0.95
AB	35	14.29	55.72±0.95
Total	245	F=18.385; df=3.15; P<0.01	F= 0.128; df=3.232; P=0.943

Effect of Blood Sources on *Aedes albopictus* Fecundity

In total 60 mosquitoes were selected from 4 different cages fed from each blood group. On average 55 eggs were laid per female after blood feeding and no statistical difference was observed in the number of eggs laid after blood feeding from different blood groups (F=0.128; df=3,232; P=0.943).

Discussion

This study demonstrated that laboratory reared *Ae. albopictus* mosquitoes preferred O blood group compared to the other blood groups in artificial membrane feeding and that the different blood types did not affect fertility of the mosquito. The ecology and behavior of this and other mosquito species has been studied in the past to better understand disease transmission and control. It has been shown that blood feeding behavior varies and can be quite complex with different species displaying varying degrees of pattern, preference and specificity on a wide range of invertebrate and vertebrate hosts, and even preference variation between individuals and populations of a single host [38,39]. Several studies have assessed the blood feeding and host finding behavior of several important mosquito species [21,24,25,31,40-47] but few have focused on the effects of blood type preference on mosquito choice. Shirai et al.[25] determined the landing preference of *Ae. albopictus* females on forearms of human with different blood types. They showed that *Ae. albopictus* landed more on the forearm of volunteers with O blood group than B, A, and AB blood groups. In another study Prasadini et al.[31] reported that *Ae. aegypti* also displayed a significant preference for O blood when offered different blood groups. The authors suggested that blood meal choice differs with the availability of hosts and the time. Takken & Verhulst [21] hypothesized that blood type influences mosquito host choice. At first these researchers suggested that the disaccharide group (antigen H) on red cell membranes of O group was responsible for mosquito attraction. But data collected after their experiment did not explain the preference for O-type blood hence there may be other unknown influences underlying blood preferences. Based on these studies it seems blood typing has no clear effects on mosquito preference due to the interference of factors such as odor when using human participants instead of blood in in vitro tests. Hence other studies like the present studies and Prasadini et al.[31] are recommended where only the individual blood types are used.

Some common hypotheses have been put forth regarding blood preference of mosquitoes. Blood choice may mostly depend on what is available and accessibility to feed from. *Ae. albopictus* species originated from Asia, where there is a higher prevalence of A-type blood in the population, than O-type blood [21-25]. Tursen et al.[34] suggested that even though mosquito eyesight and color perception, allows them to successfully explore their surroundings, they are unlikely to play a role in host preference and choice.

ABO blood groups contain different von Willerbrand factors (VWFs), which are glycoproteins in the blood plasma associated with blood hemostasis and are responsible for blood coagulation [35]. People with O blood group possess 20-30% less VWF levels than other blood group types [36]. In addition VWF molecules of blood group type O have a short survival rate and cleared faster than other molecules linked with the other three blood groups [37].

Blood feeding behavior of female mosquitos is directly connected to reproductive activities. It has been reported that serum proteins are important blood components linked to oviposition. After taking a blood meal, serum proteins are broken down into amino acids like isoleucine in the digestive tract that have a huge effect on the vitellogenesis process of mosquitoes [26]. Hence, this study reasoned that besides oviposition, the result from this study will help in understanding the behavioral patterns of *Ae. albopictus* and provide additional information to improve vector control programs.

Since blood group O individuals are more likely to be bitten by vectors, they are more likely to be infected with *Ae. albopictus*-transmitted diseases. Similarly, other mosquito species like *Ae. aegypti* [32] and *Anopheles gambiae* [24] show a significant preference for O blood group. This might be a concern about personal protection measures effectively to prevent mosquito bites. Identification of the

connection between mosquito physiology or behavior and blood group preference can be useful for national vector control programs conducting vector control to effectively to control *Aedes*-transmitted diseases in Türkiye. Also, the link between dengue incidences and ABO blood group distribution in the humans is vital to infer the relationship of asymptomatic and symptomatic dengue infections in different individuals. On the contrary, Khan et al.[33] showed that *An. stephensi* displayed a strong preference for individuals with B blood group when on the search for hosts. An *in vivo* study Anjomruz et al.[24] demonstrated that *An. stephensi* species however prefers AB blood type. It was then suggested that mosquito behavior is mediated by semiochemicals and that possibly the first females who came into contact with AB-type blood individuals produced an aggregation pheromone that attracted other mosquitoes to that same host.

Individuals with blood groups more preferred by vectors are more prone to be bitten and should be protected more than others if vectors have specific ABO host choice. The most preferred blood type of *Ae. albopictus* is O according to the current analysis. The fecundity of these mosquitoes was not affected by the blood type. The existence of different factors that influence mosquito human blood preferences is a fact, however, this topic still needs more conclusive data that could be used to prevent diseases transmitted by these vectors. Also, if more studies are carried out on the blood meal preference of other mosquito species, personal protection will be easier. Since personal protection is one of the effective control measures to prevent the diseases that this species is vector, it will be very important to choose the blood group of female mosquitoes in personal protection measures against mosquito bites.

Availability of Data and Materials

All data sets collected and analyzed during the current study are available from the corresponding author (F. B.) on reasonable request.

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Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

F. B. and F. M. S. designed and planned the work. F. B. drafted the experiment and analyzed data. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

Comparison Effects of Pre-emptive Gabapentin and Meloxicam for Postoperative Pain in White New Zealand Rabbits Undergoing Ovariohysterectomy Using the Grimace Scale

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ABSTRACT

The study aimed to evaluate the effectiveness of Gabapentin in controlling postoperative pain in New Zealand white rabbits after ovariohysterectomy. Twenty sexually mature and healthy female rabbits were divided into four groups: a negative control, Meloxicam treatment, Gabapentin treatment, and Meloxicam plus Gabapentin treatment. After the surgery, the rabbits' pain levels were assessed using the Grimace Scale at various time points. The Gabapentin group consistently had the lowest Grimace Scale scores. Statistical analysis showed significant differences between the Meloxicam group and the negative control, the Gabapentin plus Meloxicam group and the negative control, the Gabapentin group and the negative control, and the Gabapentin group and the Gabapentin plus Meloxicam group. Postoperative analgesia was significantly better in the Meloxicam, Gabapentin, or combined treatment groups compared to the negative control. Gabapentin was found to be equally effective as Meloxicam in controlling pain. However, the combination of Meloxicam and Gabapentin was not as effective as Gabapentin alone. In conclusion, Gabapentin showed preventive efficacy in controlling postoperative pain after ovariohysterectomy in New Zealand white rabbits. These findings suggest that Gabapentin could be a valuable analgesic option for surgeons to provide adequate pain control in this surgical context. Further research is needed to explore optimal dosing and potential synergistic effects when combining Gabapentin with other analgesics.

Keywords: Postoperative Pain, Grimace scale, Gabapentin, Meloxicam, Ovariohysterectomy, Rabbit

INTRODUCTION

Surgical interventions are linked to central and peripheral sensitization [1]. Postoperative pain may prolong recovery time, hospital stay, and time to mobilization for patients recovering from surgery and anesthesia. In postoperative care, preventing and treating postoperative pain and its consequences, including nausea and vomiting, remains a significant concern. Opioids are routinely used to treat pain, but they have a number of adverse side effects that limit their use. A multimodal approach has been proposed to improve postoperative analgesia and prevent opioid-related adverse effects. An important area of acute pain research involves testing novel analgesics and combinations of analgesics in an attempt to decrease the need for opioids [2,3].

Gabapentin is a structural analog to aminobutyric acid (GABA), a medication that was first used as an anti-epileptic drug. It is a well tolerable anticonvulsant drug with limited side effects and drug interactions. It binds to the voltage-gated calcium channel's 2-protein subunit, which is found throughout the central (CNS) and peripheral (PNS) parts of the nervous system. This modulates excitatory neurotransmitters, such as glutamate, release and suppresses calcium influx in pain pathways [4]. Gabapentin also, promotes amino acid release in the dorsal horn of the spinal cord and reduces reactivity to neural stimuli, thus lowering or stabilizing the activity of injured nerves [5]. There are also some other possible pathways for Gabapentin, including recruiting the descending noradrenergic system [6], activating potassium channels [7], and inhibiting α2δ-1-NMDAR complexes [8]. Gabapentin can thus be used to treat chronic pain diseases such as fibromyalgia [9], diabetic neuropathy [10], postherpetic neuralgia [11], and other neuropathic conditions [12]. Gabapentin's analgesic effects

have been extensively studied in surgical settings in recent years. According to the findings of these investigations, Gabapentin has analgesic qualities in the treatment of postoperative pain [13-15].

Meloxicam, an NSAID, was originally approved for oral use in the United States in the late 20th century. Meloxicam is a member of the oxicam family of compounds that suppresses cyclooxygenase-2 more than cyclooxygenase-1, resulting in fewer gastrointestinal side effects and no interference with platelet function as compared to non-selective NSAIDs [16,17]. Its efficacy and safety have been tested in several randomized controlled studies (RCTs) after procedures such as abdominal hysterectomy, abdominoplasty, dental surgery, and other major operations [18].

We aimed to assess the preemptive efficacy of Gabapentin in controlling postoperative pain in New Zealand White Rabbits after hysterectomy and ovariohysterectomy.

Material and Methods

Ethical Statement

All procedures were carried out with the approval of the Ethical Committee of Islamic Azad University - Karaj Branch (Approval ID: IR.IAU.K.REC.1400.005).

Animals

Twenty adult female white New Zealand rabbits (Razi Institute, Karaj, Iran) entered this study. They were housed individually in suspended cages (970 × 895 × 1718 mm) and acclimatized for two weeks before the start of the experiment. There was no physical contact between the rabbits. Animals were kept on a 12:12 light-dark cycle (lights on at 06:30 AM), the room temperature was between 19-21°C, and humidity was 45±10%. Each rabbit had *ad libitum* access to water and standard rabbit food (Pellet diet, Razi Institute, Karaj, Iran). The protocol used for anesthesia in all cases included: an intramuscular injection of 35 mg/kg 10% ketamine HCl plus 5 mg/kg Xylazine HCl (K-X protocol).

Study Groups

A total of twenty white New Zealand rabbits were included into the study which were divided into four groups and each group consist of five rabbits. The rabbits were randomly assigned in one of four groups seven days before surgery. The first group, the negative control group (NG), received anesthesia according to the K-X protocol. They received no other drugs and underwent surgery. The second group received K-X anesthesia and subcutaneous Meloxicam 2% at 0.5 mg/kg one hour before surgery as a positive control group (MG). The third group received K-X anesthesia and oral Gabapentin every 12 h for five days before surgery at 10 mg/kg (GG). The last group received Meloxicam 2% (0.5 mg/kg) one hour before surgery in addition to the protocol of the third group (GMG).

Surgical Procedure

Animals were placed in a supine position, hair was removed from the area, and a median laparotomy was performed under aseptic conditions. A routine ovariohysterectomy was conducted on each animal using a No. 15 scalpel blade and a ventral midline abdominal incision that began approximately 2 cm caudal to the umbilicus. Once the uterus had been located, the fat around each ovarian pedicle was meticulously removed to allow the ovarian vessels to be identified. The broad ligament was then perforated and accessed, and the suspensory ligament was severed. The uterine horn was then made fully accessible, an incision was made in the broad ligament, and a 3-0 absorbable suture was tied around the blood vessels of the ovary and uterine horn. Uterine vessels were sutured and removed after both uterus horns were clamped and dragged out of the abdominal cavity. After ligation, each horn was removed cranial to the cervix. In the same way, the other horn was cut, and then both horns were taken and pulled slightly outwards. They were tied in order to ligate the uterine arteries located in the body of the uterus. The white line was closed with bites in a simple interrupted pattern by the use of 3-0 absorbable suture. For skin closure, an intradermal suture pattern with a 3-0 non-absorbable silk was used.

Postoperative Measurements

Finally, the surgical site was cleaned with sterile gauze and serum. The antibiotic Enrofloxacin was used prophylactically. The pain was measured using the evaluation of the rabbit grimace scale [19]. Five characteristics of rabbits, including orbital tightening, flattening of the cheeks, shape of the nostril, whisker shape and position, and ear shape and position, were considered observational indicators and were examined at eight-time points after the surgery by scoring from zero to two. The average of these scores for each rabbit was then calculated as the Grimace Scale Score (GSC).

Statistical Analysis

We used SPSS version 23 for data analysis. Numerical data was expressed as means and standard deviations. To see if a variable is normally distributed, we utilized the Shapiro-Wilk test. The equality of variances for a variable calculated for two or more groups was assessed using Levene's test. In a repeated measures ANOVA, Mauchly's test of sphericity was employed to see if the assumption of sphericity was met. If the sphericity was accepted, we directly used Pillai's trace, Wilks' Lambda, Hotelling trace, and Roy's Largest Root tests. If not, we applied Greenhouse-Geisser, Huynh-

Feldt, and Lower Bound corrections. P≤0.05 considered significant.

Results

The preoperative weight of rabbits in NG, MG, GG, and GMG was 2.33±0.06, 2.29±0.28, 2.34±0.10, and 2.31±0.17, respectively.

In all analyses, Levene's test confirmed equality of variances and the Shapiro-Wilk test confirmed normal distribution of data.

Orbital Tightening

Table 1 shows the mean and SD of orbital tightening at different time points. Mauchly's test approved the sphericity

Table 1. *Mean and SD of orbital tightening, nostril shape, cheek flattening, ear shape and position, whisker shape and position, and grimace scale score in each study groups*

Groups	After Anesthesia	Few Hours After Surgery	Night of Surgery	One Day After Surgery	Two Days After Surgery	Three Days After Surgery	Four Days After Surgery	Five Days After Surgery
Orbital Tightening								
Negative control	1.40±0.54	1.00±0.00	0.80±0.44	0	0	0	0	0
Meloxicam	1.00±0.00	0.60±0.54	0.20±0.44	0	0	0	0	0
Gabapentin	0.60±0.54	0.60±0.54	0.20±0.44	0	0	0	0	0
Meloxicam + Gabapentin	1.10±0.22	0.80±0.57	0.20±0.27	0	0	0	0	0
Nostril Shape								
Negative control	2.00±0.00	1.60±0.54	1.00±0.00	1.60±0.54	1.40±0.54	1.20±0.57	0.80±0.27	0.40±0.54
Meloxicam	1.00±0.70	0.60±0.54	0.80±0.44	1.10±0.22	1.10±0.41	0.20±0.44	0.30±0.44	0.10±0.22
Gabapentin	1.00±0.70	0.60±0.54	0.60±0.54	1.00±0.35	0.80±0.27	0.60±0.65	0.30±0.44	0
Meloxicam + Gabapentin	1.60±0.54	1.60±0.54	0.80±0.44	1.30±0.44	1.20±0.44	1.00±0.00	0.80±0.27	0.10±0.22
Cheek Flattening								
Negative control	1.20±1.20	1.00±1.00	1.20±1.20	0.80±0.80	1.00±1.00	0.80±0.80	0.70±0.70	0
Meloxicam	0.40±0.54	0.40±0.54	0.60±0.54	0.50±0.35	0.50±0.00	0.50±0.35	0.30±0.44	0
Gabapentin	0.60±0.54	0.60±0.54	0.40±0.54	0.20±0.44	0	0	0	0
Meloxicam + Gabapentin	1.00±0.00	0.60±0.54	1.00±0.70	0.60±0.54	0.50±0.61	0	0	0
Ear Shape and Position								
Negative control	2.00±0.00	1.40±0.54	0.80±0.44	0.70±0.44	0	0	0	0
Meloxicam	1.40±0.54	0.80±0.27	0.20±0.27	0.20±0.27	0	0	0	0
Gabapentin	1.40±0.30	0.20±0.20	0.20±0.20	0.20±0.07	0	0	0	0
Meloxicam + Gabapentin	2.00±0.00	1.00±0.00	0.50±0.35	0.20±0.27	0	0	0	0
Whisker Shape and Position								
Negative control	2.00±0.00	2.00±0.00	1.00±0.70	1.00±0.00	0.90±0.22	0.40±0.41	0	0
Meloxicam	2.00±0.00	1.60±0.54	0.60±0.54	0.50±0.35	0.30±0.44	0	0	0
Gabapentin	1.60±0.54	0.90±0.22	0.40±0.54	0.30±0.44	0.10±0.22	0	0	0
Meloxicam + Gabapentin	2.00±0.00	1.60±0.54	0.70±0.44	0.70±0.44	0.40±0.54	0	0	0
Grimace Scale Score								
Negative control	1.72±0.10	1.40±0.28	0.96±0.21	0.82±0.13	0.66±0.15	0.48±0.19	0.30±0.10	0.08±0.10
Meloxicam	1.16±0.16	0.80±0.23	0.48±0.31	0.46±0.15	0.38±0.16	0.14±0.11	0.12±0.17	0.02±0.04
Gabapentin	1.04±0.16	0.50±0.17	0.36±0.26	0.34±0.16	0.18±0.08	0.12±0.13	0.06±0.08	0
Meloxicam + Gabapentin	1.54±0.13	1.12±0.23	0.64±0.15	0.56±0.15	0.42±0.25	0.20±0.00	0.16±0.05	0.02±0.04

Table 2. *Repeated measures ANOVA for the orbital tightening, nostril shape, cheek flattening, ear shape and position, whisker shape and position, and grimace scale score over time*

Effect		Test	Value	F	Df1	Df2	P-value
Orbital Tightening	Time	Pillai's trace	0.63	13	2	15	0.001
		Wilks' Lambda	0.36	13	2	15	0.001
		Hotelling trace	1.73	13	2	15	0.001
		Roy's Largest Root	1.73	13	2	15	0.001
Nostril Shape	Time	Pillai's trace	0.93	22.01	7	10	0.0001
		Wilks' Lambda	0.06	22.01	7	10	0.0001
		Hotelling trace	15.40	22.01	7	10	0.0001
		Roy's Largest Root	15.40	22.01	7	10	0.0001
Cheek Flattening	Time	Pillai's trace	0.71	4.48	6	11	0.015
		Wilks' Lambda	0.29	4.48	6	11	0.015
		Hotelling trace	2.44	4.48	6	11	0.015
		Roy's Largest Root	2.44	4.48	6	11	0.015
Ear Shape and Position	Time	Pillai's trace	0.92	53.79	3	14	0.0001
		Wilks' Lambda	0.08	53.79	3	14	0.0001
		Hotelling trace	11.52	53.79	3	14	0.0001
		Roy's Largest Root	11.52	53.79	3	14	0.0001
Whisker Shape and Position	Time	Pillai's trace	0.97	110.43	5	12	0.0001
		Wilks' Lambda	0.02	110.43	5	12	0.0001
		Hotelling trace	46.01	110.43	5	12	0.0001
		Roy's Largest Root	46.01	110.43	5	12	0.0001
Grimace Scale Score	Time	Pillai's trace	0.98	127.80	7	10	0.0001
		Wilks' Lambda	0.01	127.80	7	10	0.0001
		Hotelling trace	89.46	127.80	7	10	0.0001
		Roy's Largest Root	89.46	127.80	7	10	0.0001

Table 3. *Repeated measure ANOVA test results for orbital tightening, nostril shape, cheek flattening, ear shape and position, whisker shape and position, and grimace scale score*

Parameter	Effect	Sum of Square	Df	Mean of Square	F	P	Eta Squared	Power
Orbital Tightening	Intercept	30.10	1	30.10	180.62	0.0001	0.91	1
	Group	2.97	3	0.99	5.95	0.006	0.52	1
Nostril Shape	Intercept	126.91	1	126.91	200.06	0.0001	0.92	1
	Group	11.59	3	3.86	6.09	0.006	0.53	0.90
Cheek Flattening	Intercept	42.35	1	42.35	159.70	0.0001	0.90	1
	Group	9.12	3	3.04	11.46	0.0001	0.68	1
Ear Shape and Position	Intercept	54.45	1	54.45	221.96	0.0001	0.93	1
	Group	6.12	3	2.04	8.32	0.001	0.60	0.97
Whisker Shape and Position	Intercept	91.87	1	91.87	456.99	0.0001	0.96	1
	Group	6.74	3	2.24	11.17	0.0001	0.67	1
Grimace Scale Score	Intercept	46.87	1	46.87	817.39	0.0001	0.98	1
	Group	4.87	3	1.62	28.31	0.0001	0.84	1

of orbital tightening scores (P=0.81). Pillai's trace, Wilks' Lambda, Hotelling trace, and Roy's Largest Root tests showed the effects of time on orbital tightening (P<0.001 for all) *(Table 2)*. Repeated measures ANOVA between groups to evaluate the effect of oral Gabapentin on orbital tightening showed significant changes (P<0.0001) *(Table 3)*. The post hoc Tukey test demonstrated that there was a significant difference between Meloxicam versus negative control (MD=0.46±0.14; P=0.03) and Gabapentin versus negative control (MD=0.60±0.14; P=0.005) *(Table 4)*. As shown in *Fig. 1*, orbital tightening changes were less in the Gabapentin group than in the other groups.

Nostril Shape

The sphericity of the nostril bulging scores was confirmed by Mauchly's test (P=0.48). Further tests showed the effects of time on nostril shape (P<0.0001 for all) *(Table 2)*. The effect of oral Gabapentin on nostril shape was evaluated using a repeated measures ANOVA between groups, which revealed significant differences (P<0.0001). Meloxicam versus negative control (MD=0.60±0.17; P=0.018) and Gabapentin versus negative control (MD=0.63±0.17; P=0.01) showed a significant difference *(Table 4)*. As illustrated in *Fig. 1*, the Gabapentin group had fewer nostril shape alterations than the other groups.

Cheek Flattening

The sphericity of cheek flattening alterations was verified by Mauchly's test (P=0.71). Further testing revealed that time had an influence on cheek flattening (P<0.015 for all). Repeated measures ANOVA showed significant differences between groups (P<0.0001). Post hoc Tukey test demonstrated significant difference between Meloxicam versus negative control (MD=0.50±0.12; P=0.005), Gabapentin plus Meloxicam versus negative control (MD=0.42±0.12; P=0.01), Gabapentin versus negative control (MD=0.70±0.12; P=0.0001), and Gabapentin plus Meloxicam versus Meloxicam (MD = -0.7±0.12; P=0.005) *(Table 4)*. The level of this index in the Meloxicam group was lower than in the other groups until the second time point of the experiment (a few hours after surgery). At the third time point (the night of surgery) the index in the Gabapentin group was lower than in the other groups, and the index in the Gabapentin group reached zero two days after surgery.

Ear Shape and Position

After Mauchly's test approval (P=0.45), further analysis showed the effects of time (P<0.0001 for all). Also, ANOVA showed significant changes and the post hoc Tukey test mentioned a significant difference between Meloxicam versus negative control (MD=0.57±0.15; P=0.01) and Gabapentin versus negative control (MD=0.72±0.15; P=0.001) *(Table 4)*. Ear shape and position changes were less in the Gabapentin group than in the other groups *(Fig. 1)*. Two days after surgery, all groups' ear shape and position scores reached zero.

Table 4. *Results of post hoc tukey tests for orbital tightening, nostril shape, cheek flattening, ear shape and position, whisker shape and position, and grimace scale score*

Parameter	Groups	Mean Difference ± Standard Deviation	P
Orbital Tightening	MG-NG	0.46±0.14	0.03
	GMG-NG	0.36±0.14	0.10
	GG-NG	0.60±0.14	0.005
	GMG-MG	-0.10±0.14	0.90
	GG-MG	0.13±0.14	0.80
	GG-GMG	0.23±0.14	0.42
Nostril Shape	MG-NG	0.60±0.17	0.018
	GMG-NG	0.20±0.17	0.68
	GG-NG	0.63±0.17	0.01
	GMG-MG	-0.40±0.17	0.15
	GG-MG	0.03±0.17	0.99
	GG-GMG	0.43±0.17	0.10
Cheek Flattening	MG-NG	0.50±0.12	0.005
	GMG-NG	0.42±0.12	0.01
	GG-NG	0.70±0.12	0.0001
	GMG-MG	-0.70±0.12	0.005
	GG-MG	0.20±0.12	0.39
	GG-GMG	0.27±0.12	0.16
Ear Shape and Position	MG-NG	0.57±0.15	0.01
	GMG-NG	0.30±0.15	0.26
	GG-NG	0.72±0.15	0.001
	GMG-MG	-0.27±0.15	0.32
	GG-MG	0.15±0.15	0.77
	GG-GMG	0.42±0.15	0.06
Whisker Shape and Position	MG-NG	0.38±0.11	0.02
	GMG-NG	0.31±0.11	0.06
	GG-NG	0.66±0.11	0.0001
	GMG-MG	-0.06±0.11	0.93
	GG-MG	0.28±0.11	0.10
	GG-GMG	0.35±0.11	0.03
Grimace Scale Score	MG-NG	0.35±0.05	0.0001
	GMG-NG	0.22±0.05	0.004
	GG-NG	0.46±0.05	0.0001
	GMG-MG	0.13±0.05	0.08
	GG-MG	0.11±0.05	0.21
	GG-GMG	0.24±0.05	0.05

NG = Negative control, MG = Meloxicam, GMG = Meloxicam + Gabapentin, GG = Gabapentin

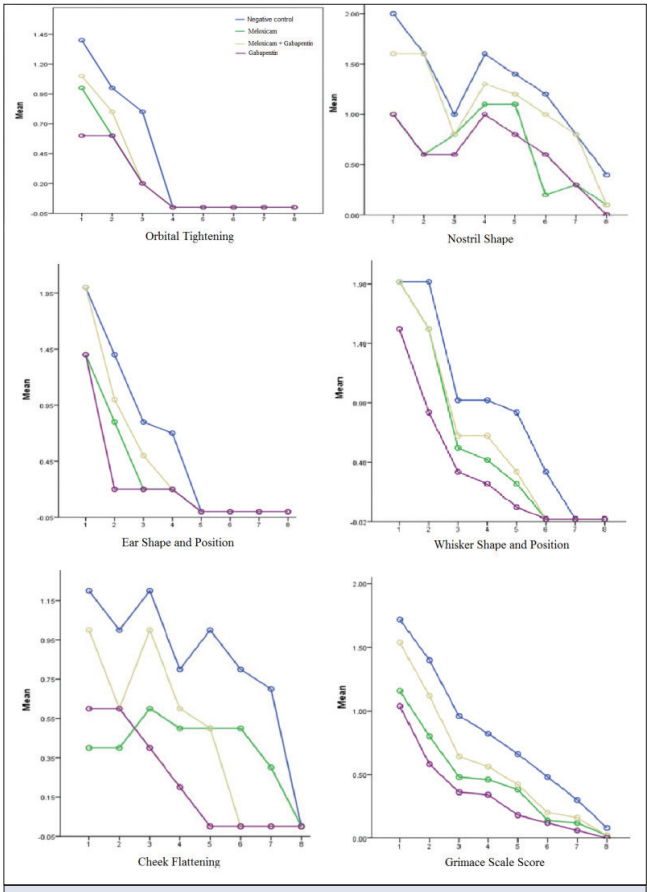

Fig 1. Mean of orbital tightening, nostril shape, cheek flattening, ear shape and position, whisker shape and position, and grimace scale score in each study groups (1: recovery period, 2: Few hours after surgery, 3: Night of surgery, 4: One day after surgery, 5: Two days after surgery, 6: Three days after surgery, 7: Four days after surgery, 8: Five days after surgery)

Table 5. *Epsilon-corrected results of whisker shape and position and Grimace Scale Score*

Whisker Shape and Position	**Corrections**	**Sum of Squares**	**Degrees of Freedom**	**Mean Sqaure**	**F**	**P-value**
Time	Sphericity	47.57	5	9.51	69.3	0.0001
	Greenhouse–Geisser	47.57	3.14	15.10	69.3	0.0001
	Huynh–Feldt	47.57	4.74	10.02	69.3	0.0001
	Lower Bound	47.57	1	47.57	69.3	0.0001
Error	Sphericity	10.98	80	0.13		
	Greenhouse–Geisser	10.98	50.38	0.21		
	Huynh–Feldt	10.98	75.92	0.14		
	Lower Bound	10.98	16	0.68		
Grimace Scale Score	**Corrections**	**Sum of Squares**	**Degrees of Freedom**	**Mean Sqaure**	**F**	**P-value**
Time	Sphericity	694.59	7	99.22	166.46	0.0001
	Greenhouse–Geisser	694.59	4.01	173.03	166.46	0.0001
	Huynh–Feldt	694.59	6.53	106.34	166.46	0.0001
	Lower Bound	694.59	1	694.59	166.46	0.0001
Error	Sphericity	66.76	112	0.59		
	Greenhouse–Geisser	66.76	64.22	1.03		
	Huynh–Feldt	66.76	104.5	0.63		
	Lower Bound	66.76	16	4.17		

Whisker Shape and Position

Mauchly's test did not approve the sphericity of whisker change scores (P=0.049). Hence, we used Greenhouse-Geisser, Huynh–Feldt, and Lower Bound corrections *(Table 5)*. Repeated measures ANOVA between groups to evaluate the effect of oral Gabapentin on whisker change showed significant changes. There was a significant difference between Meloxicam versus negative control (MD=0.38±0.11; P=0.02), Gabapentin versus negative control (MD=0.66±0.11; P=0.0001), and Gabapentin versus Gabapentin plus Meloxicam (MD=0.35±0.11; P=0.03).

Grimace Scale Score

The sphericity of grimace scale scores was not approved by Mauchly's test (P=0.046). Hence, we utilized Greenhouse-Geisser, Huynh–Feldt, and Lower Bound corrections *(Table 5)*. The effect of Gabapentin administration on grimace scale scores was evaluated using repeated measures ANOVA, which revealed significant alterations. Post hoc Tukey test demonstrated that there was a significant difference between Meloxicam versus negative control (MD=0.35±0.05; P=0.0001), Gabapentin plus Meloxicam versus negative control (MD=0.22±0.05; P=0.004), Gabapentin versus negative control (MD=0.46±0.05; P=0.0001), and Gabapentin versus Gabapentin plus Meloxicam (MD=0.24±0.05; P=0.05).

Discussion

In the present study, the analgesic effects of Gabapentin were evaluated and compared with those of Meloxicam, Gabapentin plus Meloxicam, and the negative control group. The Grimace Scale Score, as an indicator of pain, in the Meloxicam, Meloxicam plus Gabapentin, and Gabapentin groups was significantly lower than the negative control group. The Grimace Scale Score is reduced over time, and the Gabapentin group gets the lowest scores. These medications reduce opioid-related side effects and the occurrence of chronic postoperative pain [20].

The results of previous studies on postoperative pain are too controversial. An RCT on cats undergoing ovariohysterectomy compared the analgesic effects of Gabapentin-Buprenorphine, Meloxicam-Buprenorphine, and Buprenorphine alone. The two first groups did not significantly ask for rescue analgesia. However, the latter group, Buprenorphine alone, asked for more rescue analgesia [20]. Another RCT on outpatients undergoing laparoscopic cholecystectomy showed that 60-min rest pain was significantly lower with Gabapentin alone versus Meloxicam alone. Also, the combination of Meloxicam and Gabapentin did not show different results compared to Gabapentin alone. However, on postoperative days 1, 2, and 30, there were no significant effects of the treatment group on spontaneous or movement-evoked pain measures [21]. Contrary to their study, we showed that a multimodal approach is not as effective as Gabapentin alone, and Meloxicam alone showed similar efficacy to Gabapentin alone. A recent study showed that Gabapentin alone or in combination with Meloxicam could not significantly reduce neuropathic pain compared to placebo [22]. Jain et al.[23] studied the efficacy of Gabapentin (1200 mg) prior to induction of anesthesia in patients scheduled for laparoscopic cholecystectomy. They found that the pain score was significantly lower in the Gabapentin group compared to the placebo group one hour following the surgery. However, at other time points, there was no difference. Also, Karri et al.[24] showed similar results to the Jain et al.[23] study. The findings of the present study are in accordance with Karri et al.[24] and Jain et al.[23], but our study procedure was more invasive, so the pain lasted longer. A study on 100 patients who underwent laparotomy for gynecologic surgery divided them into four groups: placebo, Gabapentin 300, 600, and 1200 mg, who received drugs 2 h before surgery. The study showed that postoperative intravenous fentanyl requirement was lower with Gabapentin treatment, but there were no significant differences for the different doses [25]. Fassoulaki et al.[26] conducted a study on 60 patients undergoing abdominal hysterectomy. Patients were randomly assigned to either oral administration of 400 mg Gabapentin every 6 h for seven days plus continuous wound infusion of Ropivacaine 0.75% for 30 h or placebo. The treatment group consumed less cumulative morphine over the first 48 h and fewer lonalgal tablets on days 3-7. The visual analog score values at rest and after coughing did not differ between the groups during the first seven postoperative days. One month after the operation, fewer patients in the treatment group experienced pain than in the control group.

The current study did not look at long-term postoperative results, and this is an area where more research is needed. All pain scoring methods have limitations, and we avoided interobserver variability by having the same blinded observer perform all of the assessments throughout the trial. It's probable that having an observer around changed their behavior, as well as their pain scores and expressions.

Postoperative analgesia was significantly higher in groups that received Meloxicam, Gabapentin, or both compared to the negative control group. Gabapentin is as effective as Meloxicam. However, Meloxicam plus Gabapentin is not as effective as Gabapentin alone.

In conclusion, our results indicate that using either Gabapentin or Meloxicam as pre-operative medication can decrease post-operative pain while it may have coincidence with some adverse effects. In addition, it seems that applying these treatments can limit administration of

opioid drugs during the surgery.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (S. Mohitmafi).

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Ethical Statement

All procedures were carried out with the approval of the Ethical Committee of Islamic Azad University - Karaj Branch (Approval ID: IR.IAU.K.REC.1400.005).

Competing Interests

The authors declared that there is no conflict of interest.

Authors' Contributions

S.M and Z.A designed the study. H.M performed the laboratory analysis and wrote the paper. S.M reviewed and revised the paper. All authors have read and agreed to the published version of the paper.

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RESEARCH ARTICLE

Ixodid Ticks (Ixodoidea: Ixodidae) Infesting Wild Animals in Hatay, Türkiye

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ABSTRACT

Wild animals play an important role as amplifiers and/or reservoir hosts in the ecology of many ticks and tick-borne pathogens affecting livestock and humans. In this study, which was carried out in the Hatay province, the southernmost region of Anatolia, a total of 362 (210♀, 146 ♂, 6N) tick specimens were collected from 18 wild animals from 7 species, including white stork *(Ciconia ciconia)* (n = 1), roe deer *(Capreolus capreolus)* (n = 5), badger *(Meles meles)* (n = 2), jackal *(Canis aureus)* (n = 3), red fox *(Vulpes vulpes)* (n = 5), hare *(Lepus europaeus)* (n = 1), and wild goat *(Capra aegagrus)* (n = 1), which were obtained between 2014 and 2022. The collected ticks were identified according to morphological criteria at the level of species as *Amblyomma lepidum, Dermacentor marginatus, Haemaphysalis erinacei, Haemaphysalis inermis, Haemaphysalis kopetdaghica, Ixodes gibbosus, Ixodes kaiseri, Ixodes ricinus, Rhipicephalus kohlsi, Rhipicephalus rossicus*, and *Rhipicephalus turanicus*. With this study, *A. lepidum* was reported for the first time in Türkiye, while *R. rossicus* detected in roe deer was reported for the first time in wild animals, and the lesser-known/rare tick *H. kopetdaghica* was reported for the second time in wild goats where it was previously reported.

Keywords: *Amblyomma lepidum*, *Haemaphysalis kopetdaghica*, *Rhipicephalus rossicus*, Tick, Türkiye, Wild animals

INTRODUCTION

Human intervention in the habitats of wild animals increases contact between wild animals, livestock, and humans. Accordingly, many vector-borne bacterial, viral, and parasitic pathogens in wild animals may pose a threat to livestock and human [1]. Blood-sucking vectors, such as ticks, cause pathogens to spread among wildlife and urban life [2]. Ticks, which are the most important vector of pathogens that cause diseases in animals, are considered the most important vector of disease for humans after mosquitoes [3]. Ticks are of great importance in health research, not only because of their role in transmitting pathogenic agents to their hosts but also because they cause blood loss and tick paralysis in their hosts [4].

It is known that most wild animals are suitable hosts for ticks and play a reservoir and/or carrier role in the ecology of tick-borne pathogens affecting livestock and humans [4]. Even when their population density is low, these animals stimulate the reproduction of ticks, causing their numbers to increase and thus the spread of tick-borne pathogens [5]. Many tick-borne diseases have occurred from the past to the present, and new pathogens have been identified and continue to be identified. Lyme disease, Crimean-Congo hemorrhagic fever, tick-borne encephalitis, spotted fever group (SFG) rickettsiosis, babesiosis, theileriosis, and anaplasmosis are some of the significant tick-borne pathogens [6].

Studies and data on ticks and tick-borne pathogens in wild animals are relatively scarce. The most significant reason for this situation is that these animals, many of which (especially large mammals) are protected by law, and they are difficult to track down or catch [7]. Therefore, examining wild animals that are injured, hunted, or dead in any way in terms of tick infestations is very essential [8].

Türkiye is a country suitable for the inhabitation of ticks in terms of its sub-tropical climate zone, vegetation, domestic and wild animal diversity. The first studies reporting the presence of ticks in wild animals in Türkiye were based on individual or incidental cases. Recent studies have focused on investigating tick-host relationships [9,10] as well as pathogen relationships [6,8,11]. However, in Türkiye, where the diversity of wild animals is relatively high, there is a need for studies to reveal the presence of ticks and tick-borne pathogens [11].

This study aimed to identify tick species that infest some wild animals obtained from the Hatay province and to contribute to the tick fauna of Türkiye. First aid, treatment, intensive care, and rehabilitation services for wild animals injured or sick due to various reasons (such as traffic accidents, firearms, and trauma) in the Hatay province are carried out at Hatay Mustafa Kemal University (HMKU) Veterinary Health, Application and Research Hospital and HMKU Wild Animal Rescue and Rehabilitation Center.

Material and Methods

Ethical Approval

This study was approved by the decision of the Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (2022/07-08) and Directorate of Nature Conservation and National Parks (13/07/2023-287402).

Features of the Study Areas

Hatay, which is located at the eastern end of the Mediterranean Region and is a border province, is located between 35°52′ and 37°04′ north latitudes and 35°40′ and 36°35′ east longitudes with a surface area of 5403 km². The province is surrounded by Syria to the south and east, Gaziantep and Kilis to the northeast, Osmaniye and Adana to the north and northwest, and the Mediterranean Sea to the west [12]. Hatay province, which has a Mediterranean climate with hot and dry summers and warm and rainy winters, is surrounded by the Amik plain, in which the Asi River is located, and Amanos and Kızıl Mountains [13]. Hatay region is one of the most special areas of Türkiye in terms of biodiversity with its location and different habitat types [14].

Collection and Identification of Tick Specimens

This study was conducted in the Hatay between 2014-2022. Ixodid tick samples, which constitute the material of the study, were collected from 18 wild animals from a total of 7 species, including white stork *(Ciconia ciconia)* (n = 1), roe deer *(Capreolus capreolus)* (n = 5), badger *(Meles meles)* (n = 2), jackal *(Canis aureus)* (n = 3), red fox *(Vulpes vulpes)* (n = 5), hare *(Lepus europaeus)* (n = 1), and wild goat *(Capra aegagrus)* (n = 1) *(Table 1)*. The whole body of the wild animals brought to HMKU Veterinary Health, Application and Research Hospital and Wild Animal Rescue and Rehabilitation Center as injured, sick, or dead was carefully examined for tick infestation. The detected ticks were collected into vials containing 70% ethyl alcohol, and then sent to Ankara University, Faculty of Veterinary Medicine, Ticks and Tick-Borne Diseases Research Laboratory for species identification. The obtained ticks were diagnosed at the species level using their morphological characteristics under a stereomicroscope (Stemi 2000-C, Zeiss, Germany) equipped with an AxioCam digital camera and ZEN software with the help of species-specific taxonomic keys [15-21].

Results

A total of 362 tick samples [210♀, 146♂, 6 nymph (N)] were collected: 1 from the white stork, 131 from roe deer, 13 from badgers, 159 from jackals, 23 from red foxes, 27 from hares, and 8 from wild goats. According to the results of morphological analysis, 11 tick species were identified, including, *Amblyomma lepidum* (1♂), *Dermacentor marginatus* (1♀), *Haemaphysalis erinacei* (1♂), *Haemaphysalis inermis* (2♂, 3♀), *Haemaphysalis kopetdaghica* (1♀), *Ixodes gibbosus* (2♂, 4♀), *Ixodes kaiseri* (4N, 13♀), *Ixodes ricinus* (2N, 34♂, 56♀), *Rhipicephalus kohlsi* (1♀), *Rhipicephalus rossicus* (21♂, 12♀), and *Rhipicephalus turanicus* (85♂, 119♀) *(Fig. 1)*. Of these species belonging to the family Ixodidae, *A. lepidum* is detected only in white stork, *I. ricinus, H. inermis, R. rossicus,* and *D. marginatus* only in roe deer, *H. erinacei* only in the red fox, *I. gibbosus, H. kopetdaghica,* and *R. kohlsi* species are detected only in wild goats, while *I. kaiseri* was detected in two different hosts (badger and red fox), and *R. turanicus* species was detected in four different hosts (badger, jackal, fox, and hare). As a result, it was determined that *R. turanicus* and *I. kaiseri* from 11 tick species were infested in more than one host species, while the other 9 species were determined to be infested in only one host species.

Of the wild animals, 61.11% (11/18) were infested with a single tick species, 27.77% (5/18) with 2 tick species, and 11.11% (2/18) with 3 tick species. Those found as a single species were *A. lepidum* (stork), *R. turanicus* (hares), *I. ricinus* (1 roe deer), *R. turanicus* (1 badger), *R. turanicus* (3 jackals), and *H. erinacei, I. kaiseri, R. turanicus,* and *R. turanicus* (4 foxes). Two different species found together were *I. ricinus* + *H. inermis* (2 roe deer), *D. marginatus* + *R. rossicus* (1 roe deer), and *I. kaiseri* + *R. turanicus* (1 badger and 1 fox). Three different species found together were *I. ricinus* + *H. inermis* +*R. rossicus* (1 roe deer), and *I. gibbosus* + *H. kopetdaghica* + *R. kohlsi* (wild goat).

Rhipicephalus turanicus was the most abundant species in the study, with 56.35% (204/362) followed by *I. ricinus*

***Table 1**. Tick species, numbers and sexes infesting some wild animals in Hatay region*

Wild Animal Species and Numbers (n)	Tick Species	Tick Number and Gender	Total
Ciconia ciconia (n = 1)	*A. lepidum*	1 (1 ♂)	1
Capreolus capreolus (n = 5)	*I. ricinus* *H. inermis*	6 (4 ♂, 2 ♀) 1 (1 ♀)	7
	I. ricinus *H. inermis*	60 (2 N, 20 ♂, 38 ♀) 2 (1 ♂, 1 ♀)	62
	I. ricinus *H. inermis* *R. rossicus*	23 (10 ♂, 13 ♀) 2 (1 ♂, 1 ♀) 6 (5 ♂, 1 ♀)	31
	D. marginatus *R. rossicus*	1 (1 ♀) 27 (16 ♂, 11 ♀)	28
	I. ricinus	3 (3 ♀)	3
Meles meles (n = 2)	*I. kaiseri* *R. turanicus*	11 (4 N, 7 ♀) 1 (1 ♂)	12
	R. turanicus	1 (1 ♂)	1
Canis aureus (n = 3)	*R. turanicus*	1 (1 ♀)	1
	R. turanicus	9 (3 ♂, 6 ♀)	9
	R. turanicus	149 (59 ♂, 90 ♀)	149
Vulpes vulpes (n = 5)	*I. kaiseri* *R. turanicus*	2 (2 ♀) 1 (1 ♀)	3
	H. erinacei	1 (1 ♂)	1
	I. kaiseri	4 (4 ♀)	4
	R. turanicus	8 (3 ♂, 5 ♀)	8
	R. turanicus	7 (1 ♂, 6 ♀)	7
Lepus europaeus (n = 1)	*R. turanicus*	27 (17 ♂, 10 ♀)	27
Capra aegagus (n = 1)	*I. gibbosus* *H. kopetdaghica* *R. kohlsi*	6 (2 ♂, 4 ♀) 1 (1 ♀) 1 (1 ♀)	8
Total (n = 18)	11 tick species	210 ♀, 146 ♂, 6 N	362

N; nymph

Fig 1. Dorsal and ventral views of ticks collected in this study. *Amblyomma lepidum* (a1: male-dorsal view, a2: male-ventral view), *Dermacentor marginatus* (b1: female-dorsal view, b2: female-ventral view), *Haemaphysalis erinacei* (c1: male-dorsal view, c2: male-ventral view), *Haemaphysalis inermis* (d1: female-dorsal view, d2: female-ventral view), *Haemaphysalis kopetdaghica* (e1: female-dorsal view, e2: female-ventral view), *Ixodes gibbosus* (f1: female-dorsal view, f2: female-ventral view), *Ixodes kaiseri* (g1: female-dorsal view, g2: female-ventral view), *Ixodes ricinus* (h1: female-ventral view, h2: female-dorsal view), *Rhipicephalus rossicus* (i1: female-dorsal view, i2: female-ventral view), *Rhipicephalus turanicus* (j1: female-dorsal view, j2: female-ventral view), and *Rhipicephalus kohlsi* (k1: female-dorsal view, k2: female-ventral view)

25.41% (92/362), *R. rossicus* 9.11% (33/362), *I. kaiseri* 4.69% (17/362), *I. gibbosus* 1.65% (6/362), and *H. inermis* 1.38% (5/362), respectively. *Amblyomma lepidum, D. marginatus, H. erinacei, H. kopetdaghica,* and *R. kohlsi* species were detected as one each and became the least common species *(Table 1)*.

Discussion

Wild mammals and migratory birds may have great potential for the spread of tick and tick-borne pathogens [7,22]. Therefore, it is very significant to examine these animals, which are important parts of wildlife, in terms of ticks and tick-borne pathogens [8]. However, it is quite difficult to track and catch these animals, which are preferred as hosts by many tick species in their ecological environments. Therefore, the determination and identification of ticks infesting on wild animals are extremely valuable in terms of understanding the ecology of ticks and their vector role in the spread of pathogens [23]. Since the mammal fauna consists of species of African and desert origin and species extending along the Anatolian cross-mountain range to the peaks of the Amanos, it is the richest region of Türkiye in terms of both mammal diversity and bird diversity since it is located on an important migratory bird route in the world [14,24].

In this study sampled from the Hatay province; 362 tick samples were collected from 18 wild animals from 7 species, 6 of which were mammals and 1 of which was migratory bird, and 11 tick species were identified. These species are *A. lepidum, D. marginatus*, *H. erinacei*, *H. inermis*, *H. kopetdaghica*, *I. gibbosus*, *I. kaiseri*, *I. ricinus*, *R. kohlsi*, *R. rossicus*, and *R. turanicus*.

Rhipicephalus turanicus, the most abundant species in this study, is one of the most common tick species in Türkiye that infests humans [25] and domestic animals [26,27]. The main hosts of this tick species, which is also common in wild animals, are wild and domestic ungulates, but birds and scaly reptiles are considered exceptional hosts [28]. In studies conducted in Türkiye, *R. turanicus* species have been identified in red deer, brown bear [8], red fox [8,9], hare [6,8], wild boar [6], some mouse, hedgehog and rodent species [9]. In the current study, *R. turanicus* was determined in four wild animals namely red fox, wild rabbit, badger, and jackal, and the host range was the widest species. The presence of this tick in red foxes [8,9] and wild hares [6,8] in Türkiye has been reported in previous studies.

Roe deer, one of the areas of distribution in Türkiye is the Amanos Mountains of Hatay and is classified among the species of "Minimum concern" according to the criteria of the World Union for Conservation of Nature (IUCN)[24]. In our study, *I. ricinus, H. inermis, R. rossicus*, and *D. marginatus* species were detected only in roe deer. *Ixodes ricinus*, the second most abundant species in this study, is one of the most significant vectors in Europe and has a wide range of hosts. This species, which can select a wide variety of mammalian species including birds, reptiles, and humans as hosts, plays a role as a competent vector in the transmission of pathogens such as tick-borne encephalitis virus, *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum, Rickettsia monacensis, R. helvetica, Babesia divergens,* and *B. microti* [29]. In studies conducted in Türkiye, *I. ricinus*, which is generally detected in domestic animals, has been reported in humans [30], sheep and goats [26], cattle [27], jackals, some small mammals such as rats, mice, and squirrels, rodents [9]. A point to be emphasized about this species, whose existence was once again detected in roe deer in this study, is the possibility that the specimens identified as *I. ricinus* actually represent *Ixodes inopinatus*, which is very closely related and sister species to *I. ricinus*. A very recently characterized tick species, *I. inopinatus*, is a species in the *I. ricinus* complex and is closely related to *I. ricinus* both morphologically and molecularly [31]. It has been reported that the morphological distinction of both species cannot be made precisely and even some of the molecular markers (e.g. 16S rDNA) is not sufficient to distinguish [32,33]. Therefore, our relevant specimens in this study have been identified as *I. ricinus* at this stage to avoid any confusion in the future. However, considering that *I. inopinatus* adapted to more arid geographies than *I. ricinus* and spread especially in the Mediterranean region and North Africa, and that it could even be found sympatric with *I. ricinus* in such regions [31-33], it should be underlined that the Hatay province is also a geography quite suitable for *I. inopinatus*. To clarify all these, detailed molecular analyses of *I. ricinus* spreading in this region are needed in the near future.

Haemaphysalis inermis is a species whose vector capacity is poorly known and is considered to carry several zoonotic agents [34]. The presence of *Theileria orientalis* and *Rickettsia* sp. has been reported [27] in this tick species collected from cattle [27] and humans [35] in Türkiye. *Rhipicephalus rossicus* is one of the vectors of the Crimean-Congo hemorrhagic fever virus, *Francisella tularensis,* and *Coxiella burnetii*. The presence of *R. rossicus*, another member of the *Rhipicephalus sanguineus* group, in Türkiye, was first reported by Pomerantzev [36]. Later, this tick species was identified in the Tunceli province. A total of 3 tick samples obtained from dogs, cows, and plant vegetation from domestic animals were determined to be *R. sanguineus* s.l. in their morphological analysis and *R. rossicus* species in their molecular analysis [37]. In this study, 33 (21♂, 12♀) species were detected in roe deer and this species was reported for the first time from wild animals in Türkiye as far as we know. *Dermacentor marginatus* is a species that lives in habitats generally above 1000 m, is cold-resistant and adults can be active at temperatures above 0°C [17]. In this study, where the temperate Mediterranean climate prevails and its altitude is not high, *D. marginatus* 1 (♀) was found to be one of the least common tick species. This species, which is one of the most common tick species in Türkiye, has been reported in different regions in humans [25,35] and domesticated [26,27] and wild animals [6].

Ixodes gibbosus, H. kopetdaghica, and *R. kohlsi* species were identified together in a wild goat in this study. *Ixodes gibbosus* is a relatively common but little-known tick species. It was named "*I. ricinus var. gibbosus*" by Nuttall in 1916 in adult tick samples collected from domestic goats in İzmir, Türkiye. This species was later reported morphologically in domestic goats and sheep [17] in countries located in the Mediterranean basin, in wild sheep *(Ovis orientalis ophion)* in Cyprus [11], in humans [35] in Türkiye, and in wild goats by the molecular method in addition to morphological identification [11]. *Haemaphysalis kopetdaghica* is one of the rare tick species and has been included in the list of endangered tick species because it has been detected in limited numbers from past to present [38]. First identified by Kerbabaev in a wild goat, a leopard, and a horse in the Kopet mountains of Turkmenistan, this species was later reported in a wild goat in Iran, two wild goats and a wild sheep in the Kopet Mountains, and a sheep in Tajikistan. *Haemaphysalis kopetdaghica* was rediscovered after a long time in two wild goats in Kemaliye, a mountainous district of Erzincan province in the Eastern Anatolia region, by morphological and molecular methods [11]. *Rhipicephalus kohlsi* is one of the tick species that is mostly native to small ruminants such as sheep and goats and does not

have wide distribution. This tick, which has a single-host life cycle, was first described in domestic goats and sheep in Jordan [19]. This species, which has been reported from sheep and goats in different countries including Türkiye, has been reported in cattle, horses, mules, camels, and roe deer in Israel. Also, this tick species has been detected in wild goats in Iran and in Türkiye. The wild goat, also known as bezoar, Anatolian bezoar, or bezoar ibex, is one of the important creatures of wildlife. This species of goats, whose populations are known to be significantly reduced, is known to be on the IUCN red list. *Haemaphysalis kopetdaghica, D. raskemensis, I. gibbosus, R. bursa*, and *R. kohlsi* [11] species has been reported in wild goats in Türkiye in previous study. In this study, one of the lesser/rarest species *I. gibbosus, H. kopetdaghica,* and *R. kohlsi* have been reported once again by being detected together in a wild goat. Considering that it is included in the endangered species list, the detection of *H. kopetdaghica* species in wild goats in this study is a significant finding in terms of its presence in Türkiye.

Ixodes kaiseri was detected in one badger and two red foxes in this study, with the second largest host range. This species, first described in the common Egyptian fox [10], completes parasitic life cycles in carnivores, porcupines, and rodents [39]. This tick species has been recorded in Türkiye in red foxes [10], dogs [40], jackals [9] and cave environment [41].

Haemaphysalis erinacei is a species that generally prefers small and medium-sized mammals, mainly hedgehogs as its host [17], but it can also infest carnivores, rodents, bats, and birds [28]. This tick species has been found in Türkiye in white-breasted hedgehogs, Arabian rabbits, red foxes, brown bears, lynx, rock marten, humans, and cave environments [41-43]. *Haemaphysalis erinacei* 1 (♂), one of the least common species in this study and previously reported from red foxes in Türkiye [9], has once again been identified in the red fox.

Amblyomma lepidum, more common in African countries, is one of the species belonging to the *Amblyomma* lineage [44]. This species, which has a great variety of hosts, has been reported to infest sheep, goats, cattle, camels, donkeys, horses, dogs, cats [45], and rarely birds [46]. This tick species, which is especially common in livestock, has been detected in the United Arab Emirates, Sudan, Somalia, Uganda, Ethiopia, Kenya, Tanzania, Israel [47], Azerbaijan [48], Cyprus [22], and Iran [46].

Among the exotic (non-native) tick species carried by migratory birds from Africa to Europe, *A. lepidum* [22] as been reported in Marabou stork in Uganda *(Leptoptilos crumeniferus)* [49], blackbird in Cyprus *(Turdus merula)* [22], and Norfolk plover *(Burhinus oedicnemus)* [48] in Azerbaijan. In our study, *A. lepidum* (1 ♂) species detected in a white stork was reported for the first time in Türkiye. Hatay province is an important area where migratory birds, including storks, enter Türkiye by following the valley formed by the Sinai mountains by moving along the Nile River after spending the winter in Central and Southern Africa [50]. The occurrence of this tick species in the Hatay, which is adjacent to Türkiye's border with Syria, has led to the idea that *Amblyomma* ticks of African origin can be carried through transport hosts such as migratory birds. In this regard, it will be useful to conduct studies on the presence of *Amblyomma* ticks in the region.

As a result, while *A. lepidum* detected in white storks in Türkiye was recorded for the first time with this study, *R. rossicus* species detected in roe deer were detected for the first time in wild animals, one of the lesser known/rare tick species, *H. kopetdaghica* was reported for the second time in wild goats, where it was previously reported. We believe that these data will contribute to the tick fauna of Türkiye and the relevant literature.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (A. Zerek) on reasonable request.

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Ethical Approval

This study was approved by the decision of the Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (2022/07-08) and Directorate of Nature Conservation and National Parks (13/07/2023-287402).

Conflict of Interest

The authors have no conflicts of interest to declare.

Author Contributions

A.Z., İ.E., M.Y., M.E.A., and Ö.O.: Concept, Design, Supervision, Resources, Materials Data, Collection and/or Processing, Analysis and/or Interpretation, Literature Search, Writing and Critical Reviews

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RESEARCH ARTICLE

Effects of Dietary Protein on Milk Yield and Colostrum Whey Protein Composition of Tibetan Sheep in Modern Intensive-fed Pattern

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ABSTRACT

Colostrum protein, an essential source of dietary nutrients, could improve new-born animals' immunity, and play a vital role in mammals' early development. In order to explore the milk yield and colostrum whey protein composition of Tibetan sheep, 120 Tibetan sheep were arbitrarily separated into categories, namely treatment groups (A, B, C) and control group (D). Compositional and functional differences in milk yield and colostrum whey protein composition among different dietary proteins were compared using proteomics methods. The results showed that sheep with 14% protein level diet group (group B) had the least bodyweight loss and higher milk yield during lactation compared to the other groups. Fifty differentially expressed proteins (DEPs) were recognized using iTRAQ, these DEPs were analyzed based on cluster, GO, KEGG and PPIs analysis. GO-BP involved were Protein transmembrane transport, Protein regulation metabolic process, Biological regulation, Regulation of biological process, and Response to stimulus. Meantime, DEPs participated in many KEGG pathways, including Fatty acid metabolism, Glycerophospholipid metabolism, Protein digestion and absorption, Ras signaling pathway and Cell adhesion molecules. The treatment groups showed increase in the abundance of regulation metabolic process (especially protein metabolism and fatty acid metabolism), along with decrease in stress reaction process. Lactoferrin, Alpha-S2-casein, Superoxide dismutase [Cu-Zn], Alpha-s1-casein, Alpha globin and Lactoperoxidase appeared in the center of the PPi network intersection. Interestingly, 14% protein group (group B) had exhibited the greatest variability between biological relevance in milk composition and function, these results could increase the understanding of different dietary protein on colostrum whey protein composition of Tibetan sheep, which could provide important information and potential directions for the infant milk powder and functional food industries.

Keywords: Tibetan sheep, Dietary protein, Milk yield, Colostrum whey protein, Proteomics

INTRODUCTION

Tibetan sheep grazed grassland all year round with traditional grazing management, and the herbage and nutrients were often insufficient to maintain the normal physiological function in cold season, which would result low ewe lactation and high lamb mortality [1,2]. The study showed that dietary proteins have many nutritional and biological functions, and dietary protein bioavailability directly affects animal production performance [3,4]. There are positive effects of dietary protein on sheep productivity and reproductive performance, which could reduce body weight-loss and feeding costs, and increase economic efficiency [5]. Therefore, there is a big potential to improve sheep lactation performance and milk composition through developing protein-diet supplementary system during cold season.

Protein is the basic material of mammal living activities; dietary protein level is the limiting nutrient element that affects the sheep milk yield and lactoprotein [6]. When varying the quantitative of protein-diet supplementation in the diet, milk compositions in ruminants may fluctuate due to a change in nutritional intake [7]. Many studies reviewed that the protein-diet supplementation in the diet exhibited prominent effect on the sheep milk yield, especially in the early stages of lactation [8]. Moreover, the appropriate dietary crude protein level in the later period of pregnancy and lactation could improve milk yield and lactoprotein content [9]. Once there is a lack

of dietary protein, the dry matter intake of sheep could decrease, which results in a decrease in milk yield. Meanwhile, the high dietary protein, which exceeded the needs of maintenance and lactation, would also have a negative impact in milk yield and milk composition [10,11]. Therefore, the regulation of dietary protein nutrition was an important link in the production of sheep during lactation.

The conversion of protein in feed into available nutrients is very important for mammalian production traits, and the nitrogen deposition of high protein diet is significantly greater than that in low protein diet, which led to increase of protein deposition, promote the growth and development, and improve animal final body weight and ADG with the increase of dietary protein [12,13]. However, protein utilization rate in ruminants is usually lower than that of monogastric animals. A large proportion of dietary protein is not effectively utilized by ruminants, and the unutilized nitrogen is expelled through metabolic process, which could result in environmental nitrogen pollution [14]. In the present study, we measured and analyzed Tibetan sheep milk yield and colostrum composition with different protein-diet supplementary, the change rule and biological significance of dietary protein level on milk proteome difference, which provided basic data for Tibetan sheep feeding and development of feed products.

Material and Methods

Ethics Statement

Animal experiment was approved by the Institutional Animal Care and Use Committee (State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University) (QHDX-19-10-07-06).

Study Site

The study was done at Tibetan Autonomous Prefecture of Hainan, Qinghai Province of China, situated at south of Qinghai-Tibetan Plateau. This area is over 3200 m above the sea level and has a dry cold climate. The experimental Tibetan sheep were feed in standardization shed, and processed according to experimental design [15].

Animals and Diets

One hundred-twenty Tibetan ewes (single lamb trait) were selected, which were the same body weight (43.39±2.20 kg) and age (3-4 years old, 2nd birth orders). Before this study, individuals grazed only natural pasture and were not offered supplements. The experiment lasted 75 days from December, 2017 to March, 2018 (15 days before parturition was an adjustment period and the following 60 days was for sample collection period). Different levels of dietary protein supplements were provided for the Tibetan sheep, which were grazed on the natural grassland until the end of the experiment. All ewes were allotted randomly into four groups. Group A, B and C were fed 12%, 14% and 16% dietary protein, respectively (Treatment Group, A, B and C). Group D was fed no supplement (Control Group, CON). The dietary formula and nutritional components were showed in *Table 1*. The experimental diets were formulated according to the nutrient requirements of an ewe weighing 40 kg (NRC 2007 and Standards for Feeding Sheep of China NYT816-2004). Diets were composed of the most popular feed resources in Qinghai-Tibetan plateau as feeding standard, 1.8 kg (dry matter) per individual and day of a total mixed ration. The nutrient composition of mixed-feed was analyzed or tested by'Feed Analysis and Quality Test Technology'. [16].

Measurement of Samples

The ewes were weighed at day 1 and 60 of lactation period by using platform scale before feeding in the morning.

The ewes were milked twice daily at 08:00 and 18:00 via a milking machine, daily individual milk yield was recorded and then kept at the dairy laboratory of Key of

Table 1. *Ingredients and chemical composition of the experimental diets (DM)*

Items	**Proportion (%)**			
Ingredients	**A**	**B**	**C**	**D**
Corn	52.00	47.00	41.50	
Soybean meal	2.00	6.50	13.00	
Cottonseed meal	3.00	5.00	5.00	
Bran	1.50	1.00	0.50	
Rapeseed meal	7.00	5.80	5.00	
Oaten Hay	10.00	10.00	10.00	
Ensiling Corn	20.00	20.00	20.00	
Limestone	0.50	0.70	1.00	
Premix[1]	4.00	4.00	4.00	
Total	100.00	100.00	100.00	
Nutrient Content				
DM	72.47	72.48	72.40	94.51
CP	12.23	14.12	16.13	5.33
DE (MJ/Kg) [2]	12.13	12.11	12.09	4.12
EE	2.93	2.81	2.71	2.69
ADF	12.83	13.26	13.54	42.17
NDF	23.53	23.79	23.79	60.23
Ca	0.35	0.43	0.55	3.77
P	0.34	0.36	0.37	0.03

[1] *The premix provided the following per kg of dietary: Vit. A: 12.000 IU, Vit. D: 2000 IU, Vit. E: 30 IU, Cu: 12 mg, Fe: 64 mg, Mn: 56 mg, Zn: 60 mg, I: 1.2 mg, Se: 0.4 mg, Co: 0.4 mg*
[2] *Digestible Energy (DE) was the calculated value, and others were the measured values*

Laboratory of Plateau Ecology and Agriculture (Qinghai University).

Individual milk samples from 08:00 and 18:00 were collected at lactation period (1d, 3d, 5d, 10d, 15d, 20d, 25d, 28d, 30d, 33d, 35d, 40d, 45d, 50d, 55d and 60d) and collected 5 repeats of each milk sample. All the milk samples were carried to the laboratory and processed in order to assess the normal composition of nutrition, such as fat, skim solids, lactose, protein and milk density, by using milk composition measuring instrument (MT-100, China). The colostrum (1-10 day postpartum) samples were centrifuged at 4000 r/min for 20 min at 4ºC. Fat fractions of the milk samples were carefully removed. The skim milk samples were centrifuged at 12000 r/min for 60 min at 4ºC to detect the protein concentration by Bradford method.

The whey was collected to determine the milk proteome difference of the 4 groups by iTRAQ technology (Isobaric Tags for Relative and Absolute Quantification), and whey proteomic analysis was outsourced to Shanghai Majorbio Bio-pharm Technology (SMBPT) Co., Ltd (China, Shanghai). Protein digestion was performed according to the filter-aided sample preparation (FASP) procedure, described by Wisniewski, and the resulting peptide mixture was labeled using the 4-plex iTRAQ reagent (AB SCIEX, Foster City, CA, USA), according to the manufacturer's instructions. A total of 30-μg peptide mixture was labeled with iTRAQ reagents according to the manufacturer instructions (Applied Biosystems, USA). Group 1 samples were labeled with reagent 114, group 2 with reagent 115, group 3 with reagent 116, and group 4 with reagent 117. The labeling reaction was performed by 1-h incubation at room temperature. The iTRAQ-labeled peptides were fractionated by SCX chromatography using the AKTA Purifier system (GE Healthcare, Fairfield, CT, USA). Experiments were performed on a Q Exactive mass spectrometer that was coupled to an Easy nLC (ThermoFisher Scientific, Waltham, MA, USA). The MS/MSspectra were searched using the MASCOT search engine (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.4 (Thermo Electron, San Jose, CA, USA) against the uniport database (91,245 sequences, download at 20171210) and decoy database. Differentially expressed proteins (DEPs) were based on standards of a 1.2-fold change in abundance (ratio ≥1.20 or ≤0.833) and $P<0.05$.

Statistical Analysis

Bioinformatics was analyzed via R language toolkit [17]. Functional annotation and classification of all identified proteins were determined using the Blast2GO and InterProScan program against the Uniprot database (uniprotsheep 515149.fasta). Pathway analyses were extracted using the search pathway tool of the KEGG mapper platform *(http://www.genome.jp/kegg/)* and BLAST program. Pathway enrichment statistics were conducted by the Fisher's exact test, and the pathways with a corrected $P<0.05$ were defined as the most significant pathways. The STRING program *(http://string-db.org/)* for the retrieval of interacting genes/proteins database for the prediction of the physical and functional interactions was used to analyze the PPIs. The graphical visualization and analysis of the interaction network were performed in Cytoscape software.

The data were expressed as mean ± standard deviation (SD). Duncan's post hoc test was used to determine any significant differences among 4 groups. Differences were considered significant at $P<0.05$ and extremely significant at $P<0.1$.

Results

Body Weight Changes During Lactation Period

The initial body weights, final weights and live weight gain of Tibetan ewes during lactation period in different dietary protein level were presented in *Table 2*. The data indicated that the live weight gain was positive increase in treatment groups and a decrease in control group during lactation period. The final weights can be significantly increased in treatment groups than in control group ($P<0.05$). As far as treatment groups were concerned, the effect of group B and C were significantly better than group A in live weight gain ($P<0.05$). There was no significant difference between group B and group C ($P>0.05$), while the 14% protein group (group B) had more obvious effect in live weight gain.

Milk Yield and Lactation Regularity

The results of milk yield and lactation regularity were summarized in *Fig. 1* and *Table 3*. The present results showed that administered dietary protein levels significantly affect milk yield. Maximum milk yield (30d) and average milk yield of various stages (10d, 0-60d, 0-10d, 10-30d and 30-60d) of Tibetan ewes during lactation period were significantly higher in treatment groups than those of control group ($P<0.05$). The milk yield of 14% protein level diet was significantly higher than 12% and 16% protein level diet during whole lactation period ($P<0.05$). All of the groups had same lactation regularity, the milk yield showed a downward trend from 0 to 10 days, then showed a rising trend from 10d to 30d, and milk yield reached highest point on the 30d, and then showed a downward trend from 30 to 60 days. And 14% protein level diet group (group B) was greater milk yield than other groups during the whole lactation period ($P<0.05$). The results of milk composition analyses at the 0-10d were summarized in *Table 4*. Compared to control

Table 2. *The body weight changes of Tibetan ewes in different dietary protein levels*

Groups	N	Initial Weight (kg)	Final Weight (kg)	Live Weight Gain (kg)
A	30	43.53±0.96	49.88±0.85[b]	6.35±0.45[b]
B	30	43.62±1.15	54.10±1.07[a]	10.48±0.69[a]
C	30	44.39±1.20	53.40±1.11[a]	9.01±1.03[a]
D	30	44.18±1.08	42.86±1.94[c]	-1.32±0.57[c]

[1] *Group A was fed the 12% dietary protein level group, Group B was fed the 14% dietary protein level group, Group C was fed the 16% dietary protein level group, Group D was the control group*
[2] *The initial weight was measured on December 31, 2017, the final weight was measured on March 1, 2018*

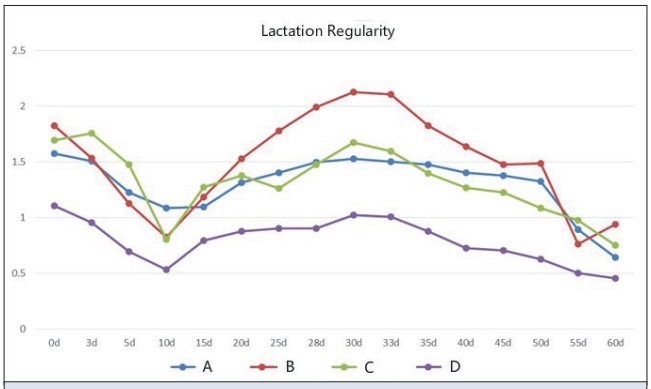

Fig 1. The lactation regularity of Tibetan ewes in different dietary protein levels

Table 3. *The milk yield of Tibetan ewes in different dietary protein levels (kg)*

Groups	Average	10d	30d	0-10d	10-30d	30-60d
A	1.302[b]	1.202[a]	1.525[b]	1.348[a]	1.367[b]	1.23[b]
B	1.509[a]	0.772[b]	2.125[a]	1.328[a]	1.72[a]	1.461[a]
C	1.318[b]	0.769[b]	1.675[b]	1.433[a]	1.412[b]	1.184[b]
D	0.793[c]	0.502[c]	1.025[c]	0.823[b]	0.901[c]	0.699[c]

[1] *Group A was fed the 12% dietary protein level group, Group B was fed the 14% dietary protein level group, Group C was fed the 16% dietary protein level group, Group D was the control group*

group, the increases in lactose of treatment groups were significantly (P<0.05). No significant difference existed in the proportions of milk fat, skim solids, protein and milk density at any time point (P>0.05).

Table 4. *The 0-10d milk composition of Tibetan ewes in different dietary protein levels (%)*

Groups	Fat	Skim Solids	Lactose	Protein	Density
A	4.414	11.212	6.737[a]	4.762	33.842
B	4.466	11.606	6.626[a]	4.974	33.773
C	4.382	11.131	6.483a	4.922	33.847
D	4.406	11.396	5.039[b]	4.836	33.258

[1] *Group A was fed the 12% dietary protein level group, Group B was fed the 14% dietary protein level group, Group C was fed the 16% dietary protein level group, Group D was the control group*

Changes in Proteome Profiles During Different Dietary Protein Levels

Three hundred and forty six non-redundant proteins with *Ovis aries* database were successfully identified via Mascot and iTRAQ method. Then we applied a manual thresholding approach and a probabilistic prediction algorithm, yielding 310 high-confidence candidates. A total of 50 differentially expressed proteins in different groups using 1.2-fold and a P-value <0.05 of differentially expressed protein were identified from the 310 proteins. The expression levels of 37 proteins were up-regulated in samples in treatment groups and 13 were down-regulated (*Fig. 2*). Interestingly, 14% protein group showed the highest up-regulated/down-regulated trends in up-regulated proteins and down-regulated proteins.

Sheep milk differentially expression proteins were directly subjected to hierarchical clustering by Cluster 3.0 software,

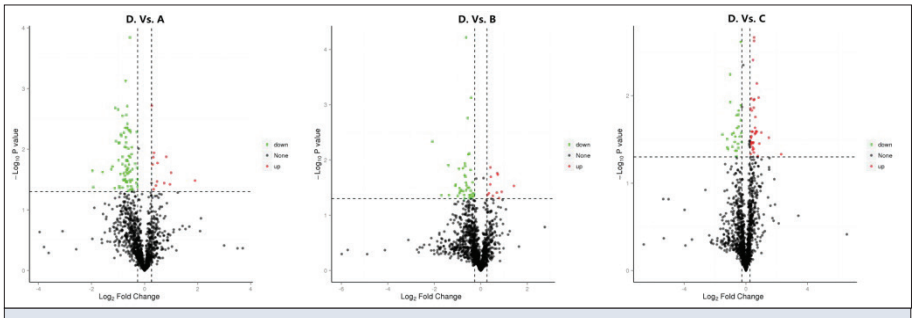

Fig 2. Differentially expressed proteins, including 3 replicates with Tibetan sheep colostrum whey during treatment groups (A, B, C) and control group (D). The image presents the relative abundance of proteins using different colors, where deeper red represents higher intensity and blue represents lower intensity

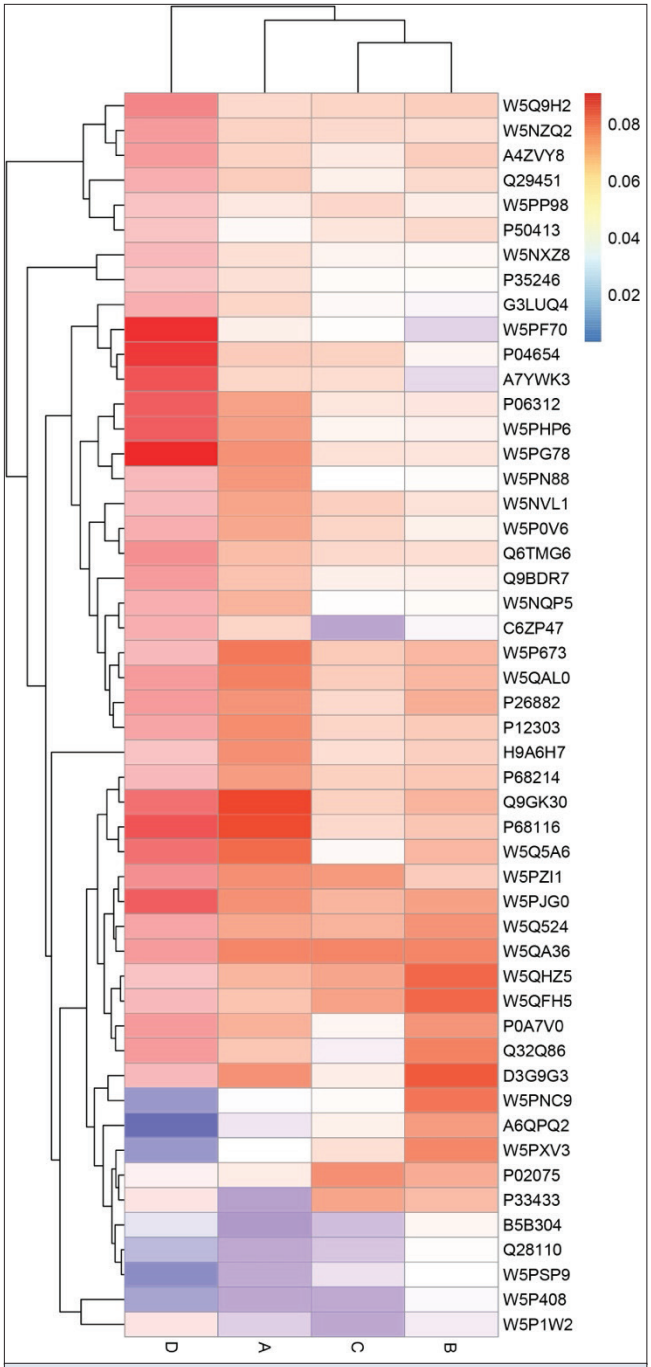

Fig 3. Differentially Expressed Proteins (DEPs) Cluster Evaluation. Down was the DEPs of treatment group were less than control group; Up was the DEPs of treatment group were more than control group; None was the DEPs of treatment group were no significant differences than control group

which yielded a pattern consisting of two major sample clusters. The sheep milk of treatment groups shared similar proteomic patterns and 14% protein dietary level and 16% protein dietary level milk comprised one sub cluster and 12% protein dietary level milk joined this group to constitute another sub cluster. The sheep milk of control group was comprised of another sub cluster *(Fig. 3)*. Although the sheep milk of treatment groups was the same differentially expression proteins patterns, hierarchical clustering analysis revealed differences in sheep milk of different protein level dietary. These differentially expression proteins are clearly presented in the hierarchical clustering map via view tree software.

We further performed biological function process and signaling pathways to investigate the function of these differentially expression proteins. BLAST2GO software and KAAS database were used to participate the 30 biological function process (GO-BP) and 20 signaling pathway of 50 differentially expression proteins *(Fig. 4, Fig. 5)*. The top 5 biological process categories were: Protein transmembrane transport (21.0%), Protein regulation metabolic process (15.6%), Biological regulation (9.1%), Regulation of biological process (6.9%), and Response to stimulus (6.4%). And top 5 pathways categories were: Fatty acid metabolism (P=0.06), Glycerophospholipid metabolism (P=0.10), Protein digestion and absorption (P=0.13), Ras signaling pathway (P=0.14) and Cell adhesion molecules (P=0.18). The treatment groups showed increase in the abundance of regulation metabolic process (especially protein metabolism and fatty acid metabolism), along with decrease in stress reaction process.

The protein-protein interaction network was produced for the 50 differentially expressed proteins via the database at www.string-db.org *(Fig. 6)*. As expected, the target proteins constituted a complex and strong PPi network, and those results of this analysis identified that Lactoferrin, Alpha-S2-casein, Superoxide dismutase [Cu-Zn], Alpha-s1-casein, Alpha globin and Lactoperoxidase appeared in the center of the PPi network intersection indicating their important role in the protein interactions. Hence, DEPs could be vital for function and physiological operation in addition to the protein composition of the Tibetan sheep colostrum whey. Interestingly, to provide further insights into the biological processes identified by this approach, we took Fishers' test (Significance A/B Test) for target proteins, those sixproteins from our results were not only at the center of the functional network intersection, but also exhibited the greatest variability between biological relevance in milk composition.

To elucidate the correspondence between the transcript level of mRNA and abundance of protein species, transcriptional analysis of 6 differentially expression protein were performed by qPCR *(Fig. 7)*. The transcript levels of four genes displayed the same trend with the abundance of the corresponding protein species, such as Lactoferrin, Superoxide dismutase [Cu-Zn], Alpha globin and Lactoperoxidase. In contrast, the expression level of two genes (Alpha-S2-casein and Alpha-s1-casein) showed the opposite trend with the abundance of their corresponding protein species. The discrepancy between the transcription level of the two genes and the

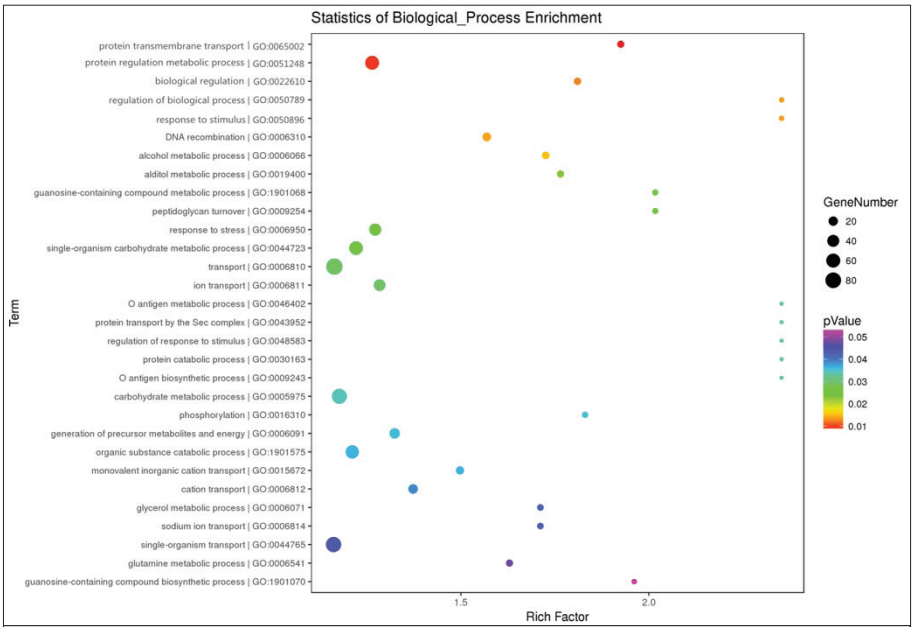

Fig 4. Differentially Expressed Proteins (DEPs) GO-BP Enrichment Evaluation

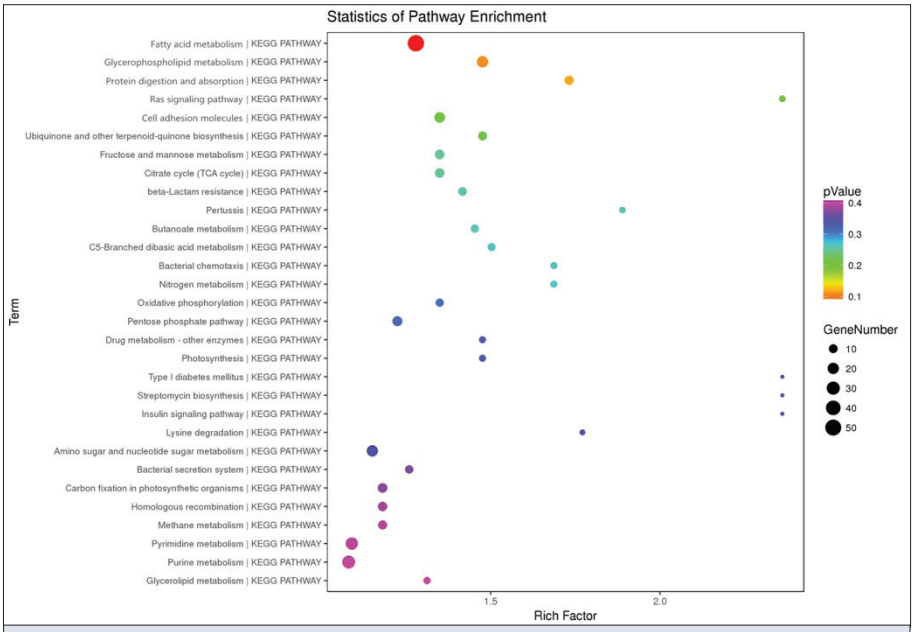

Fig 5. Differentially Expressed Proteins (DEPs) KEGG Enrichment Evaluation

abundance of the corresponding protein species probably resulted from various posttranslational modifications under different dietary protein level stress, such as protein phosphorylation and glycosylation.

Discussion

Proteins are substances for basis of life and specific practitioners of life activities [18]. Dietary protein was the limiting nutrient element that affects mammal milk yield and lactoprotein, and milk protein intake was the key factor for the survival of newborn lambs [19]. The major challenge for ruminant milk researchers was the complexity of the milk composition response to dietary composition [20]. Some researchers showed that reducing dietary protein content in a certain range could improve milk nitrogen efficiency and reduce nitrogen excretion through feces and urine [21]. Although milk composition has been extensively studied in the fields of proteomics, epigenetics, transcriptomics, and molecular biology, the mechanisms by which dietary protein composition affects milk composition still need to be further elucidated.

Colostrum intake was the key factor for the survival of newborn mammals, which contained many special

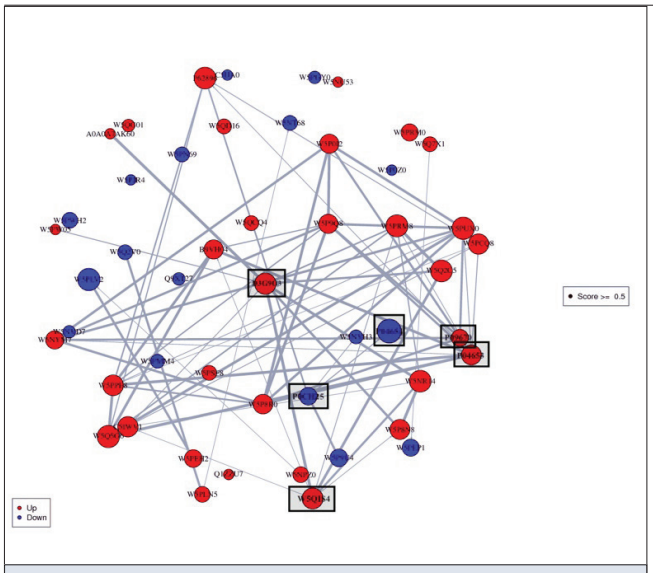

Fig 6. Differentially Expressed Proteins (DEPs) PPIs Evaluation. The marked zone was enriched DEPs, the red points were up-regulated DEPs. and the blue points were down-regulated DEPs

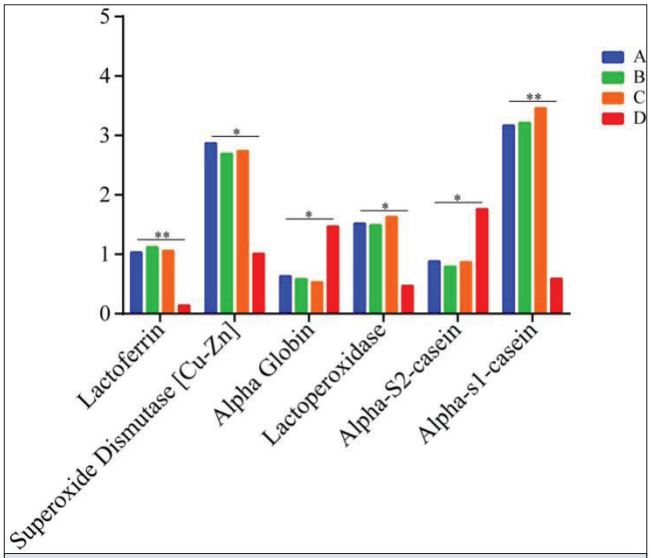

Fig 7. Effects of different dietary protein levels on the differentially expressed proteins coding genes in Tibetan sheep colostrum whey during treatment groups (A, B, C) and control group (D)

function proteins to promote development of gastrointestinal tract and improve immunity [22]. The milk nutrients could be obtained in the biosynthesis of mammary gland epithelial cells by using the raw materials in the blood [23,24]. Therefore, the various components of milk derive from the blood, and the nutrients in the blood were provided by the feed. In present study, we screened 50 differentially expressed proteins that demonstrated differential expression in sheep milk with different protein level dietary using iTRAQ technology, we found that 6 proteins (Lactoferrin, αS1-casein, Superoxide dismutase [Cu-Zn], αS2-casein, Alpha globin and Lactoperoxidase) had exhibited the greatest variability between biological relevance in milk composition and warrant further study. Previous studies have demonstrated that dietary protein levels determine αS1-casein protein synthesis and are particularly significant in high-protein diets [25]. This coincides with our findings.

NRC estimated the effect of diet protein on milk yield based on 393 data from 82 experiments showed the mammal can make better use of protein in low protein diet condition, but high protein diet had little effect on milk performance, increased nitrogen excretion, and resulted in low protein utilization efficiency [14]. The six candidate target proteins played an important role in improving Tibetan sheep milk yield and colostrum whey protein composition in our experiment. Superoxide dismutase [Cu-Zn], like Alpha globin, was an active substance derived, which could eliminate harmful substances produced in the process of metabolism. It could regulate the body immunity, which is closely related to the early pregnancy and immune tolerance of mammals. The intake of colostrum could not only increase the ewes' content of immune protein, but also increase the content of lambs' non-immune protein [26]. Lactoferrin (IF) was a kind of non heme iron binding glycoprotein with high biological activity, which was rich in mammalian colostrum [27]. In mammalian body, LF had the functions of balancing the iron element promoting the growth of intestinal beneficial bacteria, enhancing the immunity, broad-spectrum antibacterial and antiviral, regulating body metabolism, and acting as the activator of cell growth promoting factor. Meanwhile, it could also be used as a transcription activator or trans-activator to bind cell receptor, and participate in mitogen activated protein kinase/extracellular signal regulated kinase interference and nuclear factor NK-κB immune response pathway to promote the maturation of T lymphocytes [28], activate the activity of natural killer cells, release IL-1 and IL-2 to play the role of immune regulation [29]. Casein was the main protein in sheep milk, which had high nutritional value. αS1-casein and αS2-casein were the main components of casein. αS1-casein and αS2-casein were the highly phosphorylated proteins [30]. After enzymatic hydrolysis and phosphorylation, they could combine with calcium, magnesium, iron, zinc and copper to form soluble phosphopeptides, thus promoting the absorption of metal ions by the body [31]. The phosphorylated casein in sheep milk could not only resist the hydrolysis of various enzymes in the digestive tract, but also form soluble substances with calcium to prevent calcium from forming calcium phosphate precipitation [32]. Meanwhile, it could effectively prolong the retention time of calcium in the body, promote the absorption of mineral elements in the intestine, promote in vitro fertilization of animals, enhance immunity and induce apoptosis. lactoglobulin was a member of the lipid transporter family [33]. The

milk specific protein synthesized by mammary epithelial cells was a high-quality protein with the best proportion of amino acids and high content of branched chain amino acids [34]. For newborn lambs, lactoglobulin had the function similar to immunoglobulin, and had the physiological activities such as antibacterial, antiviral, antioxidant, etc. for example, when vitamin E was deficient in the body, and the protein could improve the content of reduced glutathione in the liver, thus enhancing the antioxidant capacity of cell membrane [35]. In our study, 14% protein level supplementary feed could optimize sheep milk Superoxide dismutase [Cu-Zn], Alpha globin, LF, αS2-casein, αS1-casein and Lactoglobulin content, which would be helpful for lamb's growth and development. The casein result of qPCR was contrary to iTRAQ, which might be caused by the inconsistency of mRNA and protein expression due to the post transcriptional phosphorylation of α-casein. Previous studies have demonstrated that α-caseind expression is regulated by transcription factors [36].

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (J. Jia).

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Conflicts of Interest

The authors declare no conflict of interest.

Ethics Statement

Animal experiment was approved by the Institutional Animal Care and Use Committee (State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University) (QHDX-19-10-07-06).

Authors' Contributions

Hao REN and Qian CHEN: the hypothesis of this study; Hao REN and Yingying ZHANG: work management, article writing; Qian CHEN and Huaixia ZHANG: experimental procedure follow-up, statistical analysis; Qian CHEN and Yingying ZHANG: literature review, review of results; Jianlei JIA: final decision, funding support.

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RESEARCH ARTICLE

Isolation and Amplification of the *phy* Gene Coding Phytase from *Bacillus sp.*

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ABSTRACT

Poultry are unable to metabolize phytic acid in feed due to no enzyme to digest and degrade it. Therefore, phytic acid becomes an anti-nutritional substance in poultry feed which can result in decreased feed efficiency, decreased nutrient intake which gave a negative impact on health and production. The addition of phytase in the poultry ration is necessary to degrade phytic acid. However, the availability of phytase in Indonesia is still limited because technology in Indonesia is still inadequate to produce phytase, so Indonesia cannot produce it itself and still imports phytase at quite high prices. This study aims to isolate and amplify the *phy* gene encoding phytase derived from native microbe's genus *Bacillus* from Indonesia. The method used in present study was to isolate the genome of *Bacillus* using cell lysis method, then amplify the genome using polymerase chain reaction (PCR) method. The results of this study were that genome isolation results from *Bacillus* gene sources were obtained that the purity of A_{260}/A_{280} from *B. sp* 6, *B. sp* **7,** and *B. licheniformis* was 1.86 µg/mL, 1.95 µg/mL and 1.86 µg/mL, respectively. Furthermore, the present study showed that 0.4 µM/µL primer concentration was able to amplify the *phy* gene. 200-400 ng/µL DNA template concentration can produce optimal DNA bands during amplification of the *phy* gene in *Bacillus sp***.** In conclusion, amplification was successfully carried out on *B. sp* and *B. licheniformis***.** The length of the *phy* gene in *B. sp* is 1149 bp and the *phy* gene in *B. licheniformis* is 1146 bp. The genus *Bacillus* native to Indonesia has the potential to be continued as a source of phytase producing genes.

Keywords: Amplification, *Bacillus*, Isolation, PCR, Phytase

INTRODUCTION

In general, poultry feed is formulated from cereals, grains, wheat, and legumes [1]. One of the important elements contained in these plants is phytic acid. Phytic acid *(myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate)* is the dominant phosphorus storage compound in plants [2]. Some animal feed raw materials such as rice bran, soybean meal and pollard contain lots of phytic acid. It is known that poultry is unable to metabolize phytic acid due to the lack of phytate hydrolytic enzymes inside digestive system [3]. Therefore, phytic acid is an anti-nutritional agent in poultry because it can chelate mineral elements needed by poultry such as calcium, magnesium, copper and zinc [4].

Anti-nutritional substances contained in animal feed raw materials can result in a decrease in feed efficiency due to a decrease in nutrient absorption that affects digestion, health and livestock production. Phosphorus is an important mineral for the growth and development of poultry bones [5]. Phosphorus deficient in poultry lead to detrimental effects such as bone deformities and disruption of metabolic processes as well as low performances [5]. In order for poultry phosphorus needs to be met, it is necessary to add inorganic phosphate to poultry feed. However, phosphorus excreted through feces is the main cause of environmental pollution problems [6].

Phytase has been used as feed additive to help degrade phytic acid in feed. Phytase *(mio-inositol hexaxisphosphate phosphohydrolase)* is an enzyme capable of hydrolysis the reaction of a phosphodiester bond in phytic acid and produces inorganic phosphate and phosphate esters

from lower mio-inositol [4]. Phytase is generally used as an enzyme in monogastric animal feed to increase the bioavailability of phosphorus phytate and other nutrients [7].

The availability of phytase enzymes in Indonesia is limited. Indonesia is still importing phytase at quite high prices because technology and human resources in Indonesia are still inadequate to produce phytase its own phytase. Therefore, it is important to carry out various efforts to overcome and fulfill these needs. One of the efforts made is to over-express through genetic engineering which can be sourced from various sources. Phytase can be sourced from plants or microorganisms [1]. Indonesia is rich in biological natural resources that have the potential source to produce phytase like native microbial gene sources in Indonesia. A previous study showed that there was a prominent bacteria like *Bacillus* genus [4]. In this study the phytase-type *β-propeller phytases* (BPPs) were characterized, mainly from *Bacillus sp*. considered as potential candidate based on its characteristics [1]. The BPP have good thermostability and usually maintain their maximum activity at neutral or alkaline conditions [3]. BPP phytase is a potential candidate as a feed additive in the aquaculture industry and environmental applications because it has a neutral to alkaline pH profile and higher thermal stability [1].

Considering that there are many benefits from phytase, such as increasing phosphorus absorption, reducing dependence on phosphorus supplements, increasing feed efficiency, and reducing water and soil pollution [8], production of the phytase enzyme is important, especially in Indonesia, which currently cannot produce its own. Before producing phytase, the *phy* gene is needed, which is obtained by isolating the gene in a microbe to obtain DNA from the microbe. Then it is amplified to produce many copies of the DNA sequence using the polymerase chain reaction (PCR) method [9]. We hypothesize that native Indonesian *Bacillus* sp. has significant potential as a commercial bacterial phytase because of its biological characteristics, including substrate selectivity, thermophilicity, resistance to proteolysis, and catalytic efficiency. Therefore, the objective of this study is to isolate and amplify the *phy* gene encoding phytase derived from microbes native to Indonesia from the genus *Bacillus*.

Material and Methods

Ethical Statement

All experimental procedures were approved by the Research Ethics Committee Universitas Padjadjaran.

Materials

The study was using bacteria from the genus *Bacillus* as a gene source isolate. The bacteria were obtained from the National Research and Innovation Agency (BRIN) InaCC collection, Indonesia. There were 3 isolates from different *Bacillus* species according to the code and origin. *Bacillus* sp. 6 (InaCC B348) originating from tauco, Satonda Island in West Nusa Tenggara, Indonesia, *Bacillus* sp. 7 (InaCC B694) originating from stone, Solok in West Sumatra, Indonesia and *Bacillus licheniformis* (InaCC B1088) originating from marine sediments, in Rambut Island, Seribu, Indonesia. The bacteria were rejuvenated in liquid nutrient broth (NB) media and incubated for 16 h (overnight) in a shaker incubator at 37°C until the growth was homogeneous.

Genome Isolation

The bacterial genome was isolated using a modified cell lysis method based on the kit protocol used for genome isolation, namely the *Presto™ Mini gDNA Bacteria Kit* (Geneaid). Genome isolation methods include pre-lysis, cell lysis, DNA binding, washing, and DNA elution.

After the genome has been successfully isolated, the results of the genome isolation are checked using a DNA electrophoresis gel agarose 1%. DNA concentration and purity were measured using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

Primer Design

Gene sequence data of *Bacillus* sp. and *B. licheniformis* was obtained from the gene bank, namely the National Center for Biotechnology Information (NCBI) *(https://www.ncbi.nlm.nih.gov/)* which was then used for primary design. Specific primers for the *phy* gene were used to amplify the phytase encoding gene from isolated genomic DNA from *Bacillus* sp. and *B. licheniformis* designed manually. Primer design was carried out using several software, namely *Oligo Calculator*, *Fast PCR*, and *SnapGene*. Each primer consists of a forward primer (F) and a reverse primer (R).

Amplification *phy* Gene

The PCR parameters in this study consisted of 5 stages, these are pre-denaturation, denaturation, annealing, elongation, and final elongation [10]. Amplification of the *phy* gene using the *My Taq™ HS Red Mix PCR Kit* (Meridian Bioscience, Bioline) was carried out according to the protocol in the kit. The PCR program consisted of (1) initial denaturation for 3 min at 95°C, followed by 35 cycles of (2) denaturation for 15 sec at 95°C, (3) annealing for 15 sec with different temperatures, (4) elongation for 1 min at 72°C, and (5) final elongation for 5 min at 72°C. The third stage of PCR, which is a crucial stage to determine amplification result. Gradient annealing temperature (Ta) for *B. licheniformis* varies in the range 53-61°C. Ta in *Bacillus* sp. *6* and *Bacillus* sp. 7 varies in the range 53-59°C. In the end, the PCR results were checked by DNA electrophoresis.

Results

We obtained that the concentration and absorbance ratio of DNA in the genome of each *Bacillus* species varied. It was found that the concentration of *Bacillus* sp. 6 was 69.1 ng/µL with an absorbance ratio of 1.86 µg/mL (A_{260}/A_{280}). The concentration of *Bacillus* sp. 7 was 184.1 ng/µL with an absorbance ratio of 1.95 µg/mL (A_{260}/A_{280}). The concentration of *B. licheniformis* was 208.2 ng/µL with an absorbance ratio of 1.86 µg/mL (A_{260}/A_{280}). In this study, the genome isolation results were examined. Muzuni [11] reported that the value of the genomic DNA concentration of *Bacillus* sp. *Rh* is 1,460 µg/µL. DNA concentration is categorized as high concentration if it has a concentration value of 1000 µg/µL. A good DNA concentration can vary depending on the purpose and type of analysis to be performed. In molecular applications, generally the DNA concentration that is considered good ranges from 50 to 500 ng/µL. Olmedo [12] reported that the DNA concentration of *Bacillus* spp. of 40 ng/µL. The study in line with Sambrook & Russell [13], the purity of a sample can be declared pure if it has an A_{260}/A_{280}-A_{260}/A_{230} ratio of around 1.8-2.0 µg/mL. Even though the DNA concentration is slightly low, this does not affect its purity.

Based on gene bank data, *Bacillus* sp. have the *phy* gene encoding phytase. The number of sequences found was 19 data with the length of the *phy* gene being 1149 base pairs (bp) and 7 *phy* gene sequences from *B. licheniformis* with length 1146 bp. Specific primers for the *phy* gene were used to amplify the phytase encoding gene from isolated genomic DNA from *Bacillus* sp. designed manually. Each primer consists of a forward primer (F) and a reverse primer (R). The length of the *Bacillus* sp. primer bases for the forward primer is 30 bases (5'-ATGAAGGTTTCAAAAACAATGCTGCTAAGC-3') with melting temperature (Tm) 57.5°C and 37% GC content and the reverse is 18 bases (5'-CTAGCCGTC CAGAACGGTC 3') with Tm 52.6°C and 61% GC content. The length of the *B. licheniformis* primer bases for the forward primer is 23 bases (5'-ATGAACTTT TACAAAACGCTCGC-3') with Tm 51.7°C and 39% GC content and the reverse is 28 bases (5'-TTATTT GGCTCGTTTTTTCAGTTTTCGG-3') with Tm 55.5°C and 36% GC content. The primers design results have been tested in silico using *Fast PCR* and *SnapGene* applications. *Fast PCR* results show that the primer can attach 100% to the template and was able to amplify the gene as a whole (100%). PCR simulations using the *SnapGene* application showed that primers can amplify the *phy* gene.

Amplification was measured using the PCR method and examined using DNA electrophoresis. It was found that the *phy* genes of *Bacillus* sp. 6, *Bacillus* sp. 7, and *B. licheniformis* had been successfully amplified. We obtained that a primer concentration of 0.4 µM/µL was able to amplify the *phy* gene and varying DNA template concentrations (200-400 ng/µL) in each species could produce optimal DNA bands for use in amplifying the *phy* gene in *Bacillus sp*.

An important step during the PCR process is determining variations in annealing temperature (Ta gradient). In *Bacillus* sp. 6 and *Bacillus* sp. 7, the annealing temperature varied over a Ta gradient from 53-59°C and all showed positive results. The best annealing temperature for *Bacillus* sp. 6 is 54.98°C and for *Bacillus* sp. 7 is 56.42°C. The *B. licheniformis phy* gene was successfully amplified at an annealing temperature of 53.54; 53.72; 55.64; 54.98 and 56.42°C.

Discussion

Considering the limited availability of the phytase enzyme in Indonesia, we are trying to utilize biological resources by isolating and amplifying the *phy* gene encoding phytase originating from *Bacillus* native to Indonesia as the first step in genetic engineering for phytase production. According to Saadi et al.[4], phytase derived from *Bacillus* genes has been widely studied and is a type of phytase that has unique characteristics, as well as the feasibility of mass production for application in animal nutrition. This study was conducted to test the potential of native Indonesian *Bacillus* as a *phy* gene encoding phytase.

The principle of genome isolation is to destroy the cell wall without damaging the target DNA [3]. In the present study, 1 kb DNA ladder marker was used as a marker indicating that the genome of *Bacillus* sp. *B. licheniformis* and has a size above 10000 bp. These results showed positive results, which means that DNA has been successfully isolated. Genome isolation aims to obtain pure DNA [14]. The quality of DNA in genome isolation is very important because it affects the success and accuracy of various analyzes and experiments involving genomic DNA. In this study, all *Bacillus* genome isolation results were obtained with good DNA quality. We obtained that the purity of A_{260}/A_{280} from *Bacillus* sp. 6, *Bacillus* sp. 7, and *B. licheniformis* was 1.86 µg/mL, 1.95 µg/mL and 1.86 µg/mL, respectively. DNA can be declared pure since it has an absorbance ratio A_{260}/A_{280} in the range of 1.8-2.0 µg/mL. This study in line with research conducted by Ni'mah [15] namely The Quantity and Quality Comparison of *Bacillus sp*. DNA between Heat Treatment and Filter Based Kit and in line with research conducted by Bonis et al.[16], who isolated *Bacillus thuringiensis* DNA using the DNeasy Blood and Tissue Kit (Qiagen) with an absorbance ratio of A_{260}/A_{280} and A_{260}/A_{230} between 1.5 and 2.5. If the purity A_{260}/A_{280} value is below 1.8 then the DNA resulting from the isolation carried out is likely to be contaminated with protein, and if the purity value is above 2.1 then it can be suspected

that the DNA resulting from the isolation carried out is contaminated with RNA [17,18].

The design of the primers aims to obtain primers that will be used when amplifying the DNA of the *phy* gene by the PCR method. The length of the primer base of *Bacillus sp*. for forward (F), that was 30 bases and 18 bases for reverse (R). The length of the primer base of *B. licheniformis* is 23 (F) and 28 (R) bases. The primer pair serves to limit the target DNA fragment to be amplified [3]. The ideal primary length range was between 18-30 bp [1]. Moreover, Saadi et al.[4]'s research used *Bacillus subtilis* to design primers for the *phyC* gene, in lenght 28 (F) and 31 (R) bases.

The optimal Tm for primers ranges from 52 to 58°C [2]. Tm *Bacillus sp*. for the forward primer at 57.5°C and for the reverse primer at 52.6°C. The temperature difference is one of the considerations in the primary design. Primer pairs that have a difference in Tm exceeding 5°C can reduce the amplification process, and may even have the potential to prevent the amplification process [19]. The difference in Tm in the primer *Bacillus* sp. and *B. licheniformis* was 4.9°C and 3.8°C, respectively.

The GC contents in the primer *Bacillus* sp. were (F) 37% and (R) 61%. The GC content in the primer *B. licheniformis* were 39% (F) and 36% (R). The base content of GC was detected to range between 40% and 60% [19]. The study in line with [28] that the optimum G+C base composition is in the range of 35-60% with the difference in GC content between the two primers being in the range of 5%. The higher the GC content in the primer, the better it will be because the adhesion will be much stronger. Guanine and Cytosine have 3 hydrogen bonds which means the bond is stronger when compared to Adenine and Thymine which only have 2 hydrogen bonds. The GC content is sought to be at the 3' end sequence therefore the primary attachment bond is not easily separated [20]. The high GC content will affect the Tm value [21].

Amplification is the process of taking genes from chromosomal DNA in vitro using the polymerase chain reaction (PCR) method. PCR is a method used to amplify millions of DNA segments in a short time [20]. The *phy* gene amplification process using the PCR method needs to pay attention to the composition, concentration, and parameters of the PCR. PCR composition includes DNA templates, gene-specific primers, dNTPs, PCR and $MgCl_2$ buffers, as well as DNA polymerase enzymes [20].

The DNA template concentration used was (Sp 6.1) 276.4 ng/µL, (Sp 7.1) 368.2 ng/µL, and (L1) 416.4 ng/µL with as much volume 2 µL for all sample. The primary concentration used was 0.4 µM/µL. There are 5 parameters observed in the PCR process [22]. These parameters include initial denaturation, denaturation, annealing, elongation, and final elongation [22]. The PCR method has a working principle that mimics DNA replication in cells *in vitro* therefore it can be used in this study to amplify DNA in the *phy* gene. The PCR process carried out lasts up to 35 cycles.

The annealing temperature will vary within a predetermined range when designing the primer. Ta on *Bacillus* sp. varies in the range 53-59°C. Ta optimum for *Bacillus* sp.6 is 54.98°C, for *Bacillus* sp. 7 is 56.42°C and for *B. licheniformis* is 54-56°C. According to Yusuf [23], the annealing temperature ranges between 36-72°C, but the temperature generally used is between 50-60°C. Moreover, Sugiarti et al.[24] in their research the PCR process using an annealing temperature of 45-50°C from *B. subtilis* and 65°C from *B. licheniformis* [24]. This difference is because the longer the primary size, the higher the temperature [23].

The amplification results were checked by DNA electrophoresis gel agarose 1%. *Bacillus* sp. was successfully amplified with a base length of 1149 bp. The results of our study are in line with Rao [25] that the length of the *phy* gene from *Bacillus sp* (DECSR1) is 1149 bp. In accordance with data at NCBI that *Bacillus sp*. has a length of 1149 bp which can translate 383 amino acids. *B. licheniformis* was successfully amplified with a base length of 1146 bp. The results are in line with Li [26] dan Liu [27] that the length of the *phy* gene from *B. licheniformis* is 1146 bp.

In conclusion, the results of genome isolation of native *Bacillus* sp. 6, *Bacillus* sp. 7 and *B. licheniformis* from Indonesia were pure and can be continued for amplification. The annealing temperature that can be used in PCR for *Bacillus* sp. 6 and *Bacillus* sp. 7 varies with the optimum temperature of 54.98°C and 56.42°C respectively. The optimum Ta for *Bacillus licheniformis* is in the temperature range of 54-56°C. Amplification was successfully carried out on *Bacillus* sp. 6, *Bacillus* sp. 7 and *B. licheniformis*. Therefore, the three *Bacillus* species native to Indonesia has the potential to be continued as a source of phytase producing genes.

Availability of Data and Materials

The data supporting this study's findings are available from the corresponding author (N. Mayasari) upon reasonable request.

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Ethical Statement

All experimental procedures were approved by the Research Ethics

Committee Universitas Padjadjaran.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributıons

DAS, YW, NH, AH, ASHAI, LT, and NM compiled and planned the experiment. DAS, LT, and NH conducted data collection and analysis. DAS, LT, and NM contributed to the interpretation of the results. DAS led the script writing. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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RESEARCH ARTICLE

Evaluation of Methodological and Reporting Quality of Systematic Reviews and Meta-Analyses Published in Veterinary Journals with AMSTAR

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ABSTRACT

The complete and transparent reporting of systematic reviews and meta-analyses increases the quality of such studies. Although there are different tools to examine methodological quality, little research has been conducted on the quality of these studies in animal health. The objective of this study was to evaluate the complete reporting of systematic reviews and meta-analyses published in veterinary journals with "**A** **M**ea**S**urement **T**ool to **A**ssess Systematic **R**eviews" (AMSTAR). The journal's impact factor, the number of authors, the number of studies included, and the research period were extracted as article characteristics. Total quality scores were calculated according to the AMSTAR tool, and scores were compared using the aspects of the articles. This study assessed 207 systematic reviews and meta-analyses published in 130 veterinary journals. AMSTAR quality scores were higher for meta-analyses with fewer than five authors compared to studies with five or more authors (P=0.009). Our findings indicate that about half of all studies (51.2%) were of moderate quality regarding methodology and reporting. According to the evaluation with AMSTAR, 64.6% of systematic reviews and meta-analyses were of high quality. In conclusion, the reporting quality of the studies was good, but generally, there was insufficient information on assessing publication bias.

Keywords: AMSTAR, Meta-analysis, Reporting quality, Systematic review, Veterinary medicine

INTRODUCTION

Evidence-based studies are critical to clinical decision-making in human and veterinary medicine, as they integrate the best research evidence with clinical expertise and patient values. According to the hierarchy of studies, systematic reviews and meta-analyses are classified as the highest level of evidence [1,2]. A systematic review synthesizes the results from all available studies on a particular subject and comprehensively analyzes the collated studies' results, strengths, and limitations. A meta-analysis, on the other hand, is a statistical method that combines the findings of independent studies on the same subject, which is suitable for systematic review [3]. Therefore, animal health clinical decision-making should be based on aggregating the best evidence rather than the results of individual studies [4].

Systematic reviews and meta-analyses are essential for clinical decisions in veterinary sciences as they allow the quantitative evaluation of treatment effects and uncertainty [1,5-8]. Using such studies as a decision-making tool in clinical practice is not new, yet these studies are becoming increasingly more common in the literature [2,9].

The quality and reliability of systematic reviews and meta-analyses depend on many concepts, such as research questions, comprehensive literature review, and quality of the original studies. Excellent systematic reviews and meta-analyses should have a detailed methodology that can be reproduced [3]. Although clear methodology is a feature of systematic review and meta-analysis, the methodology may be incompletely or inadequately reported. Poor reporting and methodological quality may hinder the provision of appropriate information to clinicians [9].

Evaluating the quality of the systematic reviews and meta-analyses is essential and recommended in animal health to improve the reporting quality of these studies [7,10,11]. The increasing number of such studies has brought along the implementation of various standards to increase the quality of these studies. Some journals refer to specific reporting guidelines for systematic reviews and meta-analyses and recommend that authors follow these guidelines. Various

guidelines, checklists, and assessment tools for different study designs have been developed to improve and evaluate the quality of such studies [12-17]. These guidelines lead authors to report their results as fully as possible, thus providing more transparency in the reporting process. These tools, with checklists, are frameworks developed to improve the quality of systematic reviews and meta-analyses by checking whether the authors report the results adequately and transparently. **A** **M**ea**S**urement **T**ool to **A**ssess **S**ystematic **R**eviews (AMSTAR), one of these tools, is the only recently developed assessment tool with proven reliability and validity designed to evaluate the methodological quality of systematic reviews and meta-analyses [7,18,19]. The AMSTAR tool is an evaluation criterion rather than a reporting guide [18]. It consists of eleven questions of denominated items that examine the creation of a literature review plan, literature selection, and data extraction, inclusion and exclusion criteria, a list of included studies and their evaluation criteria, appropriateness of methods used to combine individual research results and conflict of interest information [7].

Despite the increase in systematic reviews and meta-analyses published in veterinary journals, few studies have evaluated the methodological quality of such studies. Therefore, this study aimed to assess the completeness of reporting systematic reviews and meta-analyses published in veterinary journals using AMSTAR.

Material and Methods

Data Collection

Systematic reviews and meta-analyses published in the Thomson Reuters Clarivate Analytics database containing the word "veterinary" in the journal title were selected for data collection. Of the journals found, the authors scanned articles published between 2016 and 2021 independently using the following search terms: "meta-analysis" and "systematic review" in the article title. The inclusion criteria were: (1) studies written in English and (2) studies available in full text. Exclusion criteria were: (1) abstracts or conference proceedings, (2) protocol or guidelines, and (3) narrative reviews. Data collection took place between 31 January and 25 February 2022.

Assessment of Reporting Quality

The AMSTAR tool was used for methodological quality assessment. The AMSTAR tool developed by Shea et al.[18] consists of eleven question-denominated items. For each question on the presence of a quality item, two possible actions can be performed: "Yes," if the quality item is present, 1 point will be assigned; "No/Not Applicable" for reviews not reporting this quality item, 0 points will be given. In AMSTAR, the aggregated quality score is calculated as the sum of the scores of all items (0-4 points: Low level; 5-8 points: Moderate level; 9-11 points: High level) [7].

Data Extraction

To examine the characteristics of the included studies, the following information was extracted: publication year, journal index class (SCI-expanded, not SCI-expanded), journal's impact factor, number of studies included, research period, information about research funding, and number of authors. The research period refers to the time between the publication years of the studies included in the meta-analysis.

Study Procedure and Data Analysis

The characteristics of included studies were summarized descriptively, with n (%) and median (minimum-maximum) following the data type (categorical and continuous). All reviewed articles were divided into systematic reviews only (SR), systematic reviews and meta-analyses (SR/MA), and meta-analyses only (MA) based on their titles. The methodological quality of each included study was evaluated by two authors independently using the AMSTAR tool. Any disagreements between the authors were resolved by consensus. Final AMSTAR scores were obtained by summing the scores assigned to each item. First, the total quality scores were compared according to the study's characteristics in each study type. The median was used as the threshold value for grouping studies to ensure a similar sample size to compare characteristics as described above. Then, the total AMSTAR score was compared between SR, MA, and SR/MA without separating them according to the relevant characteristics. Because the data did not meet parametric test assumptions, the Kruskal-Wallis test was used to compare more than two groups, and the Mann-Whitney U test was used to compare groups in pairs. Journal index class (Science Citation Index-expanded or not) feature was not compared due to insufficient sample size in the groups. IBM SPSS v23.0 software was used for data analysis, and P values less than 0.05 were considered significant.

Results

One hundred thirty journals included 'veterinary' in the Thomson Reuters Clarivate Analytics database. During the search period, two hundred and seventeen individual studies were identified, including "systematic review" and/or "meta-analysis" words in the title. Further, ten studies were excluded for the following reasons: conference paper or abstract (n=2), protocol or guide describing the implementation of the meta-analysis (n=2), and narrative reviews (n=6). Thus, two hundred and seven studies were included, fully meeting the specified inclusion/exclusion criteria.

Table 1. Descriptive statistics of the included studies published in veterinary journals between 2016-2021 (n=207) (categorical variables)

Study Characteristics	Categories	n	%
Study type	SR/MA SR MA	79 84 44	38.2 40.6 21.3
Publication year (all study types)	2016	20	9.7
	2017	22	10.6
	2018	28	13.5
	2019	27	13.0
	2020	45	21.7
	2021	65	31.4
Journal Index (all study types)	SCI-expanded Not SCI-expanded	181 26	87.4 12.6
Funding support (all study types)	Yes	91	44.0
	No	116	56.0

SR: Systematic Review, MA: Meta-analysis, SR/MA: Systematic review and meta-analysis

Table 2. Descriptive statistics of the included studies published in veterinary journals between 2016-2021 (n=207) (continuous variables)

Study Characteristics	Median	Minimum	Maximum
Journal Impact Factor*	2.67	0.05	3.69
Number of included studies	28	4	578
Research period of included studies (year)	21	1	117
Number of authors	5	1	23

* *n=204 (Information about the impact factor of one journal in which only three articles were published was not available)*

Characteristics of the Included Studies

The descriptive statistics of the characteristics of the included studies are given in *Table 1* and *Table 2*. The number of SR (40.6%) included in the study was observed to be higher than MA and SR/MA. As a result of the literature review covering 2016-2021, an increase has been observed in the number of SR/MA in animal health. At the same time, most (87.4%) of the journals in which these articles were published were covered by the Science Citation Index (SCI-expanded). The impact factor values of the journals varied between 0.05 and 3.69. The number of studies included in the evaluated SR, SR/MA, and MA ranged from 4 to 578, with study periods ranging from 1 to 117 years. The number of authors in the included studies ranged from 1 to 23.

Methodological Quality Assessment

The summary of the methodological quality assessment results of the studies examined according to the AMSTAR tool is provided in *Fig. 1*. The AMSTAR score for 79 SR/MA studies ranged from 4 to 11, with a median of 9.00. One study (1.3%) was rated as "low," twenty-seven studies (34.2%) were rated as "moderate," and fifty-one studies (64.6%) were rated as "high" according to the methodological quality. For 84 SR, the final AMSTAR score ranged from 2 to 11, with a median value of 7.00. Twelve (14.3%) of these SR studies were rated as low, fifty-eight studies (69.0%) were rated as moderate, and 14 (16.7%) studies were rated as high quality. As for the 44 MA studies, the final AMSTAR score ranged from 1 to 11, with a median value of 7.00. Twelve (27.3%) of SR were rated as low, twenty-one (47.7%) of these were rated

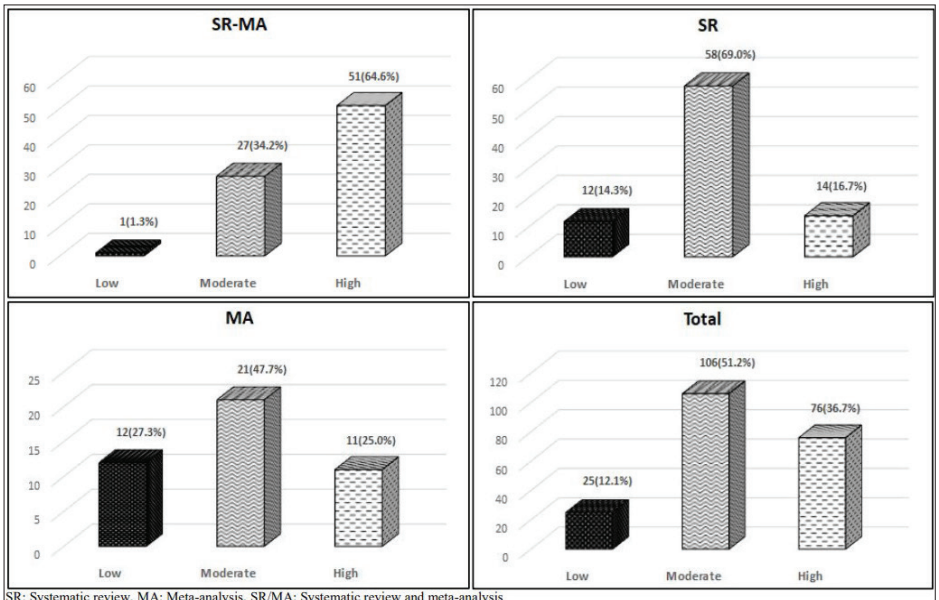

Fig 1. Quality assessment results according to AMSTAR of included studies published in veterinary journals between 2016 and 2021

Table 3. *Question content of AMSTAR [18] and summary of quality assessment criteria for included studies published in veterinary journals between 2016 and 2021*

Item	Status	SR (n=84)		MA (n=44)		SR/MA (n=79)		Total (n=207)	
		n	%	n	%	n	%	n	%
1. Was an 'a priori' design provided?	Yes	61	72.6	29	65.9	62	78.5	152	73.4
	No/not applicable	23	27.4	15	34.1	17	21.5	55	26.6
2. Was there a duplicate study selection and data extraction?	Yes	43	51.2	13	29.5	59	74.7	115	55.6
	No/not applicable	41	48.8	31	70.5	20	25.3	92	44.4
3. Was a comprehensive literature search performed?	Yes	78	92.9	34	77.3	79	100.0	191	92.3
	No/not applicable	6	7.1	10	22.7	0	0.0	16	7.7
4. Was the status of publication (i.e., grey literature) used as an inclusion criterion?	Yes	70	83.3	23	52.3	71	89.9	164	79.2
	No/not applicable	14	16.7	21	47.7	8	10.1	43	20.8
5. Was a list of studies (included and excluded) provided?	Yes	72	85.7	33	75.0	72	91.1	177	85.5
	No/not applicable	12	14.3	11	25.0	7	8.9	30	14.5
6. Were the characteristics of the included studies provided?	Yes	66	78.6	33	75.0	69	87.3	168	81.2
	No/not applicable	18	21.4	11	25.0	10	12.7	39	18.8
7. Was the scientific quality of the included studies assessed and documented?	Yes	41	48.8	18	40.9	50	63.3	109	52.7
	No/not applicable	43	51.2	26	59.1	29	36.7	98	47.3
8. Was the scientific quality of the included studies used appropriately in formulating conclusions?	Yes	44	52.4	10	22.7	52	65.8	106	51.2
	No/not applicable	40	47.6	34	77.3	27	34.2	101	48.8
9. Were the methods used to combine the findings of studies appropriate?	Yes	7	8.3	29	65.9	73	92.4	109	52.7
	No/not applicable	77	91.7	15	34.1	6	7.6	98	47.3
10. Was the likelihood of publication bias assessed?	Yes	2	2.4	22	50.0	52	65.8	76	36.7
	No/not applicable	82	97.6	22	50.0	27	34.2	131	63.3
11. Was the conflict of interest stated?	Yes	61	72.6	34	77.3	64	81.0	159	76.8
	No/not applicable	23	27.4	10	22.7	15	19.0	48	23.2

SR: Systematic Review, MA: Meta-analysis, SR/MA: Systematic review and meta-analysis

as moderate, and eleven studies (25.0%) were rated as high quality. For the total of the studies reviewed (n=207), AMSTAR scores ranged from 1 to 11, with a median of 8.00. Twenty-five (12.1%) of these studies were rated as low quality, 106 (51.2%) were rated as moderate quality, and 76 (36.7%) studies were rated as high quality.

The question contents of AMSTAR and the frequency distribution with percentages are given in *Table 3*. The comparison results and descriptive statistics of the AMSTAR quality scores given jointly by the two researchers according to the study characteristics are shown in *Table 4*. The results are reported separately for SR, MA, and SR/MA, as well as for all studies. As shown in *Table 4*, AMSTAR quality scores were higher for MA with fewer than five authors than studies with five or more authors (P=0.009). Similarly, SR with fewer than five authors had higher AMSTAR scores than studies with five or more authors (P=0.028). Considering the total results, the AMSTAR score of the articles published in journals with an impact factor of 2.67 or more was higher than the studies in journals with an impact factor of less than 2.67 (P=0.018). AMSTAR scores did not differ between the groups formed in other evaluation criteria.

The total AMSTAR score differs significantly between study types (P<0.001). Only the difference between SR and MA was not significant (P=0.869).

Discussion

In the current study, we evaluated the methodological quality of the systematic reviews and meta-analyses published in veterinary journals with the AMSTAR tool. Within the scope of this study, 207 systematic reviews and meta-analyses studies were found suitable for this research. The main features of the reviewed studies were examined. More studies were published in 2021 than in any other publication year; the current research presents up-to-date data. In terms of the index, most of the studies were included in SCI-expanded indexed journals. This

Table 4. *Comparison of results of AMSTAR quality scores according to the characteristics of the included studies published in veterinary journals of the Clarivate group between 2016 and 2021*

Study Characteristics	SR (n=84)			MA (n=44)			SR/MA (n=79)			Total (n=207)		
	n (%)	Median (min-max)	P-value	n (%)	Median (min-max)	P-value	n (%)	Median (min-max)	P-value	n (%)	Median (min-max)	P-value
Publication year												
2016-2019	41 (48.8)	6 (3-9)	0.779	25 (56.8)	7 (1-10)	0.458	31 (39.2)	9 (5-11)	0.131	97 (46.9)	8 (1-11)	0.102
2020-2021	43 (51.2)	7 (2-11)		19 (43.2)	7 (1-11)		48 (60.8)	9.5 (4-11)		110 (53.1)	8 (1-11)	
Journal Impact Factor												
< 2.67	39 (47.0)	7 (2-9)	0.521	24 (55.8)	7(1-10)	0.361	25 (32.1)	9 (4-11)	0.599	88 (43.1)	7 (1-11)	0.018
2.67 and more	44 (53.0)	7 (2-11)		19 (44.2)	7 (2-11)		53 (67.1)	9 (5-11)		116 (56.9)	8 (2-11)	
Number of studies												
< 28	34 (40.5)	7 (2-9)	0.289	31 (70.5)	7 (1-11)	0.959	38 (48.1)	9.5 (5-11)	0.187	103(49.8)	8 (1-11)	0.268
28 and more	50 (59.5)	6 (2-11)		13 (29.5)	7 (3-9)		41 (51.9)	9 (4-11)		104 (50.2)	8 (2-11)	
Funding support												
Yes information	42 (50.0)	6 (2-11)	0.337	22 (50.0)	7 (1-10)	0.813	27 (34.2)	9 (4-11)	0.962	91 (44.0)	7 (1-11)	0.097
No information	42 (50.0)	7 (2-9)		22 50.0)	7 (1-11)		52 (65.8)	9 (5-11)		116 (56.0)	8 (1-11)	
The research period of included studies												
< 21 years	44 (52.4)	7 (3-9)	0.221	22 (50.0)	7 (1-10)	0.794	33 (41.8)	9 (5-11)	0.368	99 (47.8)	7(1-11)	0.051
21 years and more	40 (47.6)	7 (2-11)		22 (50.0)	6 (1-11)		46 (58.2)	9 (4-11)		108 (52.2)	8 (1-11)	
Number of authors												
< 5	40 (47.6)	7.5 (2-9)	0.028	22 (50.0)	8 (1-11)	0.009	28 (35.4)	9 (4-11)	0.356	90 (43.5)	8 (1-11)	0.451
Five and more	44 (52.4)	6 (2-11)		22 (50.0)	5 (1-10)		51 (64.6)	9 (5-11)		117 (56.5)	7 (1-11)	
Total score	84	7 (2-11)[b]	-	44	7 (1-11)[b]	-	79	9 (4-11)[a]	-	207	8 (1-11)	<0.001*

*SR: Systematic Review, MA: Meta-analysis, SR/MA: Systematic review and meta-analysis; *Kruskal Wallis test result*
Different letters on the row indicate the difference between study types for the total AMSTAR score

means that most of the studies were published in high-quality journals.

Researchers prefer different tools in studies to methodologically evaluate the quality of systematic reviews and meta-analyses. While Buczinski et al.[20] used AMSTAR and PRISMA, Toews [21] used only seven items of PRISMA related to literature search reporting features. Vriezen et al.[11] used only AMSTAR 2 (an enhanced version of AMSTAR consisting of 16 items) to assess the studies' quality. There are studies in which the AMSTAR tool was used outside the field of veterinary medicine [7,9,22]. In these studies, different tools were used together with AMSTAR. AMSTAR is widely used as a valuable tool to evaluate the quality of systematic reviews and meta-analyses conducted in any academic field and is an open tool that can be used without special consent. Since this tool's calculation and interpretation of methodological quality scores are more transparent and understandable, AMSTAR was preferred in this study.

In this study, according to the evaluation results obtained with the AMSTAR tool for SR, it has been observed that the number of studies that meet item 9 ("Were the methods used to combine the findings of studies appropriate?") and item 10 ("Was the likelihood of publication bias assessed?") were low *(Table 3)*. The studies that provided information on items 9 and 10 were 8.3% and 2.4%, respectively. These low numbers indicate that the statistical methods for combining and evaluating the individual study results for heterogeneity and publication bias tests were not performed or reported insufficiently in the SR examined. Contrarily, it was determined that the items related to the comprehensive literature search (item 3), the status of publication used as an inclusion criterion (item 4), and the list of studies provided (item 5) were reported in most of the SR examined. The large number of studies providing these items indicates that the authors tend to provide explanatory information, especially on literature review, inclusion criteria, and a list of included (and excluded) studies. For MA studies, the number of studies that met item 2 ("Was there a duplicate study selection and data extraction?") and item 8 ("Was the scientific quality of the included studies used appropriately in formulating conclusions?") were low. To ensure item 2,

there should be at least two independent data extractors, and a consensus procedure for disagreements should be in place. However, it was found that there was only one evaluator in most of the examined MA or that consensus procedure information was not reported for two or more evaluators. Similarly, the results of the methodological rigor and scientific quality considered in the analysis and the review's conclusions required for item 8 were poorly reported in MA. The number of MA that met items about literature search (item 3) and conflict of interest (item 11) was high. For SR/MA, it was observed that most of the items examined were reported often. The item related to the literature search (item 3) was even provided in the SR/MA. It means that at least two electronic sources were searched, and the information reported in all SR/MA included years, keywords, and databases. The least met item in the SR/MA was item 7 (63.3%), where information about the scientific quality of the included studies assessed and documented is reported. SR/MA mainly provides the relevant AMSTAR items, suggesting that writing these studies is better.

In the 207 studies reviewed, we observed that the item related to publication bias (item 10) was reported the least (36.7%), and the item related to literature search (item 3) was declared the most (92.3%). Insufficient reporting of item 10 revealed in the general evaluation was also seen in SR. The low number of studies that meet item 10 indicates that using graphical representations or statistical tests to evaluate publication bias is low, especially in SR. The reporting rates of the item related to the comprehensive literature search (item 3) were high for SR, MA, and SR/MA, as well as in the general evaluation. Of the 79 SR/MA reviewed, 11 (13.9%) fully provided all AMSTAR items. This result was seen in only one SR (1.2%) and one MA (2.3%). Of all the evaluated studies, 6.3% scored total points on the AMSTAR tool.

The AMSTAR quality scores significantly differed between the studies for SR only and MA only, based on the author number being fewer than five or more. All studies (n=207) showed a significant difference in AMSTAR scores between the journal's low or high-impact factors. The higher AMSTAR score of journals with a high impact factor compared to journals with a low impact factor may indicate that SR/MA studies are better reported in these journals. While the quality of 64.6% of the SR/MA was high, 69.0% of the SR only and 47.7% of the MA only were moderate. For the 207 articles, the overall quality was moderate with AMSTAR *(Fig. 1)*.

When the studies that methodologically evaluated the quality of systematic reviews and meta-analyses are examined, these studies seem to focus on whether the items of the quality assessment tool were generally met. Data are commonly expressed in these studies as frequencies (n) and percentages (%). Quality scores have been calculated only in a limited number of studies conducted. When the features' results were examined in the current study, the scores obtained for SR/MA did not differ statistically between the specified groups. This result means that the quality scores of the SR/MA examined do not vary based on the study characteristics.

Systematic reviews and meta-analyses are powerful and essential tools used in veterinary medicine to summarize available information, predict treatment effects more precisely, and make evidence-based decisions [5]. Accurate, transparent, and complete reporting of the systematic reviews and meta-analyses is essential and increases the methodological quality. The number of studies that evaluate the quality of systematic reviews and meta-analyses in veterinary medicine is limited. Vriezen et al.[11] assessed the quality of systematic reviews and meta-analyses examining preventive antibiotics designed to prevent disease in farm animals. In another study, Buczinski et al.[20] evaluated the quality of systematic reviews and meta-analyses available for bovine and equine veterinarians. In addition, Toews [21] examined the quality of meta-analysis studies published in veterinary journals between 2011 and 2015. Sargeant et al.[23] have similarly examined the completeness of reporting in published systematic animal health reviews with some PRISMA items.

The current study examined the quality of systematic reviews and meta-analyses published in veterinary journals. As a main result of this study, we found that the SR/MA in veterinary journals were generally of moderate quality; however, some information was reported insufficiently. These deficiencies vary according to the type of study. It was determined that the information about publication bias in SR and the evaluation of the scientific quality of the included studies in MA and SR/MA were underreported. Writing and publishing systematic reviews and meta-analysis studies with critical methodological deficiencies or flaws may cause researchers to misunderstand and misinterpret these studies. An excellent systematic review and meta-analysis should include a comprehensive and critical discussion of the results and be reported fully and transparently. Studies that methodologically evaluate the quality of systematic reviews and meta-analyses will increase the quality of these studies in veterinary medicine. Additionally, this study may help to raise awareness of the AMSTAR tool and highlight deficiencies in the current conduct and reporting of systematic reviews and meta-analyses. Thus, such studies may guide researchers and veterinary healthcare professionals in clinical decision-making.

In conclusion, the reporting quality of the studies was good, but generally, there was insufficient information

on assessing publication bias. It is essential to report systematic reviews and meta-analyses thoroughly and transparently to improve the quality. It is recommended to use the relevant procedures and evaluation tools in performing this type of study. This comprehensive research evaluates SR, MA, and SR/MA studies on animal health in veterinary medicine, both individually and as a whole, according to their types, with the AMSTAR assessment tool. What distinguishes this study from other similar studies is that it examines systematic review and meta-analysis studies on all animal subjects, not just a specific animal group. In addition, not only frequency values were obtained for the findings obtained with the tool used to determine the quality of the studies, but also a quality score was calculated and the studies were classified according to these scores.

Availability of Data and Materials

The data that support the findings of this study can be available from the corresponding author upon reasonable request (E. Uzabacı).

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Competing Interests

The authors declared no conflict of interest.

Ethical Approval

The data for this research were collected from online databases, so this study does not require any ethical permission.

Author Contributions

EU: Conception and design; EU, FEC: Acquisition of data; EU, FEC: Analysis and interpretation of data; EU: Writing the manuscript; EU, FEC: Final approval of the article.

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RESEARCH ARTICLE

Correcting Angular Limb Deformities of Radius-Ulna and Tibia in Nine Dogs Using Computer-Assisted Spider Frame System

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ABSTRACT

In this study, angular deformities in 9 dogs, including 8 antebrachium and 1 tibia, were corrected using hexapod external fixators with the help of the computer-aided Spider Frame system.Preoperative planning included measurement of craniocaudal and mediolateral angular deformities, rotational deformity, length deficit, as well as determination of the source of the deformity and assembly of the frame.Joint lines and osteotomy/ostectomy lines were determined according to CORA points determined during preoperative measurements. Proximal and distal rings were placed. After the installation of the spider, radial/ tibial osteotomy/ostectomy was performed at the CORA point of the radius/tibia. After the operation, craniocaudal and mediolateral radiographs were taken in all cases. Ring dimensions, angular deformity values (including angulation, translation and rotation) and the degree of shortening were evaluated by radiographs. These data were entered into the web-based Spider software to generate correction prescriptions, after which the length and deformity correction process started on postoperative days 3-5. Functional results were excellent in seven cases and good in the other two cases. Long-term cosmesis was good to excellent in all cases.In conclusion, Spider Frame is a new generation external fixator system with many technical advantages and we recommend its use in appropriate cases.

Keywords: Dog, Angular deformity, Corrective osteotomy, Computer-assisted spider frame system

Introduction

Correction of limb deformities using the Ilizarov circular external fixator has been employed in both humans and dogs for a considerable time [1-4]. However, correcting complex deformities by means of this device poses some serious challenges, one of which is the need to modify the system to prevent residual deformity. If the deformity is to be corrected gradually, then sequential angulation, lengthening, rotation, and translation treatments must be performed [5]. When treating complex deformities, the Ilizarov circular external fixator needs to be modified and the correction period be prolonged. This explains the reason why computer-assisted use of hexapod external fixators have been in fashion for the past 15 years [6-8]. Such devices have been employed to treat both open and closed fractures, non-union and malunion cases, as well as limb deformities [9-11].

The Spider Frame is a hexapod fixator that consists of two rings connected to each other by six telescopic struts at special universal joints and is attached to the bone using half pins and/or tensioned wires. Each strut can be independently lengthened or shortened, enabling manipulation of the attached bone in six axes (anterior/ posterior, varus/valgus, lengthen/shorten) through the adjustable struts [12-15].

In this study, we attempted to correct angular deformities in dogs using hexapod external fixators with the help of the computer-assisted Spider Frame system. Therefore, the study was aimed at evaluating the effectiveness of the Spider Frame and Spider Frame Correction Software in treating shortness and different types of deformities in nine dogs

Material and Methods

Ethical Approval

Ethics committee approval was not obtained because Spider frame was applied as a clinical study in dogs with angular deformity. After the necessary information was

Table 1. *Breef summary of the cases and change in deformity in parameters*

Case	Signalement	Deformity Description before Operation
Case 1	Kangal, 35 kg 10 m, ♀	Carpal valgus, CrCd 25°Valgus ML 30°Procurvatum, External Rotation 20°
Case 2	Alabay ,50kg 11 m, ♂	carpal valgus, CrCd 20°Valgus ML 45° Procurvatum, External Rotation 30°
Case 3	Husky, 32 kg 16 m, ♂	Carpal valgus CrCd 30°Valgus ML 40° Procurvatum, External Rotation 40°
Case 4	Kangal, 48 kg 12 m, ♂	carpal valgus CrCd 18° ML 60°, External Rotation 70°
Case 5	Kangal, 43 kg 8 m, ♀	carpal valgus, CrCd 28° ML 50°, External Rotation 10°
Case 6	Kangal, 55 kg 10 m, ♂	carpal valgus, CrCd 35° ML 47°, External Rotation 30°
Case 7	Kangal, 65 kg 8.5 m, ♂	carpal valgus, CrCd 40° ML 60°, External Rotation 45°
Case 8	Kangal, 57 kg 9.5 m, ♂	CrCd 32°Valgus, ML 40°Procurvatum, External Rotation 30 °
Case 9	Kangal, 21 kg 5.5 m, ♂	CrCd Genu Valgum 30° apex medial Ext Rotat 10°

given to the patient owners, a separate consent document was obtained from each patient.

Animals

After obtaining approval from the Department of Surgery, Faculty of Veterinary Medicine, İstanbul University-Cerrahpasa, we conducted research on 9 dogs of various breeds with deformities in their extremities (8 cases involving the antebrachium and 1 case involving the tibia). Average age was 8 months (range: 5.5-16 months). Only one patient was of mature age. The breed, age, sex, breed and live weight information of the animals are given in *Table 1*.

Preoperative Clinical and Radiographic Evaluation

Preoperative planning included measuring the cranio-caudal and mediolateral angular deformities, rotational deformity, length deficit, as well as determining origin of deformity and assembly of the frame. All patients (6 males and 3 females) having limb deformities were treated using hexapod external fixators with the help of the computer-assisted Spider Frame system.

Animals were sedated with xylazine (Basilazin, Bavet, Türkiye) at a dose of 2 mg/kg by IM route before X-ray. Radiological evaluation of the deformities of all patients was performed using direct craniocaudal and mediolateral radiographs *(Fig. 1)*. CORA (Centre of Rotation of Angulation) angles were calculated by drawing proximal and distal anatomical or mechanical axes in the frontal plane. The Paley criteria, modified according to the Association for the Study and Application of Methods of Ilizarov (ASAMI) criteria, were used to evaluate the results

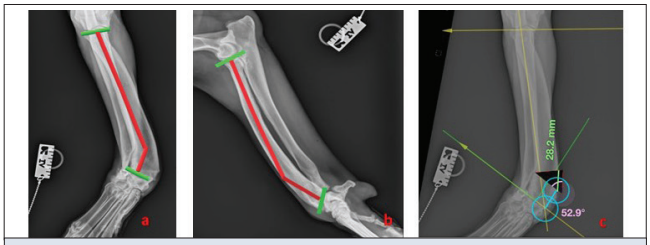

Fig 1. Preoperative radiographs of the Case 7' s antebrachium with lines showing the proximal and distal radial articular surfaces, (a) craniocaudal view and (b) mediolateral view, 90° planes from the articular surfaces and anatomical axes of the radius - CORAs. Determination of the size of the bone fragment to be removed from the radius to correct the deformity (c)

of the deformity treatment as excellent, good, fair, or poor [16,17]. In the ASAMI criteria, excellent scores indicate union, no infection, deformity under 7°, and limb-length discrepancy under 2.5 cm; good scores indicate union and two of the above criteria; fair scores indicate union and only one of the above criteria required of excellent scores; and poor scores indicate non-union, refracture, union and infection, deformity greater than 7°, or limb-length discrepancy over 2.5 cm [16,17].

The Distraction and External Fixator Indices were calculated for all patients. The Distraction Index value was determined by dividing the total length gained by the number of days spent in distraction. The External Fixator Index value was determined by dividing the total duration of fixator application by the total length gained.

Surgical Technique

Preoperative complete blood (cell) count (CBC) and biochemistry values were within normal limits in all cases. Premedication was performed with 0.5-1 mg/kg

xylazine HCl (IV), (Basilazin, Bavet, Türkiye), analgesia with 0.1-0.2 mg/kg meloxicam subcutan (SC) (Melox, Nobel Limited, Türkiye), and antibiosis with 25-30 mg/kg ceftriaxone (IV) (Novosef, Sanofi, Türkiye), and general anesthesia was induced with 5 mg/kg ketamine HCl (IV) (Alfamine, Atafen, Türkiye) and maintained with isoflurane 2-2.5% (Forane, Abbott, Italy) and 100% oxygen.

Following general anesthesia and disinfection, joint lines and osteotomy/ostectomy lines were determined based on the CORA points identified during the preliminary measurements. In cases with antebrachial deformity, distal diaphyseal ulna ostectomy was performed initially, followed by the fixation of the first ring in parallel with the proximal joint using the proximal ring as a reference) *(Fig. 2)*. Ring diameters, one of the components of the Spider frame, started from 100 mm and increased by 20 mm up to 300 mm. Ring material is made of aluminium alloy. Struts are in 5 different sizes as XXS (70- 95 mm), XS (95- 120 mm), S (120-150 mm), M (140-190 mm) and L (190-300mm). Strut material is made of titanium alloy and stainless steel. During this process, it was ensured that the rings were parallel to the joint and perpendicular to the mechanical axis of the bone. Subsequently, six struts were mounted on the proximal ring, and the distal ring was attached to the antebrachium using K-wires. These struts were then fixed and stabilized to the distal ring. No special effort was made to fix the distal ring parallel to the distal (carpal) joint. Once additional K-wires and Schanz screws were added to each ring to increase stabilization, installation of the Spider Frame was completed. Radial osteotomy/ostectomy was then performed on the CORA point of the radius *(Fig. 3)*. Radial wedge ostectomy was performed on 5 cases that had significant radial procurvatum. When osteotomy/ostectomy of the radius was performed, multiple drilling technique was employed so as to prevent bone warming.

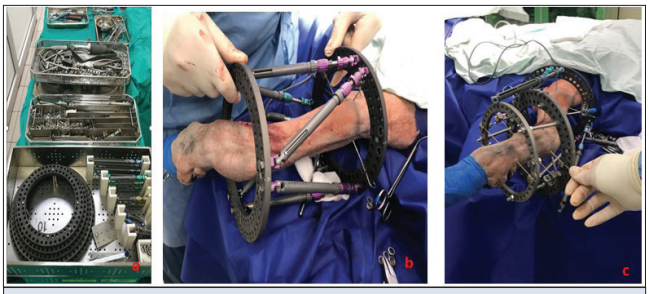

Fig 2. Components of the Spider Frame (a). Installation (b) and final view (c) of the structure of the Computer Assisted Hexapod External Fixator System

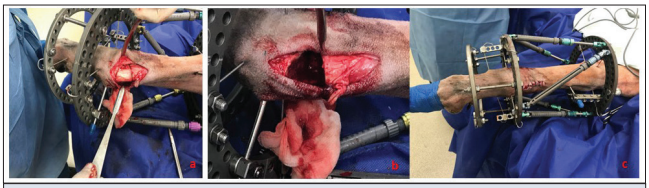

Fig 3. Closed wedge ostectomy was performed with an oscillating saw in the craniolateral approach to the radius with CORA's identified (a). Bone fragment removed from the radius after ostectomy (b). Clinical appearance after correction with spider frame (c)

The Spider Frame was fixed, attached and installed in a similar manner when correcting tibial or antebrachial deformities. Particular attention was paid to fixing the proximal ring parallel to the tibia. After installing the Spider Frame, an osteotomy was performed at the CORA point of each case. In order to avoid restricting movement in the stifle joint during the post-operative period, a fixator with a proximal ring diameter of 2/3 was used in the case with tibial deformity. Fixators with wider diameters were preferred for cases with antebrachial deformities.

Immediately following the surgical procedure, radiographs of the craniocaudal and mediolateral planes were obtained for all cases. Ring dimensions, angular deformity values (including angulation, translation, and rotation), and degree of shortening were assessed by means of radiographs. This data was entered into the web-based Spider software to generate prescriptions for correction, as shown in *Fig. 4*. Since all of our cases were owned animals, they were handed over to their owners after the operation and post-operative treatment was recommended. Control examinations were performed by calling our patients at certain intervals. Typically, the length and deformity correction process began on postoperative day 3-5. One week after the start of postoperative correction, radiography was performed to verify the accuracy of the correction, both visually and through software analysis. If residual deformity remained, a re-correction prescription was generated using the software. Struts were then changed under medical supervision. Patients were carefully monitored and treated on a daily basis during hospitalization for correction, wound care, and dressing procedures so that pin-tract infections could be prevented. The correction and extension procedures were followed by a consolidation period that lasted until callus tissue could be radiographically seen from three sides. Once bone union was complete and callus tissue could be

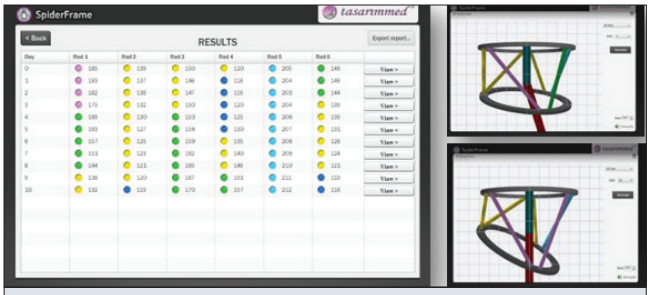

Fig 4. Spider frame correction modes

seen from all three sides, the fixator was removed. Direct input option was preferred while entering data on a daily basis into the Spider Software. This was how deformity corrections were planned and followed.

Assembly of the Spider Frame: It is mandatory to follow the Ilizarov rules. The reference ring can be placed either proximally or distally, but it should be positioned orthogonally to the bone.

If the distal ring is chosen as the reference ring during Spider Frame assembly, it must be applied orthogonally to the bone, that is, it must be perpendicular to the bone in both the anterior-posterior and lateral planes. The letter "A" engraved on the ring denotes the main tab, which should be positioned at the anterior side of the bone to serve as the zero position or reference point for the ring. If sign "A" is placed in any other position, the new position must be entered into the Spider Frame software as the reference ring parameters. The first strut should be placed on the left side of sign "A" when viewed from the front, with numbering continuing in a counterclockwise direction. The connection hole of the first strut on the reference (proximal) ring is marked with two concentric circles, while the counter connection hole is marked with a single circle on the mobile (distal) ring. Then one end of the strut is fixed to the hole with two concentric circles, and the other to the hole with a single circle *(Fig. 5)*.

Capable of solving even the most complex deformity cases, the Spider Frame utilizes the Spiderfix software (Tasarım Medical, Version 2.0.1, İstanbul) *(www.spiderframes.com)*. Of note, this software is designed to work only with Spider Frame rings and struts. Deformity corrections can be planned using Spiderfix software by choosing either of the two options: direct input or measurement. In the direct input option, the user manually enters the calculated data from a printed X-ray image, while in the measurement option, the user can calculate the deformity by drawing some basic lines as in the Picture Archiving and Communication System (PACS) software used in hospitals. When correcting deformities by utilizing the Spider Frame software, one of the three mode types can be used: Daily Mode, Speed Mode, and Advanced Mode.

The Daily Mode, as the name implies, is used for day-based correction, where the user enters the number of days necessary for the deformity to be corrected.

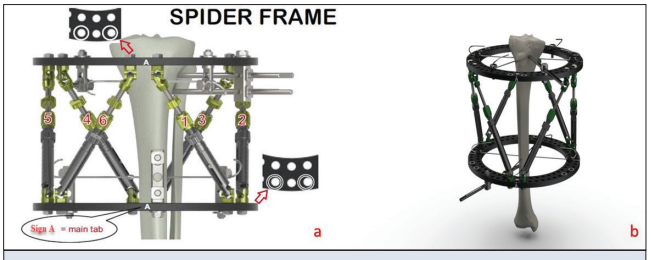

Fig 5. Schematic representation of the spider frame (a,b)

The Speed Mode allows the user to specify the distraction or compression rate, and the duration of correction is calculated by the software based on this user-generated rate.

The Advanced Mode can help correct deformity sequentially by determining the duration of correction for every single deformity type.

Result Window: Calculate button is used for getting results to correct entered deformity by defined frame.

Results

The deformities of 8 cases were located in the radius-ulna and that of 1 case in the tibia.

The cause of the antebrachial deformities was the early closure of the distal ulnar growth plate (carpal valgus) in 8 cases. Deformity in one case was due to the early closure of the lateral proximal tibial plate. Deformities were either oblique (8 cases) or uniplanar (1 case), and all cases suffered from isolated rotational deformities as well as bone shortness.

Average follow-up period was 9.7 months (range: 5-12 months), and mean duration of external application was 74.55 days (range: 53-95 days) *(Table 1)*. Mean bone lengthening was 13.17 mm (range: 6-28.5 mm), with less than 10 mm of lengthening observed in four cases and more than 10 mm in five cases. Gradual correction of deformities was performed in three cases, while six cases

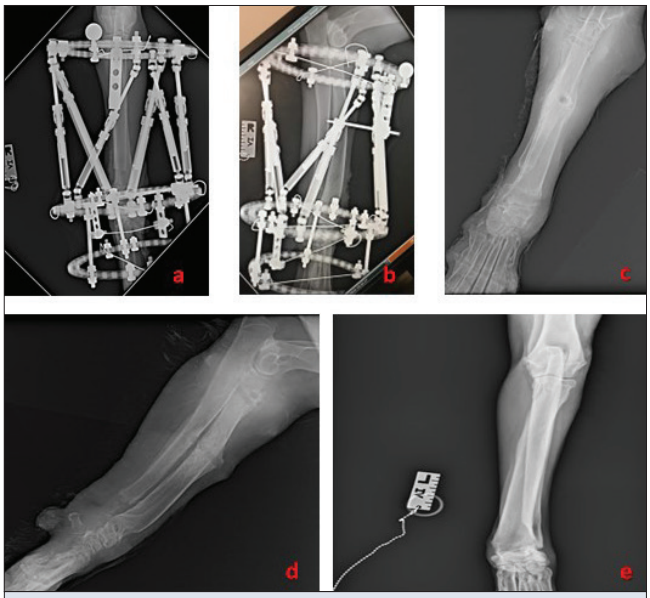

Fig 6. Postoperative radiography before planning the deformity correction of Case 7 (a,b). Craniocaudal and mediolateral radiographs taken after spider removal (c,d). Craniocaudal view of the final radiography of Case 7 (e)

Table 2. Breef summary of the cases during correction

Case No	Post- OP after SEF Placed						Distraction Time	Deformity Correction Time	Total Fixator Duration Time
	Angulation			Translation					
	CrCd	Lateral	Axial	CrCd	Lateral	Axial			
1	20°medial angulation	30° cranial angulation	5° external rotation	3 mm medial	6 mm cranial	8 mm shortness	10 D	20 D	55 D
2	Acut Correction No angulation	45° cranial	20° external	Acut Correction No angulation	Acut Correction No angulation	12 mm Shortness	14 D	38 D	95 D
3	Acut Correction No angulation	Acut Correction 10° angulatıon	20° external	Acut Correction No angulation	Acut Correction No angulation	7 mm shortness	7 D	15 D	63 D
4	8° apex	20° cranial	25° external	4 mm medial	Acut Correction No angulation	10 mm Shortness	10 D	23 D	75 D
5	Acut Correction No angulation	15° cranial	Acut correction	5 mm medial	5 mm cranial	12 mm Shortness	13 D	28 D	72 D
6	Acut Correction No angulation	15° cranial	20° external	8 mm medial	3 mm lateral	27 mm Shortness	29 D	35 D	95 D
7	Acut Correction No angulation	No angulation	25° external	3 mm medial	Acut Correction No angulation	28.5 mm Shortness	30 D	35 D	88 D
8	Acut Correction No angulation	15° apex	15° external	3 mm medial	4 mm posterior	6 mm Shortness	6 D	15 D	75 D
9	Gradual Correction 25° apex medial	—	10° external	—	—	8 mm	10 D	15 D	53 D

Table 3. Results after removal of SPİDER fixator

Case	After Fixator Removed
Case 1	CrCd angulation was decreased to 7°. Lateral angulation, external rotation, shortness and translation were corrected
Case 2	Lateral angulation was decreased to 20°.CrCd angulation, external rotation, translation and shortness were corrected
Case 3	ML and CrCd angulation,ext-ernal rotation and shortness was totally corrected
Case 4	CrCd angulation,external rotation, shortness and translation were corrected. ML angulation (procurvatum) was decreased to 10°
Case 5	CrCd angulation,external rotation, shortness and translation were corrected. ML angulation (procurvatum) was decreased to 7°
Case 6	Cranial angulation was decreased to 8°. Lateral angulation was decreased to 15°. External rotation, translation and shortness were corrected
Case 7	ML and CrCd angulation,ext-ernal rotation and shortness was totally corrected
Case 8	CrCd angulation,external rotation and shortness were corrected. Lateral angulation (procurvatum) was decreased to 13°
Case 9	CrCd angulation and shortness were corrected

with large procurvatum angles received a certain degree of acute correction on the day of the operation. This was followed by residual correction performed gradually by utilizing the software. Mean Distraction Index was 10.96 d/cm (range: 10-12.5 d/cm), and mean External Fixator Index was 70 d/cm (range: 30.88-125 d/cm). Anguler deformity osteotomy and installation times of spider frame (operation time) were 80, 90, 70, 80, 80, 100, 80, 90, 100, 80, 90, 90, 90 min respectively. Function and cosmesis were preoperatively assessed to be fair to poor in all dogs. Deformity correction started on post-operative day 3-5 and ranged from 0.5 mm to 1.0 mm twice daily. Hospitalization time ranged from 10 to 40 days. After the correction was completed, patients were discharged *(Fig. 6)*.

In the assessment of radio-ulnar correction, the modified Paley criteria, based on ASAMI criteria, were used to evaluate functional and bone results. Bone results were excellent in five cases and good in the rest four

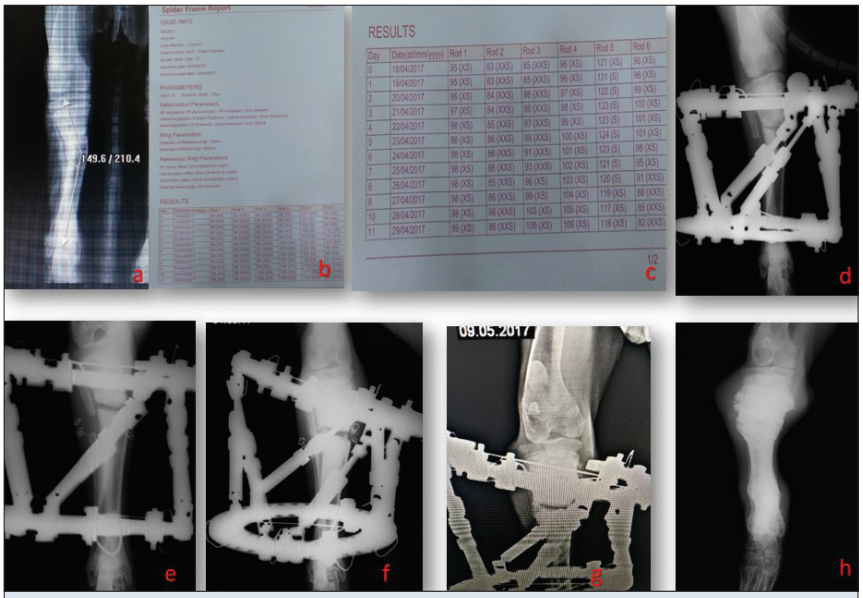

Fig 7. Case 2's clinical view (a) and preoperative Craniocaudal (b) and Mediolateral radiographs (c). Spider frame application (d) and postoperative radiographs are taken (e,f). Radiographs are taken during correction of deformity at 20th days (g,h)

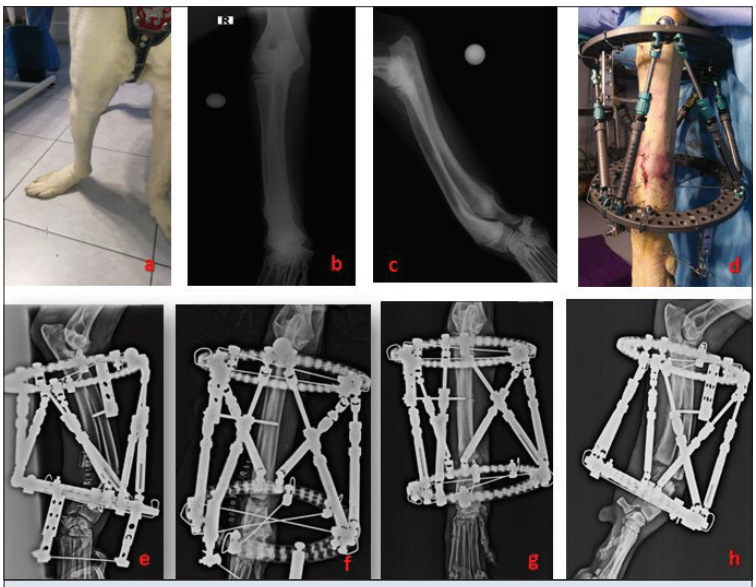

Fig 8. Radiographs of Case 2, Just before removing the frame at 95th days (a,b), Craniocaudal and mediolateral radiographs were taken after removal of the frame (c). Clinical appearance just before (d) and after removal of the spider frame (e)

(Table 2, Table 3). Functional results were excellent in seven cases and good in the rest two *(Table 2, Table 3)*. Long-term cosmesis was good to excellent in all cases. Deformities of 8 cases were in the oblique plane and that of 1 case was in the coronal plane. All cases had isolated shortness and rotational deformity. Mean cranio-caudal angulation (valgus) was 28.5° (range: 18°-40°) and mean lateral angulation was 46.5° (range: 30°-60°) in cases with antebrachial deformity. In the case with tibial deformity the cranio-caudal angulation was 30°, but no lateral angulation was observed *(Fig. 7)*. Mean external rotation angle was 31.67° (range: 10°-70°) in all cases. After the fixator was removed, mean residual cranio-caudal angulation (valgus) was calculated as 7.5° (range: 7°-8°) in two cases, and mean residual lateral angulation (procurvatum) was determined as 13° (range: 7°-20°) in five cases *(Fig. 8, Fig. 9)*. Shortness and rotational deformity were completely corrected in all cases. Superficial pin site infection was observed in most cases but successfully treated by administering local and oral antibiotics as well as by cleaning pin circumference. In two cases (cases 2, 6), a superficial infection developed around the Schanz screws attached to the proximal and distal rings, which did not respond to oral antibiotics. However, following

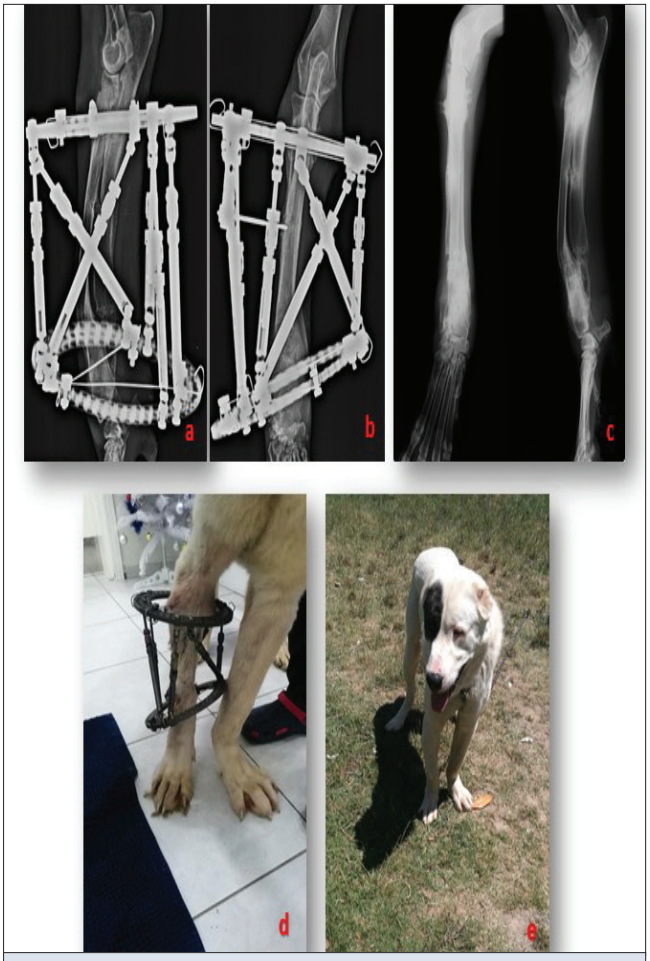

Fig 9. Preoperative Anterio- posterior radiography of the Case 9 (a). Determining the spider frame dimensions by entering the deformity into the system before the operation (b) and preparing the prescription (c). Craniocaudal radiographs taken acute postoperative (d), 8th day (e), 17th day (f), last day of correction (22th day) (g), radiography of the spider frame removed on day 53 (h)

the removal of these screws, the infection resolved during the consultation period. Temporary restriction of range of motion (ROM) in the carpal joint was observed in all cases after the circular fixators were removed. The condition resolved by physiotherapy exercises. Permanent minimal carpal joint restriction was observed in only two cases (cases 4, 6).

Discussion

External fixators are a fundamental method for treating various deformities [18-21]. Results achieved by this method are more than satisfactory; nevertheless, fixator application necessitates strict compliance with certain principles and criteria. For, even the smallest mistake during the planning stage can lead to catastrophic deformities after treatment. Computer-assisted use of circular fixators can help avoid such complications [22].

The mechanical features of the Spider fixators enable the correction of multi-axial deformities. By knowing the lengths of the telescopic rods and diameters of the rings to be used, mathematical calculations can be made to determine where one ring should be placed in relation to another. There are various computer softwares that can serve this purpose and thus provide convenience to the surgeon [23,24]. In this study, we used the Spiderfix, which is a high-tech software that allows for the correction of all deformities simultaneously.

One of the advantages of the Spider system is that the struts of the fixator can be changed without surgery. If the system is assembled properly, residual deformities can be corrected by simply changing struts, without the need for any other modification. This is especially beneficial for patients with oblique plane deformities, since the duration of distraction is shorter in this system than in traditional [25]. Partial correction was achieved in some of our cases. Struts of 6 cases had to be changed because they had greater procurvatum and larger external rotation compared to the others. Of note, all the procedures were performed under clinical conditions.

When Ilizarov or hexapod fixators are applied with proper planning, residual deformity may not occur. It is of paramount importance that if the error rate is to be minimized and permanent residual deformity prevented, the reference ring should be placed orthogonally to the bone segment and completely parallel to the joint [4]. In our cases with antebrachial deformities, the reference (proximal) ring was placed perpendicular to the bone and parallel to the elbow joint without fluoroscopy. In two cases, the deformity was not completely corrected on the desired day. CrCd and lateral X-rays of these cases were retaken, and residual deformities were corrected with the help of the software. In the case with tibial deformity, the proximal ring was selected as the reference ring and placed perpendicular to the tibia and parallel to the knee joint.

There is a relation between how long an external fixation will remain on an extremity and how rigidly it is applied to that area [4]. As well as one Schanz screw, a minimum of two K-wires were applied to each ring, with a view to increasing its stability. There was no loosening between pins and bones in any of our cases, which may be attributed to the rigid placement of the system on the extremity. The rigid assembly of the frame also contributed to its ability to remain on the extremity for a long time. We think that the application of one additional Schanz screw to each ring made it possible for the fixators to be stabilized during the whole period of correction. Of note, fixators applied to our cases remained in place for a mean period of more than 70 days.

An external fixator is a medical device used to immobilize and stabilize bone fragments and is aimed at promoting the healing process. Pin site infections and pin-bone

loosening are common complications associated with the use of external fixators, and hexapod fixators are no exception, either. To prevent pin site infections, daily cleaning with Chlorhexidine solution was performed during the postoperative period. Nevertheless, pin site infections developed in three cases, particularly at the location of the pins in the proximal ring, where there is a large muscle mass. These infections were treated with oral antibiotics (cefalexin). The use of at least one Schanz screw per ring was found to increase stability and prevent pin-bone loosening.

The rigid application of the fixator to the extremity ensures proper and radical movement of the distal fragment and associated mobile ring, leading to the correction of the deformity in the desired amount and time. Fixators were applied rigidly in all cases, and no loosening or pin-bone loosening was observed during the course of this study. However, the deformities of two cases were not fully corrected in the initial planning, thus a second planning was required. It may be that the reference ring was not placed perfectly parallel to the joint.

Different hexapod systems have been used in deformity corrections in humans, and it has been reported that mean External Fixator Index shows variations [26]. The reason for this is that there are cases with varying levels of difficulty in which external fixators are used for different durations of time. Mean External Fixator Index in our study was found to be 70 d/cm.

Sakurakichi et al.[27] reported that lengthening of less than 3 cm extended the External Fixator Index. Matsubara et al.[28] reported that Distraction Index and External Fixator Index values of cases undergoing gradual correction was lower than those undergoing acute correction. With a view to correcting the deformity quickly and shortening the duration of fixator use, partial acute correction was performed on six of our cases with severe deformities. The results indicated that mean values of Distraction Index and External Fixator Index were consistent with the literature.

When correcting complex (oblique) deformities, the surgeon must have a certain level of experience with the classic Ilizarov method [29]. The process involving the use of a computer-assisted hexapod system is shorter than the classic Ilizarov technique [30]. This explains the reason why we preferred hexagon external fixators and the Spider Frame software in planning as well as correcting oblique and externally rotated deformities in one single operation. The advantage of this technique is that procedures can be performed in a single-stage fashion and in a shorter time.

Manner et al.[31] stated that, compared to the classic Ilizarov technique, the learning curve for the use of hexapod fixators is shorter; however, success in the latter technique still depends on the surgeon's experience with the former. In the literature, successful outcomes have been reported for bone lengthening through distraction osteogenesis using both the classic Ilizarov method and various hexapod systems [7,32]. The technique of distraction osteogenesis is influenced not only by the osteotomy technique but also by the start day, rhythm, and frequency of distraction [33]. In our study, a daily lengthening of 2x0.5 mm and preservation of the blood vessels in the osteotomy area prevented non-union or delayed union. Swelling was observed in the distal fragment of a few cases with excessive external rotation. This swelling, which resolved spontaneously within one week, was thought to be due to intraoperative partial acute correction.

Although hexapod fixators have certain advantages over the classic Ilizarov external fixators, their high cost is a drawback [34]. Yet, this cost may be overlooked considering that they are easy to use, simple to understand, and have the potential to correct residual deformities.

One of the main limitations of the Ilizarov device is that the frame should be modified and deformities may not be corrected simultaneously with lengthening [35,36]. The Spider Frame actually follows the principles of the Ilizarov device but has several advantages over it [36]. To illustrate, multi-axial deformities can be corrected simultaneously by means of the polyaxial hinges of this device, which render frame modification unnecessary, except for strut changes [37]. In fact, the Spider Frame has the benefit of correcting all deformities at the same time, which saves time [38]. The struts of the Spider Frame are made of titanium alloy, which is lighter and stronger than the stainless steel Ilizarov frames. The Spider Frame software provides an advanced correction mode, which allows the surgeon to choose the ideal correction sequence and duration. For instance, the surgeon may first correct shortness, followed by translation in the CrCd, lateral translation, axial translation, rotation, and finally, angulation. Moreover, the double-sided holes on the surface of the Spider Frame ring provide more flexural strength and connecting holes compared to circular devices.

The Spider Frame also features a threaded locking mechanism to prevent uncontrolled strut movements.

The computer-assisted system allows operators to determine the duration of the deformity correction, making it easier to achieve the desired outcome. Besides, it makes postoperative interventions possible and increases physician confidence.

According to our observations, the only potential disadvantage of the Spider Frame is that the proximal and distal rings are connected to the struts by a poly-axial hinge, which can result in a flexibility of 0.5 mm in the rigidity of the fixator. In contrast, Ilizarov external fixators

use fully threaded rods instead of struts, resulting in complete rigidity of the frame. It should be emphasized that the flexibility observed in the Spider Frame may be problematic only in large and giant breed dogs. Of note, we did not encounter any complications in our cases caused by or related to this flexibility. Despite this potential disadvantage, the Spider Frame is a new generation of external fixator systems with many technical advantages and can be recommended for use in appropriate cases.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (Z. Mutlu) upon reasonable request.

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Conflict of Interest

The authors declared that there is no conflict of interest related to this study.

Author Contributions

Clinical examination of the patients was performed by ZM, MK, YA. Preoperative radiographic measurements and operation were performed by ZM, MK, YA and SSH. Postoperative controls were performed by ZM, MK, YA. The manuscript was written by ZM and YA. YA edited and uploaded the article.

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Research Article

Cardioprotective Effect of Intravenous Lipid Emulsion in Bupivacaine-Induced Experimental Cardiac Toxicity

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ABSTRACT

The intravenous lipid emulsion (ILE) therapy is known to alleviate clinical symptoms in cases of bupivacaine-induced cardiac toxicity. However, there is insufficient information regarding histopathological damage. This study aimed to investigate whether the use of ILE therapy in rats with experimentally induced bupivacaine-related cardiac toxicity can ameliorate histopathological damage. 28 Wistar albino rats were divided into four groups: control (A), lipid (B), bupivacaine (C), and bupivacaine + lipid (D). After providing monitoring in all groups, group B received 1.5 mL ILE + 0.25 µg/kg/min ILE infusion, group C received 3 µg/kg/min bupivacaine infusion, and group D received 3 µg/kg/min bupivacaine infusion followed by 1.5 mL ILE + 0.25 µg/kg/min ILE infusion after observing cardiac toxicity. Heart rate and respiratory rate were recorded. Blood samples were collected post-procedure to measure LDH, CK-MB, and troponin levels. Cardiac tissue samples were obtained for histopathological examination. There was no significant difference in baseline heart rate and respiratory rate among the groups ($P>0.05$). However, in the second measurements, heart rate and respiratory rate were higher in group D compared to group C ($P<0.05$). LDH and CK-MB levels were higher in group C compared to the other groups ($P<0.05$). Irisin and asprosin scores were higher in group D compared to the other groups ($P<0.05$). ILE was found to have a cardioprotective effect in the treatment of bupivacaine-induced cardiac toxicity, as it improved both clinical and laboratory parameters. However, histologically, cardiac damage persisted.

Keywords: Bupivacaine, Cardiac toxicity, Histopathology, Lipid emulsion

Introduction

Unlike general anesthesia, local anesthetics (LA) exert their main mechanism of action by providing temporary anesthesia through the blockade of sodium channels in the targeted operative area without affecting the patient's consciousness. LAs are commonly preferred in emergency departments, procedural rooms, and surgeries performed under local anesthesia in the daily practice of anesthesiology. LAs have a wide range of side effects. While transient and minor complications are frequently observed, local anesthetic systemic toxicity (LAST) can also occur due to accidental intravascular injection or rapid absorption from the application site. LAST is known as the most fatal complication of LAs [1,2].

LAST usually begins with nonspecific symptoms such as restlessness, agitation, nausea, and vomiting. Also, it can progress to central nervous system depression, respiratory arrest, and cardiovascular collapse if appropriate and timely intervention is not performed. In addition to essential life support, intravenous lipid emulsion (ILE) treatment has been accepted as one of the treatment options for LAST and has found its place in the LAST treatment algorithm in recent years [3].

The effectiveness of ILE in treating LAST has been reported in the literature through numerous case presentations and animal studies [4-8]. These reports have demonstrated that ILE provides hemodynamic stabilization and resolves clinical symptoms. However, the histologic effects of ILE treatment on cardiac tissue in LAST cases are not clear.

This study aimed to investigate whether ILE treatment improves both the clinical findings and histopathological damage on cardiac tissue in rats with bupivacaine-induced experimental cardiac toxicity.

Material and Methods

Ethical Approval

Ethical approval for this study was obtained from the Animal Studies Ethics Committee of Adıyaman University (ADIYAMAN-HADYEK: 25.11.2021 - 2021/050).

Animals

The study was conducted in the Animal Laboratory of Adıyaman University in 2022. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. The study was conducted in the Laboratory Animal Facility (Adıyaman, Türkiye) in 2022. Twenty-eight adult male Wistar-Albino rats weighing between 300-350 g were housed and fed with a standard diet and water ad libitum. Room temperature (22-25°C) and humidity (50-55%) were monitored daily. Lighting was provided with 12 h light-dark cycles (06:00-18:00) using cool white fluorescent lamps.

Experimental Groups

To calculate the sample size, we use data from a study that correlated myocardial bupivacaine concentration between control and lipid group (respectively, 30.0/5.0 and 22.4/4.8) [9]. Thus, in order to reproduce these findings with a maximum allowable error estimation of 5%, a statistical power of 80%, and an effect size of 1.55, a sample size of 7 rats per group would be sufficient.

In the study, all animals were anesthetized using a combination of ketamine/xylazine (50 mg/kg 20 mg/kg; i.m.). Electrocardiogram (ECG) recordings in lead II were measured in anesthetized rats using the MP36 system and AcqKnowledge software (BIOPAC Systems Inc.). Respiratory rates were countered. The animals were divided into four groups. Seven subjects were used in each group.

Group A (Sham Group): No agents were administered to the rats in this group.

Group B (Lipid Group): Rats in this group underwent cardiac monitoring under anesthesia, followed by intravenous access via the tail vein. They received a 1.5 mL/kg ILE (Intralipid 20%, Fresenius Kabi AB, Uppsala, Sweden) plus a continuous infusion of 0.25 µg/kg/min of ILE.

Group C (Bupivacaine Group): After cardiac monitoring under anesthesia, rats in this group also received intravenous access via the tail vein. A continuous infusion of 3 µg/kg/min of bupivacaine (Marcaine 0.5%. AstraZeneca Ltd, İstanbul, Türkiye) was initiated to induce experimental LAST and achieve cardiac toxicity symptoms (arrhythmia, bradycardia). The bupivacaine infusion was stopped at the onset of cardiac toxicity.

Group D (Bupivacaine + ILE Group): In addition to Group C, rats in this group received intravenous ILE infusion (1.5 mL/kg intravenous bolus + 0.25 µg/kg/min infusion for 15 min) under O_2 support after discontinuation of bupivacaine infusion. The procedure was terminated upon achieving hemodynamic stability.

At the end of the defined procedures in all groups, cardiac tissues were collected for histological examination.

Biochemical Analysis

Blood samples were collected from the extracted cardiac tissue in EDTA tubes, and creatine kinase-myocardial band (CK-MB), lactate dehydrogenase (LDH), and troponin levels were determined using the Biosite Triage Meter Plus (San Diego, USA) device.

Immunohistochemical Analyses

According to the immunohistochemical staining method using the avidin-biotin-peroxidase (ABC) complex, minor modifications were applied [10,11]. Liver tissues were blocked using this method, and sections with a 4-6 µm thickness were obtained and deparaffinized. Primary antibodies Asprosin (Rabbit polyclonal IgG antibody, Fine Test, FNab09797, China) and irisin (Rabbit polyclonal IgG antibody, Phoenix Pharmaceuticals, H-067-17, USA) were used at a dilution of 1/200 with the Thermo Scientific™ TP-015-HA commercial kit. After applying 3,3-diaminobenzidine (DAB) chromogen, staining was performed using Mayer's Hematoxylin, and the samples were examined and photographed under a light microscope using the Leica DM500 microscope.

Histoscores were created based on the prevalence (0.1: <25%, 0.4: 26-50%, 0.6: 51-75%, 0.9: 76-100%) and intensity (0: none, +0.5: very weak, +1: weak, +2: moderate, +3: intense) of immunoreactivity [12].

Statistical Analysis

Descriptive statistics, including mean, standard deviation, median, minimum, maximum, frequency, and ratio, were used for the data. The distribution of variables was tested using the Kolmogorov-Smirnov test. Non-parametric tests such as Kruskal-Wallis and Mann-Whitney U tests were used for the analysis of quantitative independent variables. SPSS 28.0 software was used for the analyses.

Results

The study initially involved 28 rats and concluded with the same number. After the monitoring phase at the beginning of the study, the minimum and maximum values for heart rate were determined as 318 and 384, respectively, while the minimum and maximum values for respiratory rate were 71 and 88, respectively. Second measurements were taken in Groups C and D, where toxicity was induced. In

Table 1. *Descriptive characteristics of groups*

Parameters		Group A	Group B	Group C	Group D
Heart rate	First measurement min-max (mean)	318-351 (340.6)	343-350 (347.6)	312-384 (339.1)	326-350 (338.4)
	Second measurement min-max (mean)			157-193 (171.9)	302-330 (317)
Respiratory rate	First measurement min-max (mean)	73-85 (79.57)	74-88 (79.86)	71-84 (75.57)	75-84 (79.29)
	Second measurement min-max (mean)			48-70 (61.71)	64-81 (72.43)
Irisin score	min-max (mean)	0.6-0.9 (0.77)	0.6-0.9 (0.79)	0.9-1.2 (1.11)	1.2-1.8 (1.46)
Asprosin score	min-max (mean)	0.8-0.9 (0.87)	0.8-0.9 (0.86)	0.8-1.2 (1.06)	1.8-2.7 (2.29)

Table 2. *Comparison statistical results of groups*

Vital Signs, Laboratory and Histopathologic Parameters			Group A	Group B	Group C	Group D	P Value	
Heart rate	First measurement	Mean.±sd	340.6±12.6	347.6±3.2	339.1±25.1	338.4±7.6	0.220	K
		Median	348.0	350.0	332.0	340.0		
	Second measurement	Mean.±sd			171.9±13.0	317.0±10.5	0.002	K
		Median			172.0	319.0		
Respiratory rate	First measurement	Mean.±sd	79.57±3.99	79.86±5.05	75.57±4.50	79.29±3.20	0.217	K
		Median	80.0	80.0	74.0	80.0		
	Second measurement	Mean.±sd			61.71±10.24	72.43±5.68	0.045	K
		Median			69.0	72.0		
LDH		Mean.±sd	1141±435	579±281	2141±411	1315±428	0.000	K
		Median	903	547	2243	1344		
CK-MB		Mean.±sd	522±165	827±278	1623±351	875±155	0.000	K
		Median	498	798	1758	820		
Troponin		Mean.±sd	1.96±2.34	0.93±0.32	4.52±2.46	3.96±2.23	0.001	K
		Median	1.20	0.83	3.4	4.2		
Irisin Score		Mean.±sd	0.77±0.13	0.79±0.09	1.11±0.12	1.46±0.32	0.000	K
		Median	0.80	0.80	1.20	1.20		
Asprosin Score		Mean.±sd	0.87±0.05	0.86±0.05	1.06±0.18	2.29±0.41	0.000	K
		Median	0.90	0.90	1.20	2.50		

[K] *Kruskal-wallis(Mann-whitney u test)*

these second measurements, the minimum and maximum values for heart rate were found to be 157 and 330, respectively, while the minimum and maximum values for respiratory rate were 48 and 81, respectively *(Table 1)*.

There were no significant differences observed in the initial values of heart rate and respiratory rate among the groups (P>0.05). However, in the second measurement, heart rate and respiratory rate were significantly higher in Group D compared to Group C (P<0.05) *(Table 2)*.

Regarding LDH and CK-MB levels, Group C showed significantly higher levels than other groups (P<0.05). Regarding troponin levels, Group C and Group D were significantly higher compared to other groups (P<0.05), but no significant difference was found between Group C and Group D (P>0.05).

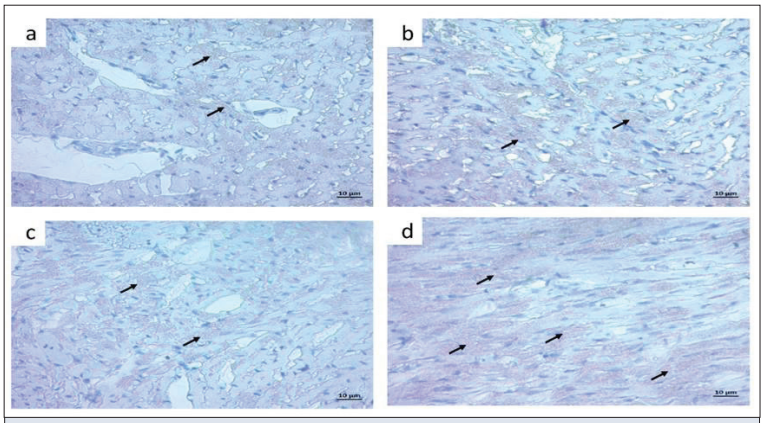

Fig 1. Irisin immunoreactivity of study groups. Scala bar: 10 µm

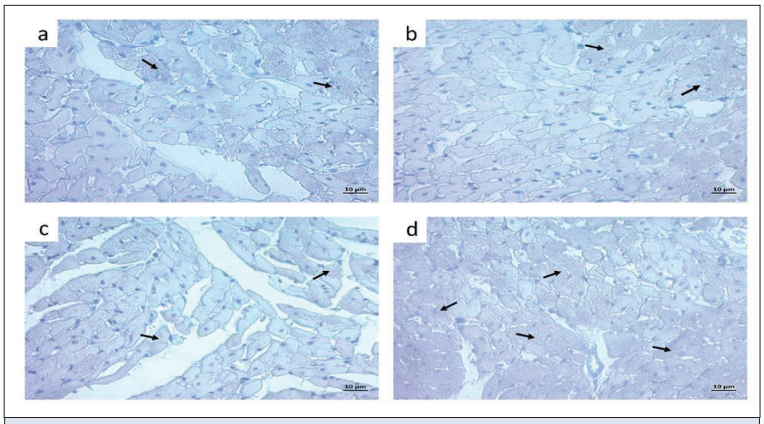

Fig 2. Asprosin immunoreactivity of studygroups. Scala bar: 10 µm

In Group D, the irisin score was significantly higher compared to all other groups (P<0.05), while no significant difference was observed between Group A and Group B (P>0.05). In Group D, asprosin score was significantly higher compared to all other groups (P<0.05), while no significant difference was observed between Group A and Group B (P>0.05) *(Table 2)*.

In the histological examination of cardiac tissues, it was observed that both asprosin and irisin immunoreactivity were higher in Group D compared to the other groups *(Fig. 1, Fig. 2)*.

Discussion

LAST typically initiates with non-specific symptoms such as restlessness, agitation, nausea, and vomiting, these cortical symptoms can be masked in cases where sedation is administered [13]. In our study, the use of sedation in rats subjected to experimental bupivacaine toxicity did not result in non-specific symptoms; instead, it presented with arrhythmia and respiratory depression.

Lazar et al.[14] found that in the group receiving Lipid + ropivacaine, there was less decrease in heart rate. Wu et al.[15] reported in their study where they investigated ILE treatment in rats with local anesthesia-induced central nervous system toxicity that ILE treatment reduced the detection of respiratory arrest and apnea, and resulted in less decline in heart rate. In our study, it was observed that ILE corrected the decrease in respiratory rate and heart rate associated with LAST.

LDH, CK-MB, and troponin are commonly used as clinical markers of cardiac damage. There are some experimental animal studies in the literature using these markers to assess cardiac damage. In these studies, elevated CK-MB, LDH and Troponin levels were associated with myocardial damage [16,17]. In our study, cardiac markers were found to be higher in the bupivacaine group compared to all other groups. When comparing the bupivacaine + lipid group to the bupivacaine group, although troponin levels were higher in the bupivacaine group, no statistically significant difference was found as observed with other cardiac markers. Unlike other markers, troponin has a later peak and a longer duration of elevation [24]. In the bupivacaine+lipid group, the lower levels of all cardiac markers compared to bupivacaine further support the association between the cardioprotective effect of ILE.

Lazar et al.[14] reported no differences in the histological evaluation of tissues obtained from the groups. In our

study, however, the irisin and asprosin scores were higher in the bupivacaine + lipid group compared to all other groups. This indicates that the highest cardiac tissue damage occurred in the bupivacaine + lipid group. In Lazar et al.[14]'s study, tissue analysis was performed after administering the determined drug doses in both the bupivacaine and bupivacaine + lipid groups without observing any toxicity. In our study, histological analyses were conducted after observing cardiac toxicity in both the bupivacaine and bupivacaine+lipid groups.

Irisin is a myokine derived from the cleavage of fibronectin type III domain-containing 5 (FNDC5). Irisin plays a role in mitochondrial energy regulation, fatty acid oxidation, and glucose metabolism. Changes in irisin levels have been shown to be associated with cardiovascular diseases and myocardial damage [18,19]. In our study, it was observed that the irisin score in the bupivacaine + ILE group was higher than that in the bupivacaine group when examining the heart tissues.

Asprosin is a newly identified centrally acting orexigenic adipokine that is secreted from white adipose tissue and regulates glucose metabolism. Increased levels of asprosin have been reported to be associated with coronary artery diseases and myocardial damage [20]. In our study, it was observed that the asprosin score in the bupivacaine + ILE group was higher than that in the bupivacaine group when examining the heart tissues.

Many studies in the literature examine the protective effects of ILE without the development of toxicity or initiate ILE treatment after the induction of cardiac arrest. In our study, ILE treatment was used in accordance with clinical practice for the treatment of cardiac toxicity related to LAST. However, the low number of experimental animals and basic-level monitoring are among the main limitations of this study. Further studies conducted with larger sample sizes and more advanced cardiac and respiratory monitoring will provide more extensive information.

In conclusion, ILE has a cardioprotective effect in the treatment of cardiac toxicity caused by bupivacaine and improves both clinical and laboratory parameters. However, it should be noted that cardiac damage still exists histologically. Therefore, continued monitored follow-up is recommended after ILE treatment in the context of LAST.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author (N. Yılmaz) on reasonable request.

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Conflict of Interest

The authors declare that they no conflict of interest.

Author Contributors

NY and MD planned the study, designed the experiments and helped manuscript writing; AT and FT helped with data analyses and bioinformatics and wrote the manuscript; NY, MD, AT and FT collected samples, conducted laboratory process and histopathologic examination; NY and FT analysed the statistics data. All authors read and approved the final manuscript.

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Research Article

Comparison of Mouse Species in an *In Vivo* SARS-CoV-2 Challenge Model

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ABSTRACT

The K18-hACE2 transgenic mice, a model animal having human ACE receptors, is employed in studies against the SARS-CoV-2 virus all over the world. Aged Balb/C mice utilized during the SARS-CoV outbreak were compared to non-T-cell, immunosuppressive Nude mice often employed in cancer research and K18-hACE2 transgenic mice used as a model animal against the SARS-CoV-2 virus challenge assay. At the same time, the role of the model animal K18-hACE2 transgenic mice in organs other than the lung was studied. The BSL3 facility was used for the challenge experiment in this study. In three groups, 105 TCID50 SARS-CoV-2 virus B.1.1.7 (the alpha variant was gavaged and intranasally administered to mice under anesthesia. The experiment was ended on the tenth day, and gross pathology was done. The viral load of SARS-CoV-2 was determined by RT-PCR after collecting the target organ lungs from all mice as well as the spleen, liver, heart, and kidneys from the K18-hACE2 transgenic mouse group. In comparison to Balb/C and Nude mice, the K18-hACE2 transgenic mouse model animal has been shown to be a suitable model against the SARS-CoV-2 virus in our study. At the same time, when the organs of K18-hACE2 transgenic mice were compared, viral load retention occurred in the target organ, the lung, with no significant retention in other organs.

Keywords: COVID-19, *In vivo* challenge, Model animals, SARS-CoV-2

Introduction

The SARS-CoV-2 virus, which infected 767 million individuals and killed 6.9 million people globally, was identified as the causal agent of COVID-19 [1-4]. Coronaviruses (CoVs) are members of the Coronaviridae family, the Nidovirales order, and the genus Coronavirus. Coronaviridae, the biggest group of viruses, is divided into two subfamilies: Coronavirinae and Torovirina. Coronavirinae is further subdivided into four generations: alpha, beta, gamma, and delta coronaviruses [5-8]. SARS-CoV, a coronavirus that emerged in 2002-2003 with a 10% mortality rate, manifested itself as a lethal disease that caused severe acute respiratory syndrome (ARDS). Middle East respiratory syndrome coronavirus (MERS-CoV), discovered in Saudi Arabia nearly a decade later, caused similar devastation and loss, with a 35% mortality rate. SARS-CoV-2, a third member of the Coronaviridae subfamily, emerged as a new deadly disease in December 2019 and was declared a pandemic by the World Health Organization [3,9].

Humans with SARS-CoV-2 infection have developed a variety of diseases, some of which are asymptomatic and occasionally show serious symptoms. Severe COVID-19 symptoms typically include progressive respiratory failure that necessitates hospitalization and ventilation. The disease's lethal state has been caused by ARDS, which is associated with inflammation and thrombosis, often resulting in multiple organ failure [10,11]. Epidemiological studies have shown that age, gender, diabetes, and obesity are all risk factors for the development of severe COVID-19 [2,12].

Based on rapidly evolving data, the National Institutes of Health (NIH) and the Infectious Diseases Society of America (IDSA) have developed the most recent COVID-19 Treatment Guidelines [13]. More than 40 vaccines, hyperimmune serums, numerous drugs, and therapeutic molecules are being studied in clinical trials, with another 150 being studied in preclinical studies [14]. Before entering the clinical stage, the final efficacy of all types of protective and therapeutic products is evaluated with an *in vivo* challenge test. Although different animal models are used in COVID-19 *in vivo* studies, mice are the most preferred model in terms of accessibility and cost. Model animal studies for Corona viruses began during the SARS-CoV and MERS-CoV epidemics. Different

mouse breeds (Balb/C, C57BL/6, B6, and 129S) have been investigated in these diseases, but the expected response could not be obtained. Balb/C mice vaccinated with SARS-CoV showed no clinical symptoms and some virus recovery, despite gaining weight. Although viral RNA was found in the lungs and intestines of these mice, there was no mortality [15,16]. SARS-CoV studies employed Balb/C mice at 21 weeks of age to capture clinical signs [17-19]. In MERS CoV experiments, however, it was found that aged Balb/C mice did not exhibit appropriate clinical signs, and the virus titer remained low. Because of the incompatibility of spike (S) protein with mouse ACE2, conventional laboratory mice did not support MERS-CoV, SARS-CoV, and SARS-CoV-2 infections, which have entered our lives. As a result, a new transgenic mouse model has been developed to replicate human disease as well as for pathogenesis investigations and the development of antiviral treatments [2,16,19,20]. K18-hACE2 transgenic mice may express the human ACE2 receptor, which is utilized by SARS-CoV-2 [21,22]. The hACE gene is expressed in epithelial cells in these transgenic mice under the control of the cytokeratin 18 promoter [23,24]. The development of model mice distinct from Balb/C and C57BL/6 mice has been critical for understanding the mechanisms of SARS-CoV-2 and developing treatment strategies.

K18-hACE2 transgenic mice are produced and sold at few sites around the world. However, many experimental animal facilities that desire to conduct SARS-CoV-2 research do not have this mouse strain. In this study, the Balb/C mouse and Nude mouse strains, both of which are widely available in Türkiye, were compared to K18-hACE2 transgenic mouse strains against the SARS-CoV-2 virus. The Balb/C mouse research carried out during the SARS and MERS CoV outbreaks was replicated in this investigation under current settings. Another aim of our research was to compare the quantity of virus uptake in various organs and the lung of a SARS-CoV-2 virus model animal, the K18-hACE2 transgenic mouse.

MATERIAL AND METHODS

Ethics Statement

All experimental procedures with animals were approved by TUBITAK (Marmara Research Center) MRC, Life Sciences, Medical Biotechnology Unit in Kocaeli, TÜRKİYE. All procedures in this study involving animals were reviewed and approved by the Institutional Biosafety Committee and Institutional Animal Care and Use Committee (HADYEK-16563500-111-3026); all the experiments were conducted in compliance with all relevant ethical regulations. The experiments were conducted in BSL3 and animal BSL3 (ABSL3) facilities at TUBITAK MRC Life Sciences.

Animals

The transgenic K18-hACE2 [B6.Cg-Tg(K18-hACE2) 2Prlmn/J] mice used in this study were provided by the Jackson Laboratory in the United States. TUBITAK MRC Life Sciences Experimental Animals Unit has rights for the production of K18-hACE2 transgenic mice. All of the experiments were conducted in a biocontainment isocage, which is part of ABSL 3. 16-18 week-old female Balb/C and Nude mice and 8-10 week-old female K18-hACE2 transgenic mice were used, and 4 mice (20-30 g body weight) were used for each group. The animals kept in an environment with a temperature of 20-24°C, with a controlled light cycle (12 h light and 12 h dark) and consumption of SPF solid food and water ad libitum throughout the experimental period. There were three groups in this study: the Balb/C mice group, the nude mice group, and the K18-hACE2 transgenic mice group.

Challenge Method for SARS-CoV-2 Infection in Mouse Models

SARS-CoV-2 virus strain B.1.1.7 (alpha variant) was isolated from patients by the Ministry of Health Directorate of Public Health and provided to us for this study. All virus growth was done in biosafety level 3 (BSL-3) labs at the TUBITAK Marmara Research Center (TUBITAK MRC), Life Sciences, and Medical Biotechnology Unit in Kocaeli, TÜRKİYE. This laboratory had all the international certificates needed to work with SARS-CoV-2.

In the BSL 3 facility, Balb/C, Nude, and K18-hACE2 mice were housed in biocontainment cages for a challenge experiment with the SARS-CoV-2 virus B.1.1.7 with a $TCID_{50}$ value of 10^5. It was kept in the laboratory for two days for adaptation to the environment. In the study's model, $1x10^5$ $TCID_{50}$ virus was administered to the animals via gavage and intranasal administration. For the first three days of the experiment, the animals were given SARS-CoV-2 virus orally via gavage, followed by 50 µL of 10^5 $TCID_{50}$ intranasally under anesthesia *(Fig. 1)*. The mice's gavage dosage was modified according to their

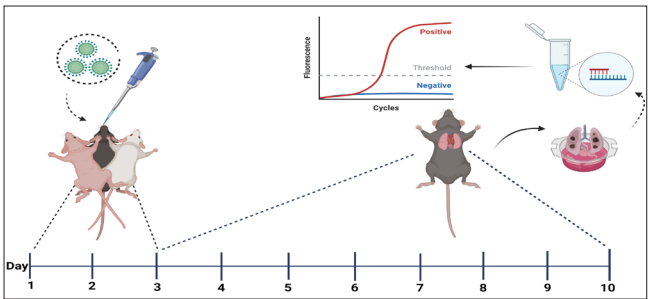

Fig 1. Representation schematic of the *in vivo* challenge experiment. Balb/C, Nude, and K18 hACE2 mice were administered the SARS-CoV-2 (Alpha) virus for three days. On the tenth day of the experiment, autopsy-collected lungs were analyzed by PCR to ascertain the amount of viral RNA (n = 4/group)

weight, however the maximum quantity they may aspirate with intranasal administration is 50 µL. As a result, all mice received the same doses of virus intranasally. The experiment lasted a total of ten days. After being infected with the virus, mice were monitored on a daily basis for morbidity (body weight) and mortality. Mice that lost more than 25% of their baseline body weight were considered to have reached the experimental endpoint and were exterminated. Pathological examinations were performed after the animals were sacrificed. All abdominal organs and thoracic cavity organs were examined with the naked eye during gross pathology. Each mouse lung was used to compare viral load by real-time PCR analysis [25-27]. During gross pathology, samples were taken to compare how much of the SARS-CoV-2 virus was absorbed into the lung, heart, spleen, liver, and kidneys of K18-hACE2 transgenic mice.

The lungs were evaluated and scored with the naked eye in terms of color, tissue integrity, appearance, and size during gross pathology. Organs that were clean and free of lesions received a score of 0 (zero), while organs with lesions such as edema, hyperemia, and pneumonia received a score ranging from 1 to 5 (one to five) [28].

Tissue Homogenization

The mice's lungs and other organs were sonicated for viral RNA analysis. The organ tissues were homogenized separately in 2 mL of PBS using an ultrasonic homogenizer at 70% amplitude for 90 sec (Bandelin HD2200.2, Germany) for viral isolation. Tissue homogenates were centrifuged at 21.500 x g for 10 min, and supernatants were collected into 15-mL falcon tubes.

Viral RNA Isolation and RT-PCR

Viral RNA was extracted with the QIAamp Viral RNA Mini Kit, Cat. No. 52906 (QIAGEN, USA) according to the instructions of the manufacturer. The viral RNA detection was performed using SARS-CoV-2 nucleocapsid-specific primers and probes detailed below with the One Step PrimeScript III RT-PCR Kit (Takara, Japan). All reactions were performed on a CFX96 Touch instrument (BioRad, USA) with the following Real-Time PCR conditions: 52°C for 5 min, 95°C for 10 sec, then 44 cycles of 95°C for 5 sec and 55°C for 30 sec. The primer and probe sequences that are used for RT-PCR are CDC-recommended and FDA-approved (EUA) NC1 and NC2 primer-probe sets whose target region is the Nucleocapsid (NC) gene of SARS-CoV-2. (Primer and probe sequences are: N1 Forward: 5'-GAC CCC AAA ATC AGC GAA AT-3', N1 Reverse: 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3, N1 Probe: 5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3 N2 Forward: 5'-TTA CAA ACA TTG GCC GCA AA-3' N2 Reverse: 5'-GCG CGA CAT TCC GAA GAA-3' N2 Probe: 5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3) [29]

Statistical Analysis

An unpaired one-way ANOVA was used for comparison between three groups. A two-sided P value <0.05 was considered statistically significant. Statistical analyzes and graphs was performed with GraphPad Prism 5 programs.

Results

Clinical Evaluation

Throughout the study, the weight and clinical symptoms of the mice in all three groups were monitored. K18-hACE2 transgenic mice were the most affected clinically. Weight loss began in virus-infected mice on the fifth day of the trial, and by the end of the experiment, approximately 17-29% live weight reduction was seen. The animals in the nude mouse group had a slight clinical effect. Two out of four mice lost approximately 6.5% of their live weight, whereas the other two mice lost no weight. The Balb/C mouse group was not clinically affected. One mouse lost 3.5% of its body weight, whereas the other mice gained weight *(Fig. 2)*.

Gross Pathology

On the tenth day of the study, the animals in the experiment were sacrificed using the cervical dislocation procedure, and gross pathology was done. Pneumonia was observed in the lungs of two mice in the K18-hACE2 transgenic mouse group, and hemorrhagic regions occurred in the lungs of the remaining two mice, depending on erythrocyte density. Hemorrhagic regions have been found in the lungs of two naked mice and one Balb/C mouse. In Nude and Balb/C mice, no abnormal signs were found in the lungs or other organs of the remaining animals. *Fig. 3-A* shows the gross pathology-related appearance of the animal lungs that were obtained, and *Fig. 3-B* shows the score graph.

Viral Load Assessment by RT-PCR

In this study, the presence of the virus was determined using RT-PCR and viral RNA from the lungs and other

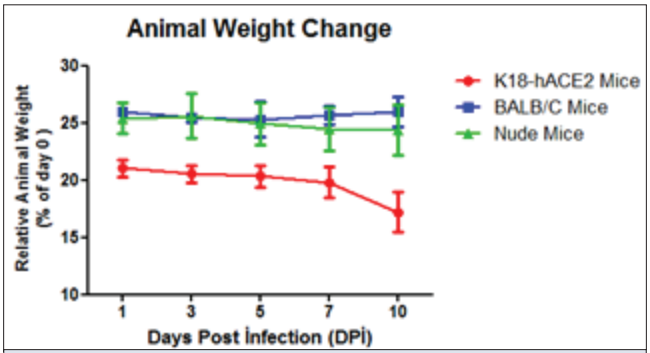

Fig 2. Graph demonstrating the mean change in body weight of challenged mice over a 10-day period. Only the K18 hACE2 group showed a significant response after seven days. One-way ANOVA test was used for statistic (P<0.0001), between-group variation was found to be significant

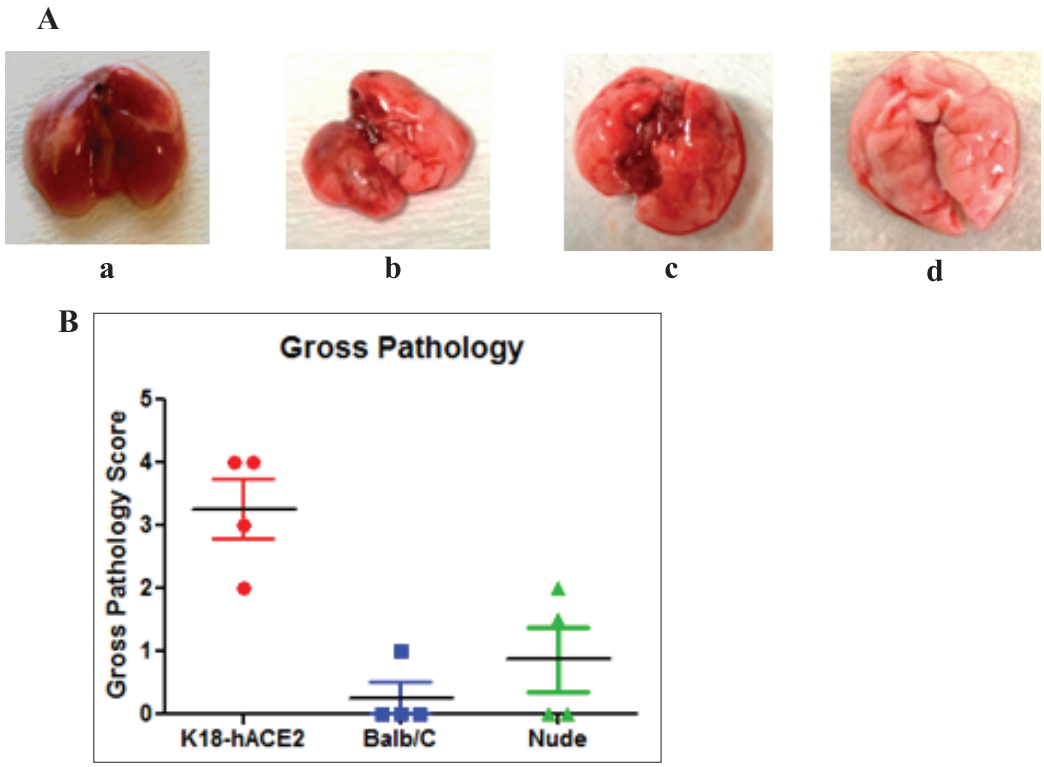

Fig 3. A) Gross pathology images of experimental mice's lungs **a)** Pneumonia-formed lung of K18 hACE2 transgenic mouse; **b)** hyperemic lung of nude mouse; **c)** Less hyperemic lung of Balb/C mouse; and **d)** intact, healthy lung of Balb/C Mouse, **B)** Gross pathological inflammation score graph of lungs from three groups of mice. Lung inflammation was graded on a scale of 0 to 5 (0 for no inflammation, 1 for low-level hyperemia, 2 for significant pathological lesion-hyperemia, and 3-5 for various stages of pneumonia). One-way ANOVA test was used for statistic (P<0.0020), but the findings were determined to be non-significant

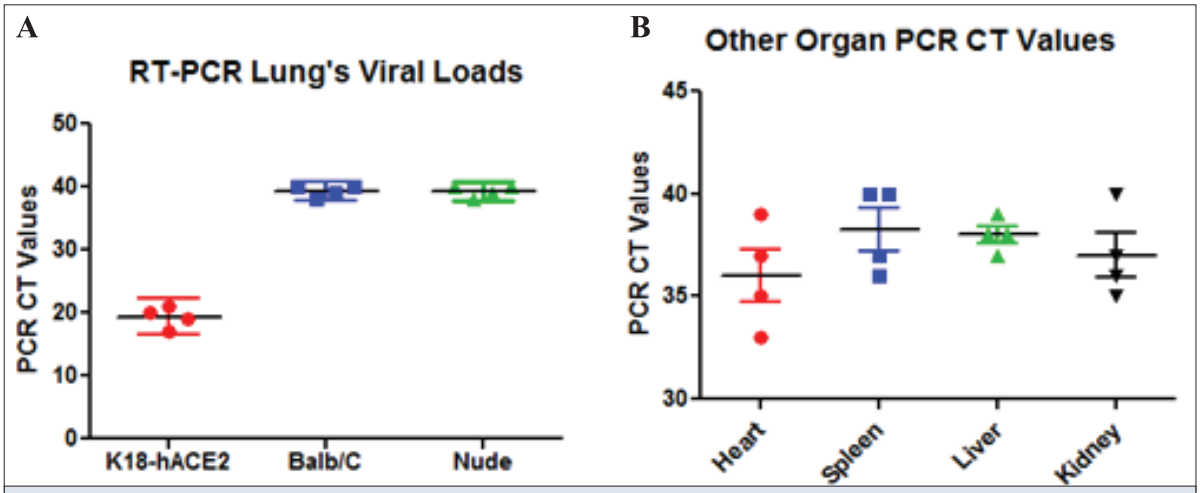

Fig 4. Viral loads in lung and other organs determined by Real Time PCR targeting two distinct regions of the SARS-CoV-2 Nucleocapsid gene. One-way ANOVA test was used for statistics. **A)** Results for the viral loads in the lung were significant (p<0.0001), according to RT-PCR, **B)** Comparing viral loads in other organs, however, was not shown to be significantly distinct (P<0.4096)

organs. CT measured between 16 to 21 in the K18-hACE2 transgenic mice group. CT measured between 37 to 40 in the Nude and Balb/C mice groups. In the K18-hACE2 transgenic group, between 33 to 40 viral CTs were acquired from different organs of mice. The CT averages for these organs are as follows: heart 36%, spleen 38%, liver 38%, and kidney 37% *(Fig.4-A,B)*.

Discussion

Many studies with K18-ACE2 transgenic mouse models

against COVID-19 disease have been published in the literature. According to Winkler et al.[23] following intranasal infections of K18-ACE2 mice at 4 dpi (Day Post İnjection) and 7 dpi, the animals lost significant weight and had high virus loads at 2 dpi, 4 dpi, and 7 dpi. According to this study, the most virus was found in the lungs of hACE2 mice at 3 dpi, followed by a decrease at 5 dpi and a continued decrease at 7 dpi [30]. The results of these studies have demonstrated that if K18-ACE2 transgenic mice are exposed to the virus every other day, the viral load begins to decline after the fifth day, becomes minimal after 7-9 days, and the virus disappears within a few days. According to the literature, we used a 3 dpi for the groups in our study [26,27]. The study contrasted the Balb/C mouse strain, which was used in the past for MERS-CoV and SARS-CoV infections, as well as the immunosuppressed CD1-nude (Foxn1null) mouse race and K18-ACE2 transgenic mice, which are model animals. Furthermore, the heart, spleen, liver, and kidneys of mice in the hACE2 group were collected, and the role of the SARS-CoV-2 virus was investigated [25,30].

When the clinical symptoms of $1x10^5$ B.1.1.7 strains and different breeds of mice were studied in this study, Balb/C mice had no effects at all, and the animals even gained weight at the end of the experiment. At the end of the investigation, naked mice lost about 1.5 g of weight, but no additional clinical signs were observed. K18-ACE2 transgenic mice, on the other hand, experienced significant weight loss as well as clinical signs such as put-off movements, abdominal breathing, stooped posture, and eye burrs.

In their study of K18 hACE2 transgenic mice, Oladunni et al.[21] investigated the histological changes and gross pathology changes of organs such as the lung, brain, liver, and spleen in 2 dpi and 4 dpi groups. They linked neutrophil and lymphocyte infiltration into the alveolar regions to the emergence of slight pneumonia at 2 dpi. At 4 dpi, the pneumonia rate increased fourfold, the alveolar gaps narrowed with hemorrhagic hemorrhages, and inflammatory cell infiltration increased. In terms of gross pathology, there is no significant pathological lesion in the lungs or other organs of Balb/C or Nude mice. Erythrocyte and lymphocyte infiltration of the lungs was seen in one Balb/C mouse and two Nude mice. In four K18-ACE2 transgenic mice, pneumonia was shown to be graded 2-4 in the lungs [28].

In this study, the presence of virus in the lungs was determined by RT-PCR, and viral density was calculated using CT values. CT ranged from 16 to 21 in the K18-hACE2 transgenic mice. Early CT values indicate a large number of viruses in this group of animals. Nude and Balb/C mice, on the other hand, produced 37-40 CT. CTs of 30 or above show that there is very little viral RNA *(Fig.4-A)*. As a result, even though CT values were found in the Nude and Balb/C groups, the results were deemed negative [26-28]. Because there was no viral load in the target organ lungs of Balb/C and Nude mice, only K18-hACE2 transgenic animals were studied for viral uptake in other organs. When comparing CT averages from organs other than the lung, low positive viral results were obtained, as in the studies of Sun et al.[25] and Johansen et al.[16]. According to our results, when we compared the viral involvement of the organs, they were heart > kidney > spleen > liver, respectively *(Fig. 4-B)*.

This investigation compared prior investigations using Balb/C, Nude, and K18-hACE2 transgenic mice groups. Older Balb/C mice employed in this study, on the other hand, displayed a substantially lower, non-significant response to the SARS-CoV-2 virus. This study showed that Balb/C experiments against Coronaviruses are not scientifically sufficient at the present time. But we don't know if this is just tied to the SARS-CoV-2 virus or if it would have responded similarly to the SARS-CoV virus today. It was revealed that CD1 nude mice, an immunosuppressive mouse strain, did not respond to SARS-CoV-2 tries as expected. With this study I demonstrated that only the K18-hACE2 transgenic mouse line should be utilized in COVID-19 mouse model experiments to produce accurate results. Despite the presence of SARS-CoV-2 virus in other organs in this study, necropsy showed that it did not cause clinical damage in these organs due to its low level virus.

Availability of Data and Materials

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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Research Article

Social Problem-Solving Skills and Empathy Levels of Veterinary Clinicians in Türkiye

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ABSTRACT

This study aimed to investigate the impact of the social problem-solving skills and empathy capacity of veterinary clinicians on their professional practice and to identify the major influential factors involved. For this purpose, 454 veterinary clinicians were enrolled in an online survey. Data was collected by applying a personal information sheet, the Social Problem Solving Inventory-Revised scale, and the Basic Empathy Scale. There was a negative correlation between clinicians' social problem-solving skills and general empathy capacity (P<0.001; r=-0.153) and emotional empathy capacity (P<0.001; r=-0, 255); a positive correlation between social problem-solving skills and cognitive empathy capacity (P<0.01; r=0.131). The study results showed that female had a greater capacity for empathy (P<0.05). It was determined that the participants' general empathy capacity (P<0.05; r=-0.098) and emotional empathy capacity (P<0.05; r=-0.102) decreased with a longer career as a veterinary clinician. The question *"If you had the chance to choose, would you opt again for being a veterinarian?"* was responded to with a *"no"* by clinicians with high emotional empathy (P<0.01) and with a *"yes"* by clinician with high social problem-solving skills (P<0.05) As a result, it could be speculated that activities such as veterinary faculty curriculum development or in-service courses on the improvement of cognitive empathy capacity would contribute to enhancing the performance of veterinary clinicians in dealing with problems.

Keywords: Cognitive empathy, Emotional empathy, Social problem solving, Türkiye, Veterinarians

Introduction

A social problem is described as the failure of a person to fulfill a task or responsibility during the course of daily life [1]. Since social problems arise from the behavioral, emotional, and social troubles a person faces in his or her living environment, they may potentially affect the environmental adaptability and quality of life of this person. Given that social problems may also negatively affect a person's interactions with other members of society, social problem-solving skills are crucial to the daily life of an individual. In social problem solving, individuals should be effective in analyzing the situation, using and developing strategies [1,2].

In order to solve a social problem, it is required that one first communicate with his or her counterpart to accurately identify the problem in question. Empathy enables us to apprehend the intention and predict the behavior of others, experience feelings, and thereby, effectively interact with people in social settings. Baron-Cohen and Wheelwright [3] describe empathy as the "glue" of the social environment, which inclines us to help others and abstain from hurting their feelings. Empathy is classified into two types: cognitive and emotional. Cognitive empathy (CE) is described as the ability to understand and accurately assess another person's perspective and emotional state, while emotional empathy (EE) is described as the ability to feel another person's emotions. While "friendly feelings" are at the forefront of emotional empathy, "perspective-taking" is important for cognitive empathy [4]. From this standpoint, empathy is considered to have a critical role in the ability to solve social problems [5,6].

In clinical practice, veterinary clinicians are in continual communication with both animals and animal owners and, thus, are frequently faced with social problems. The empathy capacity of the individual is one of the several determining factors of the smooth continuation of the communication process [6]. It is suggested that, by contributing to the solution of social problems, high empathy capacity of the clinician may increase the

satisfaction of the animal owner with the healthcare service provided, as well as the confidence of the animal owner in the clinician and his or her "customer loyalty" [7-9]. Furthermore, empathy capacity enhances the interpretation of clinical symptoms by veterinary clinicians by enabling occupational satisfaction and reducing work stress, -and thus, - can increase the success rate of the treatment employed by them [10].

While literature is available on the correlation between social problem-solving skills and empathy capacity for various professions and groups [11-14], to our knowledge, there is no previous study investigating this topic for the veterinary medical profession in Türkiye. The investigation of the impact of the social problem-solving skills and empathy capacity of veterinary clinicians could contribute to identifying shortcomings or weaknesses and actions for improvement such that potential adversities are prevented.

This study aimed to investigate the impact of the empathy capacity of veterinary clinicians on their social problem-solving skills and to demonstrate any demographic variable-based difference in empathy capacity and problem-solving skills in Türkiye.

Material and Methods

Ethical Statement

This study was approved by Fırat University Social and Human Sciences Research Ethics Committee (Approval no: 06/24-07.04.2022).

Study Design

The social problem-solving skills and empathy capacity of veterinary clinicians in Türkiye were measured using the Social Problem Solving Inventory-Revised (SPSI-R) [15] and the Basic Empathy Scale (BES) [16], respectively. Demographic variables were determined by means of a personal information sheet.

Social Problem Solving Inventory-Revised

This is a 25-item scale developed by D'Zurilla et al.[17]. Each item is rated on a 5-point Likert scale ranging from 0 (not at all true for me) to 4 (extremely true for me). The scale was adapted to Turkish, validity and reliability analyses were performed, and accordingly, the Cronbach alpha reliability coefficient of the scale was computed as 0.85 [15].

Basic Empathy Scale

Developed by Jolliffe and Farrington [18], this is a 20-item scale based on four basic feelings, namely, fear, sadness, anger, and happiness, and includes subscales of cognitive empathy and emotional empathy. Cognitive empathy occur 9 items (items 3, 6, 9, 10, 12, 14, 16, 19, 20), and emotional empathy occur 11 items (items 1, 2, 4, 5, 7, 8, 11, 13, 15, 17, 18). The items are rated on a 5-point Likert scale ranging from 1 (strongly disagree) to 5 (totally agree) [18]. The adaptation of the scale to Turkish and the validity and reliability analyses were performed by Topçu et al.[16]. Based on the analyses of this scale, the Cronbach alpha reliability coefficient was computed as 0.79 [16].

Personal Information Sheet

This form was prepared by the researchers to collect data on the sociodemographic characteristics of the respondents (age, gender, marital status, parentage of children, university of graduation, years of professional experience (career length), location of veterinary clinic, specialization area of veterinary clinic, pet ownership, whether a career in veterinary was intentionally chosen as a profession, occupational satisfaction).

The study was based on a correlational research design [19]. In order to minimize face-to-face interactions and facilitate the participation of veterinary clinicians, the survey was designed as a Google form, and data was collected online. The online survey was designed to include an informed consent form on the first page and be launched in the event that the respondent agreed to participate in the survey. Data was collected during the period between 21.04.2022-19.05.2022.

Participants

Since there is no reliable data on the number of veterinarians working in the clinics, the total number of veterinarians in Türkiye has been taken into account. The total number of veterinarians in Türkiye has been reported as 40.000 by the Turkish Veterinary Medical Association [20]. Considering that the population is at least 40.000, it is recommended that the sample size be between 378-381 [21]. Therefore, it was aimed to include at least 400 respondents in the study. Some participants were sent questionnaires directly to their phones (requested support from clinicians to reach more participants and they sent the questionnaire to other clinicians after completing), while others were contacted by veterinary groups via social media and asked to complete the questionnaire (the survey was shared in groups of veterinary clinicians). All veterinary faculty graduates in Türkiye have been reached (Afyon Kocatepe Univ., Aksaray Univ., Ankara Univ., Atatürk Univ., Aydın Adnan Menderes Univ., Balıkesir Univ., Bingöl Univ., Burdur M.A. Univ., Bursa Uludağ Univ., Çukurova Univ., Dicle Uni., Erciyes Univ., Fırat Univ., Harran Univ., Hatay Mustafa Kemal Univ., Istanbul Univ., Kafkas Univ., Kırıkkale Univ., Near East Univ., Ondokuz Mayıs Univ., Selçuk Univ., Siirt Univ., Sivas Cumhuriyet Univ., Tekirdağ Namık Kemal Univ. and Van Yüzüncü Yıl University).

Statistical Analysis

Since the scale was applied on a new sample, Explanatory Factor Analysis (EFA) and Confirmatory Factor Analysis (CFA) were performed to examine the compatibility between the scales and the data set before starting the analysis of the data. For this purpose, to understand whether there is a correlation between variables, Bartlett's test of sphericity; Kaiser–Meyer–Olkin (KMO) test was performed to measure the adequacy of the sample size. For fit assessment, fit indices X^2 (chi square)/df (degrees of freedom), Standardized Root Mean Square Residual (SRMR), Root Mean Square Error of Approximation (RMSEA) were used. JAMOVI 2.2.5 package program [22] was used in these analyzes. After revealing the compatibility between the scale and the data set, the study data was analyzed with the SPSS 22 software [23]. Frequency and percentage distribution was made for the data. Kolmogorov Smirnov test was used to examine the distribution of data. Independent t-test was used in paired group comparisons, and the Anova test was used in comparisons of 3 or more groups. Duncan and Games Howell test from post hoc tests were made according to the equality of variances. Pearson's correlation test was used to determine the level and direction of the correlation between the demographic data and scale scores.

Results

The responses of 454 of the participants were included in the study. Due to the design of the questionnaire, there is no missing data in terms of any question. As a result of EFA for SPSI-R, 5 factors with an eigenvalue above 1 were formed. The values in Bartlett's test of sphericity and KMO were calculated as P<0.001 and 0.858, respectively. The total variance explanation level was 49.4%. As a result of CFA, X^2/df value was calculated as 2.56, SRMR value was 0.067, RMSEA value was 0.058. There were 4 factors with an eigenvalue above 1 for BES. The values in Bartlett's test of sphericity and KMO were calculated as P<0.001 and 0.866, respectively. The total variance explanation level was 53.5%. As a result of CFA, X^2/df value was calculated as 3.66, SRMR value was 0.064, RMSEA value was 0.076. The Cronbach alpha coefficients of the BES and SPSI-R were calculated as 0.828 and 0.844, respectively.

The sociodemographic characteristics of the respondents are presented in *Table 1*. In the present study, the SPSI-R, BES, EE, and CE scores were determined not to display any statistically significant difference for the following variables: age, marital status, parentage of children, university of graduation, location of veterinary clinic, specialization area of veterinary clinic, pet ownership, and whether a career in veterinary medicine was intentionally chosen as a profession (P>0.05).

Table 1. *Sociodemographic characteristics of the participants*

Variables		**N**	**%**
Gender	Female	110	24.2
	Male	344	75.8
Age (years)	30 and below	187	41.2
	31-40	167	36.8
	41-50	69	15.2
	51 and above	31	6.8
Marital status	Married	288	63.4
	Single	166	36.6
Status of having children	Yes	231	50.9
	No	223	49.1
Working year as a veterinary clinician	5 and below	220	48.5
	6-10	91	20.0
	11-15	53	11.7
	16-20	37	8.1
	21-25	29	6.4
	26 and above	24	5.3
Where the clinic is located	Big city	184	40.5
	Provincial center	67	14.8
	District	183	40.3
	Village	20	4.4
Working area of the clinic	Pet	191	42.1
	Farm	137	30.2
	Mix	126	27.7
Pet ownership status	Yes	286	63.0
	No	168	37.0
Did you choose the veterinary profession willingly?	Yes	402	88.5
	No	52	11.5
If you had the chance to choose, would you opt again for being a veterinarian?	Yes	329	72.5
	No	125	27.5

The BES scores of the respondents significantly differed for the gender variable, and female were observed to score higher points (P<0.05). Furthermore, the BES- and SPSI-R-based comparison of the responses given to the question "*If you had the chance to choose, would you opt again for being a veterinarian?*" demonstrated statistically significant differences (P<0.01). Participants who had responded with a *"no"* to this question had higher BES scores, and those who had responded with a *"yes"* to the question had higher SPSI-R scores *(Table 2)*.

The breakdown of the total scores calculated according to the answers given by the participants to the sets is as follows: 65.89 (min:0, max:100) for the SPSI-R; 59.13 (min:20, max:100) for the BES; 30.13 (min:9, max:45) for CE; and 29.00 (min:11, max:55) for EE.

Table 2. *Comparison of scores obtained from BES, SPSI-R, EE and CE with sociodemographic variables*						
Variable	**Gender**	**N**	**Mean**	**t**	**df**	**P**
BES	Female	110	59.8545	2.068	452	*0.039*
	Male	344	58.9041			
Variable	***If you had the chance to choose, would you opt again for being a veterinarian?***					
BES	Yes	329	58.8055	-2.719	452	*0.007*
	No	125	60.0000			
EE	Yes	329	28.6657	-3.265	452	*0.001*
	No	125	29.8800			
CE	Yes	329	30.1398	0.093	452	0.926
	No	125	30.1200			
SPSI-R	Yes	329	66.8815	3.324	452	*0.001*
	No	125	63.2960			

BES: Basic Empathy Scale, EE: Emotinal Empathy, CE: Cognitive Empathy, SPSI-R: Social Problem Solving Inventory-Revised, N: Number, df: Degrees of freedom, P: Probability

Table 3. *Correlation results between scores obtained from BES, SPSI-R, CE and EE*			
Variables		**SPSI-R**	**Working Year as a Veterinary Clinician**
BES	Pearson correlation	**-0.153****	**-0.098***
	P	0.001	0.037
	N	454	454
CE	Pearson correlation	0.131**	-0.024
	P	0.005	0,616
	N	454	454
EE	Pearson correlation	-0.255**	-0.102*
	P	0.000	0.030
	N	454	454

**Correlation is significant at the 0.01 level, ** Correlation is significant at the 0.05 level, BES: Basic Empathy Scale, CE: Cognitive Empathy, EE: Emotinal Empathy, SPSI-R: Social Problem Solving Inventory-Revised, P: Probability, N: Number*

An assessment of the correlation between the BES scores, SPSI-R scores and variables demonstrated that the SPSI-R, score was negatively correlated with the BES and EE scores and positively correlated with the CE score (P<0.01). Furthermore, a longer career as a veterinary clinician was determined to be negatively correlated with the BES and EE scores (P<0.05) (*Table 3*).

Discussion

As time-dependent changes in attitude cannot be monitored with the use of the cross-sectional data collection technique, a longitudinal study design could be preferred in future studies for stronger speculations. Furthermore, as online communication was established with the respondents in the present study, a proportionate stratification of the sample could not be made for the sociodemographic variables. This may have led to a bias in favor of the male respondents, respondents younger than 30 years of age, and respondents with a work experience of less than 5 years.

This study was aimed at investigating the impact of the empathy capacity of veterinary clinicians working in Türkiye on their social problem-solving skills and determining the correlation between sociodemographic characteristics, social problem-solving skills, and empathy capacity.

If the Bartlett sphericity test analysis result is at a significant level (P<0.05), the data; KMO coefficient greater than 0.60 indicates that the sample is suitable for factor analysis [24]. As a result of Bartlett sphericity (for both scales: P<0.001) and KMO tests (for SPSI-R: 0.858; for BES: 0.866), it was understood that the scales were suitable for EFA. Total variance explanation level for SPSI-R and BES were calculated 49.4% and 53.5%, respectively. Total variance explanation level between 40% and 60% is considered

sufficient [23]. X^2/df values <5, SRMR and RMSEA values of ≤0.08 indicate that the model and the data set are compatible [25,26]. Values for SPSI-R were X^2/df: 2.56; SRMR: 0.067; RMSEA; 0.058 and for BES were X^2/df :3.66, SRMR: 0.064, RMSEA: 0.076. It was determined that the values for both scales were within these reference ranges and therefore the scale and the data set were compatible.

Cronbach's alpha coefficients were examined for the reliability of the scales used in the study. A scale's Cronbach's alpha coefficient above 0.80 indicates a high level of reliability [23]. As the alpha coefficients were above 0.80 (SPSI-R: 0.844, BES: 0.828), both scales were confirmed to be highly reliable.

The study results revealed that, among the variables tested, age, marital status, parentage of children, years of professional experience, location of a veterinary clinic, specialization area of a veterinary clinic, pet ownership, and whether a career in veterinary medicine was intentionally chosen as a profession did not bring about any difference in the attitudes of the respondents toward empathy capacity or problem-solving skills.

Research has demonstrated that, in general, female show greater empathy towards both humans and animals [3,27-29]. Similarly, reports indicate that, compared to their male colleagues, female veterinarians [30], and compared to male students, female students of veterinary faculties, have a more empathetic attitude [7,31-35]. Likewise, in the present study, it was determined that while the empathy capacity of the respondents significantly differed by gender, female showed a stronger empathetic attitude than male (P<0.05). This could be explained by the recently proposed empathizing-systemizing theory. Accordingly, the female brain is predominantly hard-wired for empathy, and the male brain is predominantly hard-wired for understanding and building systems [36].

Respondents who gave the answer *"no"* to the question *"If you had the chance to choose, would you opt again for being a veterinarian?"* were observed to have a stronger empathetic attitude (P<0.01). The assessment of the subscales of the BES for the responses given to this question also showed that the respondents who gave the answer *"no"* had a stronger empathetic attitude with respect to emotional empathy (P<0.01). Emotional empathy describes the ability to apprehend and feel the emotions of others, give an emotional reaction to the prevailing circumstances, identify with others, and sympathize with their states [37]. Reports suggest that, as individuals with greater emotional empathy capacity tend to feel others' emotions, they show a higher level of sympathy for negative feelings and troubles [31,38] and suffer more from personal anxiety and distress [39,40]. From this viewpoint, veterinary clinicians with a tendency to sympathize with the negative feelings and distress of animal owners may have responded *"no"* to the question "*If you had the chance to choose, would you opt again for being a veterinarian?"* as a reaction to their continuous exposure to stress.

Respondents with high social problem-solving skills were observed to have responded with a *"yes"* to the question, "*If you had the chance to choose, would you opt again for being a veterinarian?"* (P<0.05). In view of social problem-solving skills reflecting the ability of an individual to successfully achieve tasks and responsibilities in daily life [1,2], it could be said that the success veterinary clinicians achieve in their professional practice generates work satisfaction for them.

In the present study, it was observed that social problem-solving skills were negatively correlated with empathy capacity. When assessed for the subscales of empathy, social problem-solving skills were determined to be negatively correlated with emotional empathy capacity and positively correlated with cognitive empathy capacity (P<0.01). Social problem-solving is a process that requires an accurate understanding of the problem, the establishment of viable options, and the selection and application of the most reasonable option [1]. Understanding the perspective of the counterpart plays an important role in solving social problems [40]. On the other hand, cognitive empathy is described as the ability to understand and accurately assess someone's emotional state [4]. In agreement with the previous studies referred to above, the results of the present study suggest that increased cognitive empathy capacity is associated with increased social problem-solving skills. In view of the fact that emotional empathy refers to the ability to feel the emotions of others and sympathize with them [4], it is possible for veterinary clinicians with strong emotional empathy capacities to establish an emotional bond with the people they encounter, bear their feelings, and fail to make an objective evaluation of the social problems they face [41]. This may have led to the respondents with high EE scores scoring fewer points on the SPSI-R.

The present study demonstrated that a longer career as a veterinary clinician was associated with weaker empathy capacity (P<0.05). Based on the assessment of empathy subscales, this was considered to be related to emotional empathy (P<0.05). Previous studies in this area have reported that empathy capacity decreases with longer years of professional experience in veterinary clinicians [9] and with longer years of university education in students attending medical [42,43] and veterinary [44] faculties. The results of the present study were in agreement with these literature. Compassion is described as not being indifferent to the misfortunes of others, sympathizing with their sufferings, and wanting to help them, whilst the necessity and excessiveness of this state are referred to as "compassion

fatigue" [45]. Reports indicate that compassion fatigue may be observed over time, particularly in health workers over time [46], and may result in the loss of empathy [47]. Hence, it has been reported that veterinarians [48,49] and veterinary technicians [46] are prone to compassion fatigue. In a study on veterinary students, it was determined that students with a work history at a veterinary clinic suffered from a high level of compassion fatigue [50]. Therefore, in the present study, the decrease in empathy capacity observed with a longer career as a veterinary clinician suggests that the clinicians may have developed compassion fatigue over time. However, further, detailed studies are required to fully elucidate this issue.

In summary, the collective assessment of the study data demonstrated that the social problem-solving skills of veterinary clinicians were negatively affected by emotional empathy capacity, but positively affected by cognitive empathy capacity. Thus, it is suggested that the improvement of cognitive empathy capacity could improve social problem-solving skills. For this purpose, either lectures on cognitive empathy could be included in the curricula of veterinary faculties or faculty graduates could attend vocational training courses on cognitive empathy.

Availability of Data and Materials

The data supporting this study's findings are available from the corresponding author (S. Çavuş Alan) upon reasonable request.

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Competing Interests

The authors declared that there is no conflict of interest.

Ethical Statement

This study was approved by Fırat University Social and Human Sciences Research Ethics Committee (Approval no: 06/24-07.04.2022).

Author Contributions

Conception and design: RÖ, SÇA, AÖ. Data collection, analysis and interpretation: RÖ, SÇA, AÖ. Writing: RÖ and SÇA. Reviewing and editing: RÖ, SÇA, AÖ. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Research Article

The Effect of the Combination of Rosemary Extract and Green Tea Extract on Nitrosamine Content, Microbiological, Physicochemical and Sensorial Properties of Heat-Treated Sucuk

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ABSTRACT

In this study, it was aimed to determine the effect of using rosemary extract together with green tea extract (RE/GTE) on nitrosamine content and quality characteristics of heat-treated sucuk. In addition, the influence of cooking time on the formation of nitrosamines was also investigated. Four different batters were prepared as follows: control (C), 0.1% RE/GTE (RG1), 0.2% RE/GTE (RG2), and 0.3% RE/GTE (RG3). The use of RE/GTE caused a decrease in lactic acid bacteria and *Micrococcus/Staphylococcus* counts in the final product. While the use of RE/GTE did not affect the a_w value, the lowest pH value (4.80±0.05) was observed in the RG3 treatment. RE/GTE resulted in a reduction in residual nitrite, and the lowest level was determined as 12.60±0.87 mg/kg. The lower mean TBARS values were determined in the RG2 and RG3 treatments. RE/GTE did not affect the L* and a* values, however, b* value increased in the RG2 and RG3 treatments. The lowest odor, taste, and general acceptability scores were determined in the RG3 group. The use of RE/GTE had no significant effect on nitrosodiethylamine, nitrosodimethylamine, nitrosopiperidine and nitrosopyrrolidine. The levels of nitrosamines increased with cooking, but the interaction of RE/GTE and cooking time was not significant. RE/GTE treatments also caused a decrease in hexanal level and an increase in some terpene compounds.

Keywords: Fermented sausage, Green tea, Heat treated sucuk, Nitrosamine, NDMA, NDEA, NPIP, Rosemary

Introduction

Fermented sausages are widely produced meat products around the world which have different properties. Characteristic properties of these products are affected by such factors as the species and breed of animal, type of fat, degree of comminution, additives (sugar, salt, spices, curing agent), starter culture, type of casing used, fermentation and ripening/drying conditions [1]. Sucuk is a dry-fermented type of sausage that is popular in Türkiye, which is produced by using beef, water buffalo meat, and, although rarely, mutton. In addition to meat fat, sheep tail fat is also used as a source of fat. Typical spices are allspice, paprika, cumin, black pepper and, especially, garlic. The main steps in sucuk production process are preparation of sucuk batter, fermentation and ripening/drying [2,3]. Another type of fermented sausage produced in Türkiye is heat-treated sucuk (HTS). The formulation of this product is same as sucuk. However, after a short fermentation time (initial temperature 22-25°C, pH value <5.3 during fermentation), heat treatment is applied, which is followed by the drying phase [4,5]. The moisture : protein ratio and fat : protein ratio for this product must be under 3.6:1 and 2.5:1, respectively, whereas pH of the product must be at most 5.6 [6].

HTS is an industrial product and only nitrite (150 mg/kg at max) is allowed in the production [7]. Besides its antioxidant and antimicrobial properties, this curing agent also plays a positive role in formation of color and aroma, yet it is also an important factor in the formation of nitrosamines with known carcinogenic effects [8]. The level of nitrosamines in HTS and other fermented sausages

may be below the detectable limit, or may reach high levels [8-14]. The lipid and protein degradation products formed during ripening stage of fermented sausages may be a good source of nitrosamine formation [15]. As a matter of fact, Sallan [16] reported that sheep tail fat increases oxidation and is effective on nitrosamines because it contains high unsaturated fat. Many factors such as the presence of inhibitors and catalysts, cooking temperature and time, cooking method, presence of microorganisms with decarboxylase activity, residual nitrite level, and presence of precursor substances are also effective in the formation of nitrosamines [8,13,17]. In a study conducted on HTS, the effects of nitrite levels, cooking degree, the use of ascorbic acid and starter cultures on nitrosamine formation were examined and it was concluded that cooking time was the most important factor among the factors examined in terms of nitrosamines [10]. Unlike many types of fermented sausage, HTS is cooked before consumption, thus strategies to prevent nitrosamine formation in this product are of great importance.

Rosemary *(Rosmarinus officinalis* L.) and green tea *(Camellia sinensis* L.) extracts are products used in meat products for their antioxidant activities [18-21]. There is little information about the effect of these natural products on the formation of nitrosamines in meat products [22-24]. Li et al.[22] investigated the effects of green tea and grape seed polypheneols on nitrosamine formation in dry cured sausage. In the study on dry cured bacon, the effects of green tea polyphenols, grape seed extract and their combination on N-nitrosodimethylamine (NDMA) were determined [23]. Another study was carried out in cooked sausage (western-style smoked sausage) [24]. In this study, the effects of rosemary and green tea extract, which are used as antioxidants in meat products and have an important place among natural antioxidants, on nitrosamine formation, volatile compounds, microbiological, physicochemical and sensory properties in HTS were investigated.

Material and Methods

Material

For the production, lean meat and beef fat were used as raw material. The supply of raw materials was carried out at three different times, and three productions were made. The *Latilactobacillus sakei* S15 and *Staphlococcus xylosus* GM92 [25,26] were used as starter cultures, and were added to the batters at 10^7 cfu/g and 10^6 cfu/g, respectively. Rosemary extract + green tea extract (RE/GTE) (Veg stable® 721) was obtained from a commercial company (Florida Food Products, USA).

Heat Treated Sucuk Production

In the manufacture of HTS, 80% beef meat and 20% fat were used. The formulation was included per kg meat and fat: 20 g NaCl, 2.5 g allspice, 9 g cumin, 5 g black pepper, 7 g red pepper, 4 g sucrose, and 0.15 g sodium nitrite. Four different batters of HTS were produced: control: HTS without rosemary extract + green tea extract (RE/GTE), RG1: 0.1% RE/GTE, RG2: 0.2% RE/GTE, and RG3: 0.3% RE/GTE. For each treatment, three batters were prepared at three different times.

The batters prepared using a laboratory-type cutter (Mado Typ MTK 662, Dornhan, Schwarzwald) were filled into collagen casings 38 mm in diameter, Naturin GmbH Co., Weinheim, Germany) by means of a filling machine (Mado Typ MTK 591, Dornhan, Schwarzwald). Then, samples were subjected to the fermentation in an automatic climate unit (Reich, Thermoprozestechnik GmbH, Schechingen, Germany) at 22±1°C and 90±2% relative humidity for 24 h. Following the stage, heat treatment was applied in a steam cooking chamber (Mauting, Valtice, Czech Republic) up to 64°C of core temperature. The samples were then dried in the automatic climate unit for 48 h at 16±1°C and 84±2% relative humidity.

Cooking Procedure

HTS sliced (thickness: 0.5 mm) was cooked on a hot plate preheated to 180°C. The cooking time was applied as 1 min (0.5 min per side) or 3 min (1.5 min per side). The uncooked samples were considered as the control group (0 min). The samples were homogenized and taken into glass jars and frozen at -20°C.

Microbiological Analyses

For the enumeration of lactic acid bacteria number, De Man Rogosa Sharpe Agar (MRS, Merck, Darmstadt, Germany) was used. The incubation was carried out at 30°C for 2 days in anaerobic jars (Anaerocoult A, Merck, Darmstadt, Germany). To determination of *Micrococcus/Staphylococcus* number, Mannitol Salt Phenol Red Agar (MSA, Merck) was used, and the incubation was carried out at 30°C for 2 days. For Enterobacteriaceae, Violet Red Bile Dextrose Agar (VRBD, Merck) plates were subjected to incubation at 30°C for 2 days in anaerobic jars (Anaerocoult A, Merck, Darmstadt, Germany) [4].

pH and a_w Analyses

For pH analysis, 10 g samples were homogenized with 100 mL of distilled water using ultra-turrax. The pH was measured with a pH meter (Mettler Toledo, Switzerland). A water activity device (TH-500 a_w Sprint, Novasina, Pfaffikon, Switzerland) calibrated at 25°C with 6 different salt solutions was used for the a_w analysis [27].

Residual Nitrite and TBARS Analyses

To determine residual nitrite, the method of NMKL[28] was used. For extraction, 10 g sample was mixed with 50 mL

ultrapure water (50-60°C) and then, 50 mL acetonitrile was added. After stirring for 15 min, the volume was made up to 200 mL with ultrapure water. After filtration, the samples were transferred to vials. The residual nitrite content was determined using high-performance liquid chromatography (HPLC)/diode array detector (DAD) (Agilent Technology, Santa Clara, CA, USA). The flow rate, UV wavelength and injection volume were used as 2 mL/min, 220 nm and 100 µL, respectively. Results were expressed in mg/kg based on the calibration curve prepared with nitrite standard.

The method given by Lemon [29] was applied in the analysis of thiobarbituric acid reactive substance (TBARS). 2 g of homogenized sample was mixed with 12 mL of TCA solution. After filtering the homogenate through a Whatman 1 filter, 3 mL of the filtrate was added to 0.02 M thiobarbituric acid solution. The mixture was kept in a boiling water bath for 40 min. The mixture was then centrifuged at 2000 G for 5 min and the absorbance was determined at 530 nm. The TBARS value was given as mg MDA/kg sample.

Color and SensoryAnalyses

The color values (L*, a* and b*) were determined using a chroma meter (Minolta, Osaka, Japan) [4]. The sensory analysis was carried out using a structured nine-point scale (1-9 scales: 1: "dislike extremely" to 9: "like extremely"). A total of 20 semi-trained panelists, consisting of 14 females and 6 males, participated in the sensory evaluation. Prior to analysis, panelists were briefed on the application of sensory analysis.

Volatile Compound Analyses

The vial containing 5 g of sample was placed in a thermal block (Supelco, USA) at 30°C for 1 h to collect the volatile compounds. In the extraction, solid phase microextraction with a carboxen/polydimethylsiloxane fiber (CAR/PDMS, 75 µm, Supelco, USA) was used, the fibre was placed in the vial and kept for 2 h. The gas chromatography/mass spectrometry (Agilent, Santa Clara, CA, USA) was used to identify of volatile compounds. The system conditions given by Kaban [30] were used and the libraries of the mass spectrometer and standard materials were used to evaluate the results. In addition, the standard mix (Parrafine mix, 44585-U, Bellefonte, PA, US) was used to determine the Kovats index. The results were given as AUx 10^6.

Nitrosamine Analyses

Nitrosamines were extracted according to the method specified by Wang et al.[23]. GC/MS (Agilent 6890 N/ Agilent 5973, USA) was used to detect nitrosamines. Helium was used as carrier gas and DB-5MS (30 m × 0.25 mm × 0.25 µm, Agilent, USA) as column, and the system was operated in SIM mode. The oven temperature program given by Sallan [16] was applied. Nitrosamine mix (EPA 521 nitrosamine Mix, Supelco, Bellefonte, PA, USA) was used for identification and nitrosamine levels (N-Nitrosodimethylamine (NDMA), N-Nitrosodiethylamine (NDEA), N-Nitrosopiperidine (NPIP), N-Nitrosopyrrolidine(NPYR),Nitrosodipropylamine (NDPA), N-Nitrosomethylethylamine (NMEA) and N-Nitrosodibutylamine (NDBA)) were determined at µg/ kg level. The limit of dedection (LOD) and the limit of quantification (LOQ) values were determined for NDMA (LOD = 0.32, LOQ = 0.97), NDEA (LOD = 0.37, LOQ = 1.12), NPYR (LOD = 0.37, LOQ = 1.13), and NPIP (LOD = 0.32, LOQ = 0.98).

Statistical Analyses

The experiments were carried out according to the randomized complete block design. The use of rosemary extract+green tea extract (RE/GTE) was evaluated as main effect, and the replicates as a random effect. For nitrosamines, RE/GTE and cooking time were also evaluated as main effects. The differences between the means were determined using Duncan's multiple range tests at the $P<0.05$ level. The statistical analyses were carried out using the SPSS version 20 statistical program (SPSS, Chicago, IL, USA). The principal component analysis (PCA) was also performed to determine the relationship between RE/GTE and volatile compounds, and between RE/GTE, cooking time and nitrosamine using Unscrambler program (CAMO version 10.1, Oslo, Norway).

Results

The effect of rosemary extract/green tea extract (RE/GTE) treatment on lactic acid bacteria (LAB), *Micrococcus/ Staphylococcus*, pH, a_w, residual nitrite and TBARS of HTS are given in *Table 1*. The use of RE/GTE treatment caused an important reduction in the count of LAB in HTS ($P<0.05$). Micrococci and staphylococci were also affected by RE/GTE treatment, but no significant difference was observed between the control and RG1 group *(Table 1)*. On the other hand, Enterobacteriaceae were determined to be <2 log cfu/g for all treatments (data not shown).

In all treatments, the mean pH value was under 5.0. The pH value is an important factor to stabilize the HTS and ensure its safety [1]. The use of RE/GTE caused a significant change in pH in HTS. The lowest mean pH value was found RG3 treatment *(Table 1)*. In the present study, the use of RE/GTE had no significant effect on a_w, and a_w values ranged between 0.931 and 0.934 *(Table 1)*.

The use of RE/GTE caused a decrease in the residual nitrite level and the lowest value was observed in RG3 (0.3%) *(Table 1)*. In addition, the use of RE/GTE in HTS had a significant effect on the TBARS value ($P<0.01$).

Table 1. *The effects of using rosemary extract/green tea extract at the different levels on lactic acid bacteria, Micrococcus/Staphylococcus, pH, a_w, residual nitrite and TBARS of HTS (mean ± SD)*

Treatment	Lactic Acid Bacteria (log cfu/g)	*Micrococcus/ Staphylococcus* (log cfu/g)	pH	a_w	Residual Nitrite (mg/kg)	TBARS (mg MDA/kg)
Control	4.20±0.51[a]	4.81±0.29[a]	4.97±0.06[a]	0.933±0.003[a]	18.40±1.20[a]	0.83±0.05[a]
RG1	3.35±0.12[b]	4.59±0.25[ab]	4.92±0.01[ab]	0.934±0.003[a]	15.47±1.50[b]	0.75±0.02[a]
RG2	3.28±0.07[b]	4.46±0.29[b]	4.88±0.02[b]	0.931±0.003[a]	15.07±2.20[b]	0.59±0.07[b]
RG3	3.08±0.08[b]	4.28±0.37[b]	4.80±0.05[c]	0.932±0.003[a]	12.60±0.87[c]	0.62±0.03[b]
P-value	<0.01	<0.05	<0.01	>0.05	<0.01	<0.01

HTS: *Heat treated sucuk,* ***RG1:*** *0.1% RE/GTE,* ***RG2:*** *0.2% RE/GTE,* ***RG3:*** *RE/GTE,* ***a-c:*** *Means with different letters in the same column are statistically different (P<0.05)*

However, no significant difference in TBARS value was observed between RG1 treatment (0.1%) and control group. RG2 and RG3 treatments gave lower TBARS values than control and RG1 treatments *(Table 1)*.

The effect of using rosemary extract/green tea extract at the different levels on enstrumental color values of HTS (mean ± SD) is given in *Table 2*. The L* and a* values of HTS were not affected by the addition of RE/GTE (P>0.05). On the other hand, RE/GTE had a very significant effect on the b* value (P<0.01). The addition of RE/GTE to the sausage batter had a very significant effect (P<0.01) on taste, odor and overall acceptability, but not on color and texture (P>0.05) *(Table 3)*. The lowest odor score was observed in the RG3 treatment. The lowest values in terms of taste and overal acceptibility were also determined in this treatment *(Table 3)*.

The effect of using RE/GTE at the different levels on volatile compounds of heat treated sucuk (mean ± SD) is given in *Table 4*. A total of 48 compounds including 1 alcohol, 7 suphur compounds, 1 acid, 2 aldehydes, 4 aliphatic hydrocarbons, 5 aromatic hydrocarbons, 2 esters, 3 ketones, 1 furan and 22 terpens were identified *(Table 4)*. In the present study, it was also observed that terpenes were the most aboundant volatile compounds in HTS. RE/GTE had a very significant effect on 3-carene and D-limonene (P<0.01). Hexanal, o-cymene, γ-terpinene, linalol and 4-terpineol were affected by the addition of RE/GTE at level of P<0.05 *(Table 4)*.

Table 2. *The effect of using rosemary extract/green tea extract at the different levels on enstrumental color values of HTS (mean ± SD)*

Treatment	Enstrumental Color Values		
	L*	a*	b*
Control	47.38±0.21[a]	17.73±0.18[a]	15.33±0.24[b]
RG1	47.01±0.10[a]	16.98±0.65[a]	15.75±0.38[b]
RG2	46.97±0.17[a]	17.17±0.40[a]	16.65±0.71[a]
RG3	46.68±0.25[a]	16.88±0.21[a]	16.76±0.40[a]
P-value	>0.05	>0.05	<0.01

HTS: *Heat treated sucuk,* ***RG1:*** *0.1% RE/GTE,* ***RG2:*** *0.2% RE/GTE,* ***RG3:*** *RE/GTE,* ***a-b:*** *Means marked with different letters in the same column are statistically different (P<0.05)*

Table 3. *The effect of using rosemary extract/green tea extract at the different levels on sensory parameters of HTS (mean ± SD)*

Treatment	Color	Texture	Odor	Taste	Overall Acceptability
Control	7.70±0.30[a]	7.00±0.10[a]	7.43±0.15[b]	7.40±0.20[a]	7.43±0.15[a]
RG1	7.47±0.45[a]	7.30±0.40[a]	7.90±0.10[a]	7.57±0.25[a]	7.80±0.20[a]
RG2	7.33±0.47[a]	7.53±0.32[a]	7.77±0.23[ab]	7.20±0.20[a]	7.53±0.25[a]
RG3	7.07±0.31[a]	7.07±0.21[a]	6.73±0.21[c]	6.63±0.15[b]	6.53±0.25[b]
P value	>0.05	>0.05	<0.01	<0.01	<0.01

HTS: *Heat treated sucuk,* ***RG1:*** *0.1% RE/GTE,* ***RG2:*** *0.2% RE/GTE,* ***RG3:*** *RE/GTE,* ***a-c:*** *Means with different letters in the same column are statistically different (P<0.05)*

Table 4. *The effect of using rosemary extract/green tea extract at the different levels on volatile compounds of HTS (mean ± SD) (Arbitrary Units x10⁶)*

No*	Compounds	RI	KI	Control	RG1	RG2	RG3
1	Ethanol	a	539	19.77±2.43	14.37±4.71	43.95±29.44	38.61±7.94
2	Ally mercaptan	b	610	124.18±2.57	136.80±20.09	130.80±10.79	123.70±3.75
3	Acetic acid	a	717	6.30±0.97	3.26±0.74	3.50±1.26	2.16±1.15
4	Ally methyl sullfide	b	730	11.29±7.75	13.84±5.66	7.96±3.82	10.31±2.29
5	1-(methylthio)-1-propene	b	753	1.54±2.17	2.66±3.76	2.50±3.53	2.23±2.54
6	3-Hydroxy-2-butanone	b	779	2.42±0.87	3.96±0.35	2.14±0.83	2.99±2.18
7	Toluene	a	785	1.82±2.56	2.52±3.50	2.06±0.11	1.96±0.20
8	Hexanal	a	835	3.67±0.11^{a}	3.11±0.06^{b}	3.02±0.18^{b}	2.92±0.23^{b}
9	3,3'-thiobis-1-propene	b	888	25.40±1.30	31.93±5.33	19.62±6.85	22.43±4.17
10	p-Xylene	b	892	1.43±0.61	2.81±0.34	2.04±0.48	2.07±1.17
11	Styrene	b	916	1.56±0.50	2.48±1.89	1.27±0.89	1.93±0.49
12	2-Heptanone	b	931	1.06±1.49	1.54±2.18	1.08±0.11	1.69±2.39
13	α-Thujene	b	934	2.08±1.28	2.23±3.15	2.38±0.88	5.60±1.05
14	α-Pinene	a	939	3.62±1.07	7.88±0.37	8.56±7.59	7.73±1.03
15	Methyl 2-propenyl disulfide	b	946	8.64±1.35	9.48±5.52	8.51±4.70	9.98±0.37
16	β-Pinene	b	987	8.61±0.55	11.12±1.38	10.6±1.03	9.63±1.07
17	Decane	a	1000	0.51±0.04	0.98±0.04	0.79±0.23	0.81±0.08
18	β-Myrcene	b	1005	15.58±3.28	28.38±1.09	23.39±9.91	32.39±14.56
19	2-Pentyl-furan	b	1021	0.68±0.30	0.71±0.23	0.86±0.12	0.76±0.24
20	α-Phellandrene	b	1022	4.91±0.99	12.63±4.94	14.69±0.38	24.63±8.01
21	3-Carene	b	1026	8.65±1.48^{c}	14.94±0.88^{b}	15.50±2.47^{ab}	17.56±1.54^{a}
22	2,3-Octanedione	b	1027	0.55±0.77	1.98±1.22	0.32±0.45	1.12±0.15
23	α-Terpinene	a	1030	2.57±0.63	5.17±0.07	5.16±3.34	6.06±0.52
24	D-Limonene	a	1043	17.92±0.89^{c}	34.27±7.30^{b}	39.58±5.24^{ab}	43.68±4.89^{a}
25	1-Methyl-2-(1-ethyl)-benzene	b	1046	80.29±21.68	81.86±23.99	76.79±20.89	89.13±1.89
26	Eucalyptol	b	1054	2.22±0.26	3.55±0.52	3.42±0.46	3.31±0.11
27	o-Cymene	b	1059	0.81±0.22^{b}	2.11±0.01^{a}	2.34±0.40^{a}	2.29±0.25^{a}
28	γ-Terpinene	b	1071	48.35±9.16^{c}	89.01±13.35^{b}	94.46±0.28^{ab}	113.94±18.07^{a}
29	Terpinolene	b	1095	0.87±0.13	0.92±0.08	0.95±0.06	1.38±0.46
30	4-Carene	b	1097	0.84±0.08	2.47±0.45	2.40±0.62	2.46±0.44
31	Dially disulphide	a	1116	57.73±0.57	72.91±2.74	62.40±28.04	61.56±4.91
32	2-Propenyl propyl disulfide	a	1126	3.12±0.14	4.68±0.57	3.65±3.49	3.65±0.17
33	Linalol	a	1142	39.02±3.06^{b}	58.46±0.76^{a}	55.49±2.50^{a}	57.62±1.92^{a}
34	Dodecane	a	1200	0.70±0.08	1.24±0.50	0.57±0.01	0.56±0.78
35	Camphor	b	1207	0.77±0.32	1.02±0.11	1.19±0.25	1.27±0.09
36	Hexyl butanoate	a	1214	0.51±0.09	0.63±0.16	0.60±0.23	0.68±0.11
37	4-Terpineol	b	1220	2.74±0.02^{b}	3.60±0.40^{a}	3.63±0.28^{a}	3.71±0.16^{a}
38	α-Terpineol	b	1252	1.94±2.74	5.31±0.35	4.29±0.27	5.30±0.32
39	Tridecane	a	1300	0.76±0.10	1.49±0.18	1.50±0.16	1.45±0.51
40	2-Methyl-3-phenyl- propanal	b	1318	119.46±4.90	120.26±32.20	110.32±8.11	136.21±21.38
41	α-Thujenal	b	1370	10.03±1.54	9.98±2.51	7.96±3.44	9.24±1.62
42	Tetradecane	a	1400	1.84±0.54	3.35±0.16	1.46±0.45	2.79±0.61
43	α-Cubebene	c	1408	1.34±0.25	0.85±1.20	1.27±0.68	1.63±0.03
44	Hexyl hexanoate	c	1416	1.49±0.18	0.00±0.00	1.08±0.25	0.55±0.78
45	Eugenol	b	1436	1.66±0.01	2.05±0.84	1.61±0.47	1.95±0.20
46	Isocaryophylene	c	1147	1.84±0.07	2.10±0.68	1.63±0.66	1.03±1.45
47	1,2-Dimethoxy-4-(2-propenyl)-benzene	c	1457	8.78±1.72	8.49±2.28	7.25±2.27	4.57±6.46
48	Caryophyllene	b	1490	6.16±0.91	7.94±1.35	7.96±3.01	6.05±4.51

HTS: *Heat treated sucuk,* ***KI:*** *Kovats index calculated for DB-624 capillary column installed on GC/MS,* ***RI:*** *reliability of identification,* ***a:*** *mass spectrum and retention time identical with an authentic sample;* ***b:*** *mass spectrum and Kovats index from literature in accordance;* ***a-c:*** *Means with different letters in the same row are statistically different (P<0.05)*

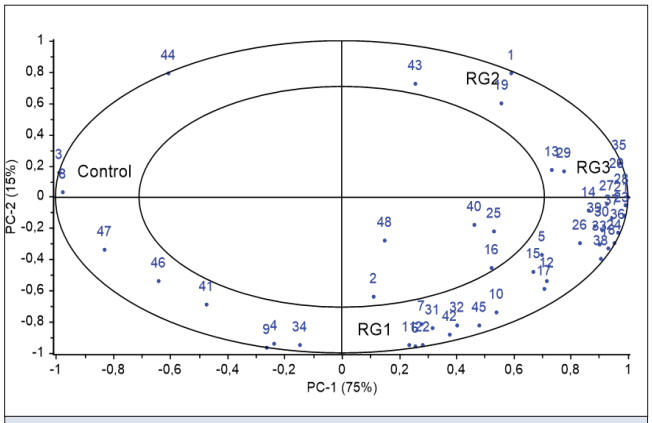

Fig 1. The principal component analysis of the relationships between factors RE/GTE treatments and volatile compounds (RG1: 0.1% RE/GTE, RG2: 0.2% RE/GTE, RG3: 0.3% RE/GTE, the numbers indicate volatile compounds in *Table 4*)

In the study, principal component analysis (PCA) was applied to assess the relationships between the treatments and volatile compounds (*Fig. 1*). The first principal component provided 75% of the total variance and separated all treatments containing RE/GTE well from the control group. In addition, most of the volatile compounds were located on the positive side of PC1 as in the RE/GTE groups. Control was found to be closely correlated with few volatile compounds while on the negative side of PC1 (*Fig. 1*).

NDMA, NDEA, NPYR and NPIP were determineted in HTS samples. NMEA, NDPA and NDBA were not dedected in any of HTS samples. The effect of using rosemary extract/green tea extract at the different levels on NDMA, NDEA, NPYR and NPIP levels of HTS is given in *Table 5*.

Table 5. *The effect of using rosemary extract/green tea extract at the different levels on NDMA, NDEA, NPYR and NPIP levels of HTS (mean ± SD) (µg/kg)*

Treatment (T)	**NDMA**	**NDEA**	**NPYR**	**NPIP**
Control	2.01±0.86[a]	1.51±0.76[a]	0.81±0.24[a]	1.05±0.41[a]
RG1	2.22±0.67[a]	1.39±0.82[a]	0.74±0.21[a]	1.00±0.26[a]
RG2	1.95±0.65[a]	1.27±0.68[a]	0.69±0.20[a]	0.98±0.24[a]
RG3	1.97±0.60[a]	1.24±0.75[a]	0.66±0.22[a]	0.92±0.25[a]
P value	> 0.05	> 0.05	> 0.05	>0.05
Cooking time (min) (CT)				
0	1.44±0.40[c]	0.78±0.26[c]	0.58±0.15[c]	0.79±0.19[b]
1	1.97±0.42[b]	1.27±0.35[b]	0.74±0.16[b]	0.94±0.16[b]
3	2.71±0.51[a]	2.01±0.81[a]	0.87±0.24[a]	1.23±0.32[a]
P-value	<0.01	<0.01	<0.01	<0.01
The interaction of T x CT	ns	ns	ns	ns

HTS: *Heat treated sucuk,* ***RG1:*** *0.1% RE/GTE,* ***RG2:*** *0.2% RE/GTE,* ***RG3:*** *RE/GTE,* ***a-c:*** *Means with different letters in the same column are statistically different (P<0.05)*

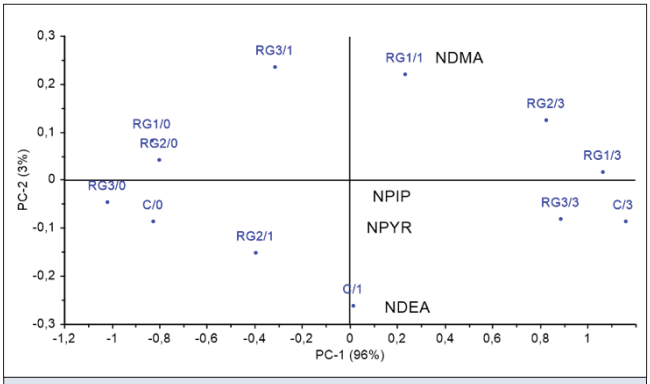

Fig 2. The principal component analysis of the relationships between RE/GTE treatments, cooking times and nitrosamines (The first number indicates the group, and the last number indicates the cooking time [min])

The principal component analysis of the relationships between RE/GTE treatments, cooking times and nitrosamines is given in *Fig. 2*. PC1 accounted for 96% of the total variance, while PC2 accounted for 3%.

Discussion

In HTS produced by subjecting to rapid ripening, LAB are important bacteria in terms of both product safety and the development of sensory properties. In industry, this product is produced using starter culture. LAB, which growth up to 10^8 cfu/g in fermentation, undergoes a certain reduction in heat treatment [4,27,31,32]. In this current study, a reduction in LAB count was also observed. However, in treatments containing RE/GTE (RG1, RG2 and RG3), the LAB count in the final product was found to be lower than in the control. In RG2 and RG3 treatments, *Micrococcus/Staphyloccus* count also decreased *(Table 1)*. It is thought that these results are probably due to the antimicrobial activity of RE/GTE. Indeed, it was reported that rosemary extract contains antimicrobial compounds such as carnosic acid, carnosol and rosmarinic acid, and green tea contains antimicrobial compounds such as epigallocatechin gallate and (-)-epicatechin gallate [33]. On the other hand, in this study, it is thought that the determination of Enterobacteriaceae number below the detectable limit is due to the drop in pH during fermentation and also the application of heat treatment [27,31].

Latilactobacillus sakei S15 strain used as a starter culture produces lactic acid during fermentation and thus the pH decreases. In this product, the pH decreases during fermentation and the subsequent heat treatment leads to an increase in the pH value, albeit slightly [31]. In the present study, the lowest pH value was observed in RG3 treatment. In a preliminary test for this study, the pH value of RE/GTE used in this study was determined as 4.91±0.01. RE/GTE lowers the initial pH value of the heat-treated sausage batter, and in this case leads to a lower pH in the final product, especially at the level of 0.3%. Jin et al.[34] reported also that rosemary extract lowers the pH value in an emulsified meat product. Similarly, Lara et al.[35] stated that rosemary extract decreased the pH value in pork patties and stated that this result was due to the fact that the active compound (carsonic acid) in this extract was an acid. On the other hand, it was reported that green tea extract did not have a significant effect on pH value in dry fermented sausages such as sucuk [20] and pepperoni [36]. Fermented sausages are environments where complex reactions take place, and their pH varies depending on various factors such as initial pH value, ingredients, buffering capacity of meat, rate and degree of acid formation of lactic acid bacteria [1,37]. On the other hand, the a_w value is a significant hurdle effect for fermented sausage. In the present study, a_w value was below 0.940, and this parameter was not affected by RE/GTE *(Table 1)*. Jin et al.[34] also stated that rosemary and thyme extracts were not effective on the a_w value of the sausages during cold storage.

HTS is a type of semi-dry fermented sausage cured with nitrite. Nitrite up to 150 mg/kg level can be used in the production of this product. Since HTS is generally consumed by cooking, the residual nitrite level draws attention as an important factor in terms of nitrosamines. In fermented sausages, the decrease in pH increases the reduction of nitrite to nitric oxide, thus reducing the amount of residual nitrite [8,27]. In this study, pH value was found below 5.0 in all groups. A similar result was observed by Sallan et al.[10] in HTS with starter culture. The low residual nitrite level in groups containing RE/GTE is thought to be due to the fact that these extracts contain antioxidant compounds [24]. In addition, the fact that the extract causes a decrease in pH also contributes to nitrite reduction [34]. In addition, in a study conducted on pepperoni, it was reported that green tea extract as a reducing agent accelerated the breakdown of nitrite [36]. Similarly, plant polyphenols (green tea or grape seed) and ascorbic acid have been found to significantly reduce residual nitrite in dry-cured sausage, and ascorbic acid is more effective [22]. On the other hand, the green tea polyphenols decreased the residual nitrite content in dry cured bacon faster than other antioxidants (grape seed extract and alpha tocopherol) [23]. However, it is emphasized that nitrite reduction depends on various factors such as the pH of the meat, ingoing nitrite level, processing and storage conditions, presence of reducing agents, and type of raw meat [23].

According to the TBARS results, the addition of 0.2% RE/GTE to the HTS batter caused sufficient to retard lipid oxidation. However, TBARS value was found below 1 mg MDA/kg even in the control group. Lin et al.[36] also found that green tea extract inhibited lipid oxidation in pepperoni. A similar result was also found in sucuk by Bozkurt [20]. Jongberg et al.[18] reported that green tea and rosemary extracts decreased the TBARS value in bologna type sausages. In another study on dry cured sausage, it was determined that green tea polyphenol was more effective in inhibiting lipid oxidation than ascorbic acid and grape seed polyphenol [22]. In addition, green tea polyphenol has been reported to have the most potent antioxidant activity in dry cured bacon production [23]. Antioxidant compounds from rosemary and green tea inhibite lipid oxidation by functioning either as free-radical scavengers or metal chelators [36,38]. Carnosic acid and carnosol components play an important role in the antioxidant activity of rosemary extract [39]. In green tea, on the other hand, catechins are active [40].

In present study, the use of RE/GTE had no significant

effect on L* and a* values *(Table 2)*. Similarly, in the studies carried out on pepperoni [36] and sucuk [20], it was reported that green tea extract did not affect the color parameters (L*, a* and b* values). Also, in a study conducted on cured pork sausage with white kimchi powder, it was reported that gree tea, rosemary and their combination did not affect the L* value compared to the control group [38]. RG2 and RG3 treatments showed higher b* values than control and RG1 *(Table 2)*. Similarly, it was stated that rosemary and green tea increased the b value in pork sausage and this result was due to the inhert pigments of plant-derived extracts [38]. Also, Jin et al.[34] reported that the use of rosemary extract in emulsified sausage increased the b* value.

In this study, the use of RG/GTE reduced the overall acceptability score. But, none of the values determined in the RG3 treatment were below 6.0. In fact, it was indicated that rosemary and green tea extracts are additives that have a positive effect on the taste and appearance of meat products, and that the effect of green tea extract on the flavor of the final product is limited [40].

HTS is a product made by applying a short-term fermentation, heat treatment and drying stages. Due to the short production time and heat treatment, reactions such as lipid oxidation and proteolysis occur to a limited extent. Therefore, terpenes have an important share in the aromatic profile of the product. The main source of terpenes is spices [4,31]. Hexanal is described as the major oxidation product, and sourced from oxidation of n-6 fatty acids such as linoleic and archidonic acids. Its high concentrations indicator flavor deterioration in meat products often resulting in a rancid aroma [30]. In this study, the use of RE/GTE resulted in a reduction in hexanal levels. However, this decline was not dependent on an increase in the RE/GTE ratio *(Table 4)*. This result is due to the antioxidant properties of rosemary and green tea extracts. This result is due to the antioxidant properties of rosemary (carnosic acid and carnosol) [41] and green tea (mainly flavonoids) extracts [39]. The levels of 3-carene, D-limonene and γ-terpinene increased due to the addition of RE/GTE. However, no difference was observed between RG2 and RG3 in terms of these compounds *(Table 4)*. It has been reported that the level of some monoterpenes, including 3-carene, increased with the increase in the amount of rosemary extract in fresh pork sausages [33]. On the other hand, the other three compounds (o-cymene, linalol and 4-terpineol) were affected by RE/GTE addition gave the highest mean value in RG3 treatment, but did not differ statistically from RG1 and RG2 *(Table 4)*. The most important source of terpenes is spices [30]. In this study, black pepper, red pepper, allspice and cumin were used as spices in the production of HTS. PCA analysis showed that the use of RE/GTE resulted in an increase in the content of terpenes in HTS.

Nitrosodimethylamine (NDMA) is defined by the International Agency for Research on Cancer as probably human carcinogen (Group 2A) [42]. NDMA is commonly determined in fermented sausages. It has been reported that NDMA in HTS varies between 1.71 and 3.71 μg/kg [9]. NDMA levels in fermented sausages in Danish and Belgian markets were found to range from undetectable to 4 μg/kg and undetectable to 7.2 μg/kg, respectively [43]. In the present study, the mean NDMA content was determined as 1.44±0.40 μg/kg in samples without cooking. The use of RE/GTE did not show a significant effect on NDMA level *(Table 5)*. Similarly, in a study conducted to determine the effects of plant polyphenols (green tea and grape seed polyphenols) and ascorbic acid on nitrosamine level in a dry fermented sausage, no significant difference was observed between the groups in terms of NDMA at the end of ripening. In the same study, the NDMA content varied between 0.56 and 0.76 μg/kg dry matter [22]. For dry-cured bacon, after three weeks of storage, it was reported that the control group and samples containing alpha-tocopherol contained more NDMA than samples containing green tea polyphenol and green tea + grape seed extract. It was also emphasized that NDMA and sodium nitrite showed a negative correlation [23]. Nevertheless, it was determined that the use of rosemary extract, grape seed extract and green tea polyphenol reduced the NDMA content in Western style smoked sausage [24]. In the present study, unlike the RE/GTE factor, the cooking time had a significant effect on NDMA *(Table 5)*. NDMA content increased as the cooking time increased. In a previous study on HTS, it was determined that the NDMA content increased as the cooking time increased [10].

NDEA is determined less frequently in fermented products than other nitrosamines such as NDMA, NPYR and NPIP. Kaban et al.[9] and Sallan et al.[10] stated that the NDEA in the heat-treated sausage samples was below the detectable limit. However, Akansel et al.[27] found that average NDEA level in HTS samples without additional heat treatment was 0.61 μg/kg. In the present study, the mean NDEA content was found to be 0.78±0.26 μg/kg for samples without cooking *(Table 5)*. As can be seen from *Table 5*, the NDEA level decreased as the RE/GTE ratio increased. However, the differences between treatments were not statistically significant. The interaction of RE/GTE and cooking time also had no significant effect on NDEA *(Table 5)*. In contrast, The NDEA content increased with the progression of cooking time. Similar results were also reported by Akansel et al.[27]. On the other hand, Li et al.[22] reported that NDEA was detected at early ripening of dry cured sausage. In the same study, it was stated that there was no significant difference in terms of NDEA between the control group and green tea polyphenol treatment on the 7th day of ripening [22].

NPYR, among the possibly (Group 2B) carcinogenic compounds [42], is an important nitrosamine for cured meat products. The level of this nitrosamine in HTS ranges from 1.65 to 7.29 µg/kg [9]. The maximum NPYR level found in salami from Belgium was 6.9 µg/kg [43]. In the present study, it was observed that the NPYR level decreased depending on the increase in the RE/GTE ratio. However, as can be seen from *Table 5*, the differences between the treatments were not found to be statistically significant. On the other hand, Li et al.[22] stated that the control group had a higher NPYR value than the groups containing green tea or grape seed polyphenols at the end of the 28 day ripening in a dry sausage. While many spices are good sources of NPYR, pyrrolidine is the major source of this nitrosamine, and putrescine is also stated to be effective in the formation of NPYR [8,15]. On the other hand, it was reported increase of ingoing nitrite level in HTS unaffected the NPYR level [10]. In the present study, cooking time had a significant effect on NPYR. In contrast, there was no relationship between RE/GTE and cooking time *(Table 5)*. Cooking is an important factor for this nitrosamine [10].

Another important nitrosamine found in fermented sausages is NPIP. Piperine and piperidine in black pepper play an important role in the formation of this compound [27]. Black pepper is also an important spice for HTS. In the Turkish market, the NPIP level in this product can reach high levels depending on the level of black pepper [9]. In this study, NPIP was not affected by the use of RE/GTE. However, cooking time had a very significant effect on this nitrosamine *(Table 5)*. Sallan et al.[10] and Yılmaz Oral [44] and Yılmaz Oral [45] reported that cooking degree was an important factor and an increase in cooking time resulted in increased NPIP. While fermented sausages are consumed raw in many countries, sucuk and HTS are generally consumed after cooking processes such as frying, barbecue and roasting in Türkiye. Therefore, cooking time or cooking degree is of great importance for reducing the risk of nitrosamines [45,46].

According to the result of PCA, PC1 provided good discrimination between treatments with different cooking times. While, all treatments of the 3 min cooked samples were placed on the positive side of PC1, the uncooked samples (0 min) were on the negative side of PC1. However, the application of 1 min cooking time differed between the treatments. Control and RG1 treatments showed a higher correlation with nitrosamines, placing on the positive side of PC1. In contrast, samples of RG2 and RG3 cooked for 1 min were on the negative side of PC1 and showed a weaker correlation with nitrosamines. It was determined in many studies that the cooking process/time is an important factor in the formation of nitrosamines.

In conclusion, the use of RE/GTE at different levels in the production of HTS, a type of semi-dry fermented sausage, caused decreases in both the pH value and the technologically important LAB and M/S numbers in the final product. However, these decreases did not occur in a way that would negatively affect product properties. RE/GTE did not cause any change in a_w value. Since the RE/GTE utilization rate was 0.2% and 0.3%, the TBARS value decreased, indicating that this extract combination slowed down lipid oxidation in HTS. While RE/GTE had no effect on the L* and a* values, it increased the b* value by 0.2% and 0.3%. In addition, due to the reducing property of the extract, it reduced the residual nitrite level, which is an important result in terms of nitrosamine formation. Sensory parameters of color and texture were not affected. A RE/GTE level of 0.3% resulted in significant reductions in both odor, taste and overall acceptability parameters. The use of RE/GTE had a limited effect on volatile compounds. It decreased hexanal level, and increased six terpenes. On the other hand, NDEA, NPYR and NPIP levels decreased as the RE/GTE ratio increased, but these decreases were not statistically significant. NDMA was also not affected by the use of this extract mixture. As the cooking time progressed, the levels of the determined nitrosamines increased. However, the use of the extract did not cause a different effect during cooking. Considering all these results, it was concluded that the RE/GTE mixture can be used up to 0.2% level and that the heat-treated sucuk should not be subjected to any additional cooking before consumption, even though the total nitrosamine level is below 10 ug/kg even in products cooked for 3 min.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author.

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Ethical Statement

Ethics approval was not required for this research due to conducting *in vitro* in the laboratory.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contribution

ZFYO: Conceptualization, methodology, validation, formal analysis, investigation, writing original draft. GK: Conceptualization, methodology, writing-review and editing. Final approval of the completed article: ZFYO and GK.

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Research Article

Reproduction Characteristics of Ouled Djellal Rams in a Semi-arid Area in Algeria

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ABSTRACT

The assessment of reproduction characteristics of an Algerian local sheep in a semi-arid zone was the objective of this study. Twenty rams of Ouled Djellal breed were used. The parameters analyzed were testosterone rate, testicular diameter, ejaculate volume, sperm concentration, mass motility, and percentage of live and dead spermatozoids (SPZ). The results obtained showed that the mean monthly testosterone rate was high throughout the year (4 ng/mL), increased in spring, peaked in summer (July) (4.9 ng/mL), and decreased in autumn and winter to reach a minimum value (January) of 1.9 ng/mL. Similarly, testicular diameter, ejaculate volume, sperm concentration and count showed high levels in spring and summer and decreased in autumn and winter. The maximum and minimum values were respectively, 7.09 cm, 1.55 mL, 4 billion/mL, 3.5, 79.37%, 21% and 5.46 cm, 0.80 mL, 2.50 billion SPZ/mL, 2.81, 77.36%, and 20.42%. Significant effects of season on serum testosterone levels, testicular conformation, ejaculate volume, and concentration were recorded in this study. Indeed, the rates of these parameters increased significantly ($P<0.05$) during spring/summer and decreased during autumn/winter except for motility, and the percentage of live and dead sperm. Testicular conformation was positively correlated with testosterone concentration, ejaculate volume, and semen concentration. To conclude, Ouled Djellal breed seemed not to be very sensitive to the photoperiod and little affected by high temperature.

Keywords: Sperm, Ram, Photoperiod, Local breed, Temperature, Season

Introduction

In Algeria, sheep breeding occupies by its numerical and socio-economic importance, a crucial place in breeding systems. It is concentrated in the steppe and constitutes an important animal resource for the country [1,2]. Mutton is the most popular consumed red meat especially in religious and traditional festivals. Eight main races have aired to date. Some of these breeds demonstrate strong adaptability to harsh environmental conditions. Among them, Ouled Djellal, also known as the white Arab race or Arbia, raised in arid and semi-arid regions and subject to a clear preference by farmers. Indeed, many studies reported the Ouled Djellal's good reproductive qualities and resistance to difficult conditions [3]. These particularly zootechnical performances contribute to the numerical productivity of the herds and therefore to obtaining good results in meat.

Improving the animal reproductive performances requires knowledge of the reproductive parameters of females and males. Ram sexual behavior can be influenced by many factors, including season, genetics, breed, hormonal condition, temperature, and nutrition. However, photoperiod is the main environmental factor affecting sheep reproduction [4]. Thus, the reproductive activity of most small ruminants, activated mainly by annual photoperiodism, exhibiting seasonal variations that extend proportionally with latitude. These variations are manifested in females by the existence of a period of seasonal anoestrus of variable duration depending on the breed and a favorable period for reproduction (sexual activity) [5]. While in the male, a period of low sexual activity could be manifested and is characterized by a decrease in the intensity of sexual behavior (libido), testicular size, hormonal secretion, and semen production both in quantity and quality [6]. The reproductive activity

Table 1. *Monthly averages of temperature, humidity, and photoperiod (daylight) during the study period*

Parameter	Month											
	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Temperature (°C)	11	11.4	14.7	14.5	23	27.9	30.3	30.5	25.9	21.2	13.4	10.6
Duration of day (h)	9.75	10.83	11.93	12.5	13.93	14.5	14.33	13.6	12.5	11.7	10.2	9.83
Humidity (%)	79.3	67.1	66.7	71.5	51.5	49	40	41	52	62	72	78

of most animals native to temperate zones shows seasonal variations. These are more or less marked depending on the species and the amplitude of variation of the photoperiod. Small ruminants show periods of non-activity in the long photoperiod and periods of activity in the short photoperiod [7]. In sheep/goats, so-called "short-day" species, the photoperiod is certainly the main cause of the seasonal variations in reproduction observed for all breeds in Northern Europe. However, in tropical and subtropical regions, variables related to temperature and nutrition also appear to affect reproductive physiology in animals [5].

In rams, during reproductive life, body weight, scrotal circumference, testosterone levels, and sperm production change under the influence of several internal and external factors. Therefore, ram reproduction data showed a complex relationship between the development of the neuroendocrine system and environmental conditions [8]. Seasonal variation in mammalian reproduction results from adaptation to annual environmental changes [9]; thus, they are an important factor influencing sperm quantity and quality. This is why the knowledge of these reproductive factors makes it possible to genetically improve the breed and increase the herd productivity [10]. The present study aimed to evaluate the influence of the season on testosterone concentration, testicular diameter, and sperm production in Ouled Djellal rams in Algerian semi-arid zone. Knowledge of this information is important insofar as it should determine the favorable or inferred periods for the spermogram, the choice and use of parents during the year and thus improve the success of insemination. This work also leads to the promotion of artificial insemination using seeds from local breeds.

Materials and Methods

Ethical Statement

All the animal studies were conducted with the utmost regard for animal welfare, and all animal rights issues were appropriately observed. No animal suffered during the course of the work. All the experiments were carried out according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA.SDA.14).

Study Area

The study was conducted at a private sheep farm located 20 km from the city of Chlef (Algeria). It is an agricultural farm, combining sheep breeding and crops (cereals and fodder). Sheep population is large and unique, around 1100 heads (350 ewes, 750 heads between rams and lambs). The entire herd is essentially of Ouled Djellal breed. The farm has 4 breeding buildings housing the different categories of animals. The area is located between 36°10′ North latitude and 1°20′ East longitude and at an altitude of 86 m. Chlef region, located in the North of Algeria (200 kilometers from the capital Algiers), is known for its semi-arid Mediterranean climate with mild little rainy winters and hot, dry summers. *Table 1* represents the average monthly variations in temperature, humidity, and photoperiod of the region during the study year (2021).

Experimental Herd

In this current work, 20 pubescent Ouled Djellal rams born on the farm and raised in the region were used. The animals selected all responded to the standard type of the Ouled Djellal breed (physical characteristics and measurements). It is an entirely white breed, with fine wool and tail, high waist and long legs, suitable for walking (*Fig. 1*). The ears are long and horizontal. The horns, present in both sexes in general, may be missing in ewes. The ram weighs up to 80 kg and the ewe 60 kg [11]. This sheep is large : height at the withers, body length, and chest circumference are respectively, 80 cm, 84 cm, and 40 cm for the male and 74 cm, 67 cm, and 35 cm for the female. The rams in our study were 3 to 4 years old, with a weight varying between 55 and 60 kg (56.8±2.1 kg). The animals

Fig 1. Ouled Djellal breed on the farm

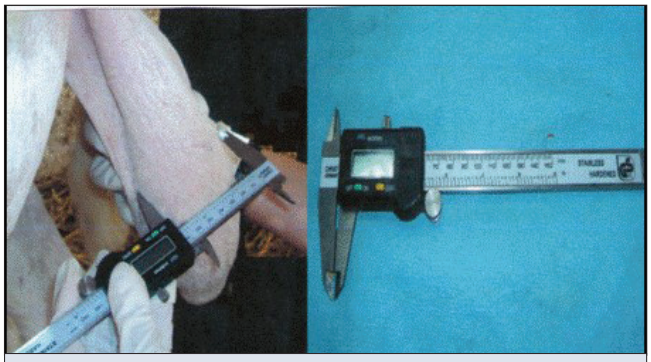

Fig 2. Testiclar anteroposterior diameter measurement

were kept in a free stall, subject to seasonal variations and environmental factors in the study region. They received a constant diet, consisting of *ad libitum* oat vetch hay and approximately 600 g of barley concentrate per head per day, and benefited from ad libitum watering. The rams were isolated from the ewes in open barns and kept out of breeding for one year. All animals were previously treated against diseases and classic parasites of sheep and were free of any pathology including brucellosis.

Blood Samples and Testosterone Assay

Blood samples were taken weekly from the jugular vein of each ram for one year (from January to December). The blood was immediately centrifuged and the serum obtained was immediately frozen at -20°C until testosterone assay. Serum testosterone concentration was obtained using the ELISA technique, as previously described [12].

Testicular Diameter Measurement

Every week and for a year, the testicular anteroposterior diameter was measured using a caliper [6,13]. These measurements were taken for each ram and always by the same operator *(Fig. 2)*.

Collection and Analysis of Semen from Rams

Semen collection was performed using an artificial vagina (made and suitable for sheep) in the presence of female in heat and attached to a special support. The rams selected were dressed for artificial vagina collection for a period of 2 to 3 months. The collections lasted a year, at a rate of one collection per week and per ram, carried out early in the morning. The characteristics of the sperm recorded were: Volume (recorded directly in the graduated glass collection tube); Concentration (spermatozoa/mL of ejaculate), measured by a spectrophotometer after 1/400 dilution in an isotonic sodium chloride solution [14]. Sperm mass motility was obtained by examining a drop of semen under a microscope equipped with a hot plate at 37°C. To evaluate the intensity of the waves formed by the movements of the spermatozoa, a score was thus assigned ranging from 0 to 5 [15]. The vitality of the spermatozoa was provided by counting after staining with Eosin-Nigrosin, to determine the percentage of living and dead spermatozoa (Eosin negative). The reading was done under a microscope. Live sperm stain white and dead stain pink or red [16].

Statistical Analysis

Results were collected monthly and presented as mean and standard deviation. ANOVA was used to determine the seasonal variations of the parameters, the correlations, and the PCA (Principal Component Analysis) to explain the variance of these factors. Pearson's significance test (P) was performed to check for variations. The differences detected were considered as highly significant when $P<0.0001$ and significant when $P<0.05$. XLSTAT software version 2002 was used for all these analyses.

Results

Seasonal Variations in Serum Testosterone

The mean baseline testosterone level of Ouled Djellal rams in this study was 3.34±0.55 ng/mL. However, over the twelve months of analysis, the concentrations were characterized by very marked monthly variations *(Table 2)*.

Indeed, a rise in concentration from February (2.98±0.26 ng/mL), to reach a peak (maximum value) in July (4.93±0.39 ng/mL) was noticed. A highly significant drop of approximately 45% was revealed in October (2.65±0.38 ng/mL), which stabilized in November-December and decreased again to reach its minimum value in January (1.92±0.29 ng/mL).

Variations in Antero-posterior Testicular Diameter

The mean value of the testicular diameter in this work was 6.23±018 cm. The previous table reported the rates

Table 2. *Monthly means of serum testosterone and testicular diameter of the 20 Ouled Djellal rams*

Parameter	Month											
	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Testosterone (ng/mL)	1.92±0.29	2.98±0.26	3.57±0.51	3.7±0.62	3.80±0.56	4.38±0.72	4.93±0.39	3.75±0.81	3.45±0.73	2.65±0.38	2.55±0.45	2.50±0.82
Testicular diameter (cm)	5.46±0.19	6.32±0.15	6.49±0.21	6.58±0.13	6.62±0.17	6.98±0.14	7.09±0.12	6.80±0.17	5.78±0.21	5.60±0.22	5.56±0.19	5.49±0.20

Table 3. *Results of ANOVA analysis*

Season	Test. (ng/mL)	TD (cm)	VOL (mL)	SC (10 ^{9}/mL)	MM	LS (%)	DS (%)
Spring	4.025b	6.7825b	1.4175a	3.9775a	3.1375a	80.95a	20.4275ab
Summer	4.7375a	7 187a	1.505a	3.8275a	3.0875a	75.45b	21.155a
Autumn	2.67c	5.39c	0.980b	2.9375b	2.6725b	77.02b	20.3975b
Winter	2.3225c	5.46c	0.9075b	2.825b	2.69b	75.46b	20.04b
R^{2-}	0.9315	0.9377	0.9285	0.9586	0.6219	0.7807	0.5110
F	54.4014	60.1677	51.9621	92.6606	6.5801	14.2370	4.1808
Pr > F	<0.0001	<0.0001	<0.0001	<0.0001	0.0070	0.0003	0.0305
Significance	HS	HS	HS	HS	VS	HS	S

Test.: Serum testosterone, TD: testicular diameter, VOL.: ejaculate volume, SC: Sperm concentration, MM: Mass Motility, LS: live Sperm, DS: Dead Sperm, HS: highly significant, VS: very significant, S: significant, R^2: coefficient of determination, F: ratio of two standard deviations, Pr: probability

of the seasonal variations in this diameter measured in Ouled Djellal rams. The variations were similar to those of blood testosterone. The seasonal influence on testicular volume and testosterone was statistically highly significant (P<0.0001) *(Table 3)*. The lowest averages were recorded in autumn/winter (September to January) and the highest in spring/summer, peaking in July.

The analysis of the monthly variations of testosterone and testicular diameter with those of the climatic factors showed a close parallelism between the profiles of seasonal evolutions of these parameters and the length of the day. It was minimum in autumn/winter then increased at the same time as the photoperiod to be maximum in spring/summer.

Seasonal Variations in Sperm Characteristics

All the rams could be sampled for semen. Thus, 45 to 48 samples were taken from each ram in this study. Semen from Ouled Djellal rams presented a milky white

Table 4. *Individual mean values of the sperm parameters*

Ram	Age (year)	LW (kg)	V (mL)	SC (x10^9/mL)	MM	LS (%)	DS (%)
1	3	56.0±0.8	1.10±0.60	2.99±0.15	2.77±0.17	79.20±10.20	27.00±5.50
2	3	56.7±0.6	1.40±0.35	3.28±0.32	4.10±0.23	80.00±15.00	17.20±4.90
3	3	60.2±0.9	1.38±0.32	3.22±0.39	3.79±0.15	89.00±9.10	9.10±5.21
4	4	55.0±1.0	1.45±0.21	2.60±0.18	3.85±0.10	76.21±12.23	28.45±3.95
5	4	57.7±0.7	1.25±0.75	4.00±0.30	2.89±0.09	59.50±10.00	45.21±3.00
6	3	58.1±1.0	1.27±0.25	2.70±0.50	3.00±0.16	56.40±19.50	30.23±6.50
7	3	59.2±0.5	1.32±0.66	3.00±0.40	3.10±0.10	90.21±15.65	8.80±7.10
8	4	60.5±2.1	1.30±0.55	3.25±0.28	3.10±0.11	88.22±12.00	13.00±6.32
9	4	55.1±1.7	1.39±0.35	3.00±0.45	2.98±0.20	70.00±17.21	23.21±6.98
10	3	55.0±0.6	1.55±0.10	3.05±0.35	3.21±0.15	73.45±18.54	22.25±4.23
11	4	56.2±0.3	1.10±0.12	2.99±0.32	3.00±0.25	90.00±8.88	8.12±4.21
12	3	58.0±0.6	1.20±0.15	3.87±0.20	3.00±0.16	92.00±7.00	8.00±3.98
13	4	59.0±0.7	1.28±0.55	4.20±0.25	4.20±0.13	68.22±22.00	25.00±2.99
14	4	55.5±0.2	1.55±0.45	4.00±0.45	3.90±0.14	49.50±12.00	40.00±3.99
15	3	55.4±0.6	1.55±0.21	3.20±0.30	3.54±0.21	87.96±12.00	26.98±6.32
16	3	57.1±0.4	1.36±0.32	3.78±0.20	2.79±0.13	89.00±10.87	15.00±5.52
17	4	57.0±0.8	1.30±0.24	3.90±0.40	4.20±0.14	91.21±9.00	8.88±5.21
18	3	59.2±1.0	1.40±0.36	2.98±0.27	2.97±0.25	79.65±8.96	20.12±4.51
19	4	60.0±0.9	1.08±0.60	3.60±0.36	2.79±0.10	85.56±12.12	12.00±5.00
20	3	60.0±0.3	1.10±0.50	3.25±0.54	3.21±0.21	80.32±10.56	16.54±3.32
Avg.	3.8±0.7	57.5±1.7	1.40±0.43	3.73±0.30	3.31±0.15	78.78±12.04	20.25±4.93

LW: Live weight, V: ejaculate volume, SC: sperm concentration, MM: mass motility, LS: live spermatozoa, DS: dead spermatozoa

Table 5. *Monthly means of the sperm values in rams*

Parameter	Month											
	Jan.	**Feb.**	**Mar.**	**Apr.**	**May**	**Jun.**	**Jul.**	**Aug.**	**Sep.**	**Oct.**	**Nov.**	**Dec.**
Volume of ejaculate (mL)	0.80±0.31	1.15±0.20	1.30±0.44	1.43±0.50	1.51±0.27	1.55±0.43	1.53±0.29	1.45±0.35	1.12±0.30	1.05±0.26	1.00±0.15	0.90±0.60
Sperm concentration ($X10^9$/mL)	2.50±0.52	3.19±0.73	3.66±0.52	3.69±0.58	3.75±0.42	3.90±0.30	4.00 ± 0.33	3.12±0.27	3.02±0.29	2.98±0.26	2.93±0.15	2.84±0.29
Mass motility	2.73±0.10	3.10±0.21	3.37±0.32	3.60±0.27	3.74±0.11	3.75±0.15	3.59±0.25	3.00±0.18	2.75±0.19	2.76±0.11	2.78±0.13	2.75±0.22
Live sperm (%)	74.00±10.05	76.20±9.25	80.90±10.02	82.23±8.77	85.00±12.21	82.00±10.00	75.10±8.95	71.00±9.90	79.50±11.10	80.10±14.05	79.40±13.20	75.00±12.00
Dead sperm (%)	20.30±5.30	20.23±4.12	19.95±6.01	20.15±7.10	20.00±6.00	21.01±4.50	22.00±3.70	22.90±3.30	21.15±6.50	20.62±3.98	20.10±4.00	20.15±6.14

Table 6. *Pearson correlation matrix between variables*

Variables	**Test.**	**TD**	**V**	**SC**	**MM**	**LS**	**DS**
Test.	**1**	0.9850	0.9897	0.9338	0.9304	0.2271	0.8790
TD	0.9850	**1**	0.9900	0.9541	0.9655	0.2653	0.8005
V	0.9897	0.9900	**1**	0.9753	0.9720	0.3529	0.8039
SC	0.9338	0.9541	0.9753	**1**	0.9942	0.5408	0.6595
MM	0.9304	0.9655	0.9720	0.9942	**1**	0.4983	0.6433
LS	0.2271	0.2653	0.3529	0.5408	0.4983	**1**	-0.1648
DS	0.8790	0.8005	0.8039	0.6595	0.6433	-0.1648	**1**

Test.: serum testosterone, DT: testicular diameter, V: ejaculate volume, SC: sperm concentration, LS: live spermatozoa, DS: dead spermatozoa

appearance in 70% of the cases, against 30% having a creamy appearance.

The mean ejaculate volume of rams was 1.40±0.43 mL (*Table 4*). It is varied between 1.10 mL (minimum value) to 1.55 mL (maximum value). This parameter also underwent highly significant seasonal variations (P<0.0001) *(Table 3)*. The volume of ejaculate was greater in spring and summer than in autumn and winter, respectively (1.4±0.4 mL, 1.5±0.4 mL versus 1.1±0.2 mL, 1.0±0.4 mL) *(Table 5)*.

The average concentration in this test was found to be 3.73±0.30 billion sperm per milliliter of semen, with respective minimum and maximum values of 2.60 and 4.1 billion sperm per milliliter of ejaculate. According to the data by month, the concentration increased from March to reach the seasonal peak in June (4.0±0.3 billion) and decreased from July reaching a minimum value in January (2.5±0.5 billion). Seasonal variations of this parameter were also highly significant (P<0.0001). It was higher when the photoperiod was increasing (spring/summer) than when it was decreasing (autumn/winter). Semen from Ouled Djellal rams was more concentrated in spring and summer than in autumn and winter.

The mean values of mass motility, percentage of live and dead spermatozoa obtained in this study were 3.31±0.15, 78.78±12.04 and 20.25±4.93, respectively *(Table 4)*.

Table 3 showed that mass motility was very significantly affected by the season (P<0.001). In fact, the highest values were recorded in spring/summer (3.58±0.23, 3.44±0.19) and the lowest in autumn/winter (2.76±0.14, 2.89±0.18).

Also, the analysis of the average monthly results in *Table 5* demonstrated a highly significant influence of the season (P<0.0001) on the percentage of live spermatozoa. The maximum value (85.71±12.21%) was observed during spring, mating period in the region and the minimum value in winter (74±10.05%). The seasonal mean results of the percentage of dead spermatozoa showed a slight variation from month to month *(Table 5)*. Indeed, the minimum extreme values in March (19.95±6.01%) and maximum in August (22.90±3.30) were revealed over the study period and did not show a very significant difference (P<0.05).

Finally, the results obtained showed that the rates of all the improved parameters evolved with the photoperiod and the temperature. Their monthly means increased from February, reaching a maximum in June (4.93±0.34 ng/mL, 7±0.12 cm, 1.55±0.43 mL, 4±0.30x10 9 Spzs/mL, 3.75±0.15, 85±10%), then decreased from September to reach their minimums in January (1.92±0.29 ng/mL, 5.46±0.19 cm, 0.8±0.31 mL, 2.50±0.52x10^9 Spzs/mL, 2.73±0.10, 73±10.5%). So, it appears that the Ouled Djellal ram produces large quantities of sperm all year round. It

is more important, more concentrated, more mobile and vital from February/March (increasing photoperiod).

Correlations

According to the PCA examination, more than 90% of the variations of the parameters were explained by the months of the year. Indeed, according to *Table 6*, the spring and summer seasons were positively correlated (r=0.80) with concentration, motility, ejaculate volume, percentage of live spermatozoa and testicular diameter. Also, the analysis of the correlation matrix of *table 3*, showed a highly significant difference (P<0.0001) between the season and the testicular diameter, the volume, the concentration, and the % of live spermatozoa and a very significant difference (P<0.001) between the season and the mass motility, while the difference between the season and the % of spermatozoa was a bit significant (P<0.05).

The relationship between the testicular measurements and the concentration of testosterone made by the correlation coefficients are presented in *Table 6*. The testicular diameter had a very high and positive correlation with the testosterone rates (r=0.98). The latter had high and positive correlations ranging from 0.93 for mass motility and sperm concentration and 0.98 for ejaculate volume. Similarly, testicular diameter showed very high and positive correlations between all sperm characteristics, with volume (r=0.99), sperm concentration (0.95), and mass motility (r=0.96).

Discussion

In this study, serum testosterone underwent significant seasonal variation. It was higher from March to July than from October to January. This pattern of variation is similar to those described by Darbeida et al.[17] and Belkadi et al.[14] in the same breed in Algeria and by Issa et al.[18] in the Fulani and Tuareg breed in the Sahelian climate.

According to our data, the testicular diameter measured in the Ouled Djellal ram was always high during the year. According to several authors, it depends on age, body weight, diet, and sexual season [12]. This result could be explained by the fact that the rams of our sample were all adults, of good body conformation and raised in good food and sanitary conditions. This parameter reflects sperm production and is a good indicator of male fertility; its heritability improves reproductive parameters [19]. Indeed, the scrotal circumference is an indicator of the amount of testicular spermatogenetic tissue, which reveals the maximum potential for sperm production. It is possible that larger testes contain more androgen-producing tissue (Leydig cells), where high levels of stimulation promote the growth and development of spermatogenic tissue [20]. These authors' conclusions were confirmed in this study by the strong positive correlations obtained between testicular diameter and sperm characteristics. Also, Belkhiri et al.[21], reported that measurement of testicular diameter in rams could be used by Breeding Centers to select suitable males for artificial breeding. Thus, any factor that influences testicular volume affects the reproductive efficiency of rams. The testicular conformation was highly affected by the season. Sexual activity peaked in spring/summer despite the increase in day length. Unlike sheep breeds in temperate zones where sexual activity increases in autumn when the photoperiod decreases. These results are in agreement with those of Kafi et al.[6] and Tabbaa et al.[22] in the Awassi breed, of Milczewski et al.[23] in the Suffolk breed, and of El Bouyahiaoui et al.[24] in the Tazegzawt ram.

Choosing an effective semen collection method is the first step in establishing a sperm cryopreservation bank. The artificial vagina collection method was used in this study because it generally results in better quality semen. The milky and creamy appearance of the semen samples collected from the Ouled Djellal rams in our work showed that the semen from this breed was mostly normal, similar to that of the majority of sheep breeds [18].

The Ouled Djellal rams produced a sperm volume included in the range of averages of 1 to 1.5 mL given for sheep [25]. In another study performed on South African indigenous rams, Ngcobo et al.[12] reported no significant difference in semen volume during the spring (0.88 mL), summer (0.91 mL), autumn (0.92 mL), and winter (0.92 mL) seasons. This may be due to the fact that Ouled Djellal rams are of significant size and testicular conformation, because the average volume of ejaculate increased according to body mass and testicular conformation. Indeed, in this study, this last parameter was found to be very strongly correlated with the volume of ejaculate [12,21].

The mean value of the spermatozoa concentration obtained is within the range of rates reported in sheep, which is between $2x10^9$-$6x10^9$ spermatozoa/mL [26,27]. This mean value is significantly higher than those of D'man [28], Noire de Thibar rams from Tunisia [29], and Djallonké from Ivory Coast [27], which were respectively $1.8x10^9$ Spz/mL, 2.9 $0x10^9$ Spz/mL, $3x10^9$ Spz/mL, and $2.55x10^9$ Spz/mL. This value is close to the concentration of semen in white Fulani and Tuareg rams, which were respectively $3.7\pm1.3x10^9$ Spz/mL, and $3.6\pm0.4x10^9$ Spz/mL [30]. This sperm production is mainly due to the high testicular weight of the animals in general, a consequence of the large format of the Ouled Djellal ram. This can also be explained by the very strong positive correlation obtained in this current work between testicular diameter and sperm production. Indeed, it is well established that the production of spermatozoa per gram of testis is a characteristic of the species and of the breed.

The average value of the mass motility recorded in this study is considered good in comparison with the value 4, from which this parameter is evaluated as acceptable [15] and the sperm in this case can be used for artificial insemination. The rate obtained in this study is close to that of European rams such as Romanov and Ile-de-France (3.5 to 4.1) [31]. However, it is higher than those reported for white Fulani and Tuareg rams from the Sahel, which were respectively 2.89±1.2 and 2.90±0.1 [30]. The determination of this parameter constitutes, in the current state of knowledge, a criterion for sorting ejaculations to be used for insemination and breeding animals [15]. Fluctuations in mass motility were not really affected by photoperiod and temperature as already shown by Colas [31] and Aller et al.[16]. This is in agreement with the finding of Ghozlane et al.[32] and Kafi et al.[6], who demonstrated in a study of Ouled Djellal rams in Algeria and Persian Karakul rams in Iran, that photoperiods and high temperatures did not influence mass motility and concluded that sperm quality was higher during and towards the end of summer.

The average monthly results of the percentage of live spermatozoa showed a significant difference between those observed during the spring/summer and autumn/winter seasons. Although the maximum average was recorded in spring/summer, the season corresponding to the breeding period in this region, the viability of the ram's spermatozoa was maintained during the year, since the average values were always above 70%. Benia et al.[33] did not note a decrease in sperm viability in Rembi breed rams (raised under arid zone conditions in Algeria) during long and short photoperiod periods. The viability of the spermatozoa of these rams, like that of the Ouled Djellal rams in our sample, was maintained throughout the year with peaks in spring/summer.

The overall average of dead spermatozoa found in the present study is within the standards accepted for good quality sperm (between 20 and 25%) [31]. This value was consistent with the findings of Aller et al.[16] who claimed that good quality sperm should not contain more than (25%) of dead sperm. If the rate exceeds 25% of the total number in an ejaculate, a reduction in fertility can be anticipated.

The values of all the sperm characteristics obtained in this work can confirm that the semen of the Ouled Djellal rams collected under the environmental conditions of Chlef region is of good quality. Indeed, the results obtained with a collection rate of once a week showed that the sperm production potential of this breed is the best compared to that of other breeds produced at the same rate. This strong production potential allows the intensification of the productivity of the sheep herds insofar as the rams will be used at a faster rate. In view of these seasonal variations in the year of study, this period (spring/summer) may seem favorable to the spermogram of the Ouled Djellal ram and it corresponds to the period of traditional struggle of sheep herds in the region. The profile of variation of all the parameters is explained as well as the strong positive correlations obtained between them, confirms the imbrication in the ram of the endocrine (hormone production) and exocrine activity of the testicle (spermatogenesis).

In this study, it was not possible to observe the effect of the age and the weight of the rams on these parameters because the animals were chosen as all adults and had on average the same age (3 to 4 years) and the same weight (55 to 60 kg).

Also, according to our data, the period from February to July appears to be favorable to the spermogram of the Ouled Djellal ram even if seasonal effects have not been observed. This variation profile has also been detected in the Djallonké ram from Ivory Coast [27], in the same breed in Algiers region [14,32], and in several rams from tropical climates [34]. Thus, the ram of the Ouled Djellal breed showed a particular behavior: a slight decrease in its sexual activity in autumn/winter, despite a theoretically favorable photoperiod and an increase in spring/summer. This indicates that contrary to what is accepted, the reproductive abilities of Ouled Djellal rams in Chlef region (latitude 36°N) are not subject to photoperiod variations. The qualities of the sperm of Ouled Djellal rams were not really degraded during the year, because the bad rates of spermogram parameters were not obtained. The registered sperm therefore retained, with regard to all the measured parameters, good fertility throughout the year. This corroborates the observation made in Ouled Djellal ewes who lamb all year round [35]. This feature highlighted in this study is different from that of most other breeds of rams living in Europe and North America. In these regions, the peaks of sexual activity occur from September to November and births at the end of winter and spring [36,37].

This low seasonality is also observed in the fat-tailed Awassi breed in Turkiye (Lat. 36 to 42°N) [38], the Romney breed in New Zealand (Lat. 40°N) [39] and the Southdown breeds, and Targhee in Ohio in the United States (Lat. 40°N) [40].

Presumably, the seasonal variations in the activity of the Ouled Djellal ram testis appear, result from parallel variations in the production of hypothalamic-pituitary-gonadal hormones and from a modification of the receptivity of the interstitial gland to pituitary stimuli. In the ram, Beltrán-Frutos et al.[41], distinguished in the annual testicular cycle, three main phases called "regression" (minimum testicular weight), "development" and "activity" (maximum testicular size) and showed that

the LH-RH peaks are fewer during the "regression" phase. Therefore, the intensity of sexual activity manifested in spring/summer in the Ouled Djellal ram can be explained by the increase in the total quantity of LH-RH released by the hypothalamus during the testicular "development" phase (in spring/summer). Also, in the Ile de France ram, very high correlations are presented between the pituitary LH content and the weights of the testicle and the accessory glands. These parameters show lower values from the end of November to the end of January (in autumn and winter) [42].

These observations made believe of that the seasonal variations of the parameters, manifested in the Ouled Djellal ram, are secondary to parallel modifications of the activity of the hypothalamic-pituitary-gonadal system. The increase in the values of the parameters taken into account in spring/summer is due to the spring reactivation of the testis (development and activity phase) caused by an increase in LH-RH and LH secretions. Also, to the possibility of seasonal variations in the sensitivity of the pituitary to LH-RH. Indeed, frequent photoperiodic manipulations have proven that the receptivity of the pituitary to the hypothalamic factor is greater in the testicular activity phase (spring/summer) than in the regression phase (autumn/winter), leading to much greater LH-RH discharges in the activity phase and the endogenous release of LH by frequent episodic peaks [12]. Therefore, it appears that the frequency of LH-RH discharges is the factor determining the cycle of variations in spermatogenesis in rams [43].

Seasonal variations in prolactin levels have also been described in Ouled Djellal rams [17], with high levels in spring/summer and much lower levels in winter. Considering the stimulating role of prolactin on the sexual sphere and in particular on the testicular function, its intervention in the determinism of the seasonal variations of the sexual activity of the ram must be granted.

Many studies have highlighted the importance of the quality and quantity of food on sexual activity. The restriction or insufficiency of the food ration produces a decrease in spermatogenesis and in testosterone production in rams [44]. Since this factor is considered to be determining in the sexual activity, in our study and because animals received throughout the duration of the experiment, a sufficient and constant diet both in quantity and in quality, the results have revealed no variation nor influence.

In this study, there is also a parallel evolution of the reproductive activity of the ram and the average monthly temperatures. However, many authors have reported that in mid-latitudes (case of the Chlef region), the thermal environment is not the main factor influencing sexual activity, and temperature fluctuations do not alter the reproductive pattern of sheep [45]. Rather, temperature effects are in general related to an increase in the animals' body temperature. But Ouled Djellal breed seems little affected by high summer temperatures and is perfectly adapted to the thermal environment of its biotope. In the other hand, other studies have shown that exposure of males to extreme temperatures can constitute heat stress and can negatively affect their fecundity [46]. This may partly explain the slight decrease in sperm parameters in the Ouled Djellal ram during the months of July and August.

At the end, Ouled Djellal rams present a little seasonal infulence on reproduction, similar to that of the ewe of the same breed. Sexual activity is higher in spring/summer and lower in autumn/winter. This finding is not in agreement with observations generally made in sheep, which known as sexually short-day species and in which photoperiod plays an essential role in the regulation of seasonal reproductive activity. This breed therefore seems not to be very sensitive to the photoperiod and little affected by high temperatures. The sperm qualities throughout the year, are compatible with normal fecundity. These results should be considered as an incentive to use Ouled Djellal rams for year-round breeding.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author (N. Mimoune) on reasonable request.

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Ethical Statement

All the experiments were carried out according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/ DGLPAG/DVA.SDA.14).

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contribution

MT, NAI, NM: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing - Original Draft, Writing - Review & Editing. DK: Supervision, Investigation.

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Short Communication

Mitogenome Characterization and Diversity of the Nangqian Grey Yak *(Bos grunniens)*

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ABSTRACT

Nangqian grey yak *(Bos grunniens)* is a unique yak population in Qinghai Province, China. In this study, the whole mitogenome sequences of 18 Nangqian grey yaks were sequenced based on the next-generation sequencing (NGS) technology and annotated. The total length of whole mitogenome sequence is between 16.323 bp and 16.325 bp, including a non-coding control region (D-loop region), 22 tRNA genes, 13 protein-coding genes and two rRNA genes (12*S rRNA* and 16*S rRNA*). Maternal genetic diversity based on the mitogenome variations **was** analyzed. A total of 12 haplotypes were identified among 18 complete mitogenome sequences, the haplotype diversity and nucleotide diversity of Nangqian grey yak were 0.948±0.033 and 0.001±0.001, respectively. Compared with the wild yak population and six other domestic yak breeds/populations in China, the haplotype diversity of Nangqian grey yak population was higher, indicating abundant maternal genetic diversity in Nangqian grey yak. The phylogenetic tree showed that Nangqian grey yak was most closely related to Tibet alpine, Xueduo, Changtai, Sibu, Zhongdian, Tianzhu white, Ashdan, Jinchuan, Jiulong, Pamir, Pali, Qinghai plateau, Huanhu, Datong, Bazhou and wild yak breeds/populations, closer to Chawula, Muli, Gannan, Niangya and Yushu yak breeds, but far away from other yak breeds (i.e. Leiwuqi and Maiwa yak).

Keywords: Grey yak, Mitogenome, Annotation, Genetic diversity

Introduction

Yak *(Bos grunniens)* is mostly found on the Qinghai-Tibetan Plateau (QTP) and nearby alpine and subalpine regions at heights ranging from 3.000 to 6.000 meters above sea level [1]. There are now 23 domestic yak breeds that are officially recognized, comprising 21 indigenous breeds and two improved breeds that are kept in China [2]. Qinghai Province of China is home to four indigenous breeds (Yushu, Qinghai-Plateau, Huanhu, and Xueduo) as well as two developed breeds (Datong and Ashdan) [2]. With the exception of the Tianzhu white yak breed, which is white, the majority of these yak breeds are black or black blown [3]. Additionally, a small number of yaks in various breeds or populations have golden, grey, or mixed coat colors [4]. It should be noted that certain individuals with the same coat color have been bred artificially over an extended period of time to form herds, such as the Nangqian grey yak [5]. Nangqian County is situated in the southern part of the Yushu Tibetan Autonomous Prefecture, Qinghai Province, China, at an average elevation of more than 4.000 meters. One of the key businesses in this County is the yak industry, and there are more than 200.000 yaks in total. The Nangqian grey yak *(Bos grunniens)* has a high degree of adaptation to resist extreme conditions such as high altitude, acute cold, and strong UV radiation. Mitochondrial DNA (mtDNA) is a circular DNA molecule that is self-replicating, non-recombinogenic, and maternal inheritance, which is characterized by rapid evolution, simple structure and straightforward sequencing [6]. It is frequently employed as an excellent molecular marker in research on the

Fig 1. Nangqian grey yak

taxonomy, phylogeny, and genetic diversity of mammals [7]. The maternal genetic diversities of wild yak and some Chinese domestic yak breeds/populations (e.g. Qilian, Pamir, Qinghai-Plateau, Huanhu, Xueduo, and Yushu yak) were recently also comprehensively analyzed based on nucleotide variations of the complete mitogenome [8-11]. According to these previously reported findings, both domestic yak breeds/populations and wild yak exhibited higher maternal genetic variations, which were clustered into three lineages with three possible maternal origins [8-11]. In this study, we sequenced and annotated the whole mitogenome sequences of 18 Nangqian grey yaks and investigated its characterization, maternal genetic diversity and phylogeny, which would be helpful for the future conservation and molecular breeding of this rare yak genetic resource.

Materials and Methods

Ethical Approval

All experiments in this study were performed following requirements of animal welfare and were based on the recommendations of the Regulations for the Administration of Affairs Concerning Experimental Animals of China. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Academy of Animal Science and Veterinary Medicine, Qinghai University.

Sample Collection

Here, the blood samples of 18 Nangqian grey yaks *(Bos grunniens)* were collected in Nangqian County of Yushu Tibetan Autonomous Prefecture, Qinghai Province, China (96°28′44”N, 32°12′26”E) *(Fig. 1)*. To ensure that the representative of yak samples, all experimental animal individuals are collected through the way of asking herdsmen, consulting the pedigree records and random sampling. The voucher specimen (Sample No.: NQ202201-202218; Approval date:20220822) was kept in the Plateau Livestock Genetic Resources Protection and Innovative Utilization Key Laboratory of Qinghai Province (Xining, Qinghai, China).

Mitochondrial Genome Extraction and Statistical Analysis

DNA Extraction Kit (Aidlab Biotechnologies Co., Ltd, China) was used to extract genomic DNA. All the data of Nangqian grey yak were obtained on the Illumina NOVA 6.000 platform with 2×150 bp paired-end reads. Then the read pairs were aligned to the yak reference mitogenome (Accession number: NC_006380) using the Burrows-Wheeler Aligner (BWA) v0.7.15, which were subsequently converted to BAM files using samtools v0.1.19 software. Indel realignment and recalibration were performed with the Genome Analysis Toolkit (GATK v3.8) [12]. Mitogenome characterization of Nangqian grey yak was identified using comparative genome approach. Furthermore, Dnasp5.10 [13] and Arlequin3.11 [14] were used to detect the polymorphic sites, the number or type, type of haplotype/haplogroups, haplotype diversity and nucleotide diversity. Taking American bison (*Bison bison*) as an outgroup, a phylogenetic tree was constructed using neighbor-joining (NJ) method in Mega 5.0 software [15] (Kimura 2-parameter model, 1000 replicates) to explore the phylogenetic relationship between Nangqian grey yak and other 23 yak breeds/populations.

Results

The annotated mitogenome haplotype sequences of Nangqian grey yak were submitted to GenBank with the accession number OP598192, OR085996~OR086006. The length of the mitogenomes was 16.323 bp~16.325 bp and it composed of noncoding control region *(D-loop)*, two rRNA subunit genes (12*S rRNA* and 16*S rRNA*), 22 tRNA genes and 13 protein coding genes *(Fig. 2, Table 1)*. Noncoding control region *(D-loop)* was located between the *tRNA*^{Pro} and *tRNA*^{Phe}, which was the control region for the mitochondrial genome transcription and replication. The two rRNA genes were between 957 bp (12*S rRNA)*

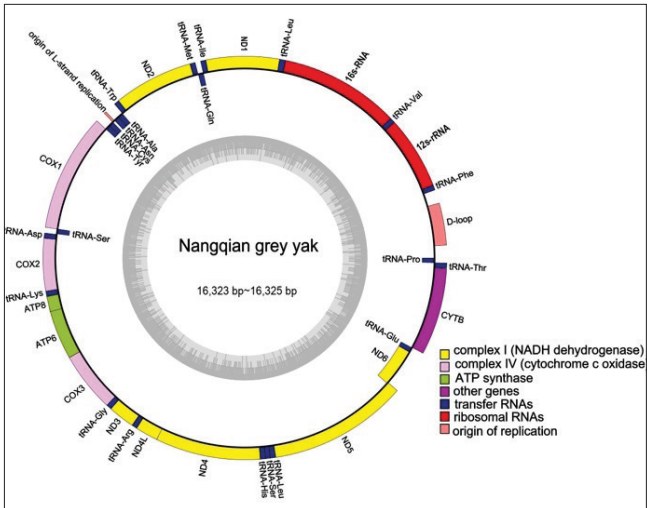

Fig 2. Pattern map of mitogenome of Nangqian grey yak

Table 1. *Structural characteristics of mitogenomes of Nanngqian grey yak (Bos grunniens)*

Gene	Position		Size(bp)	Start Codon	Stop Codon	Strand
	From	To				
D-loop	166~168	717~718	551~552			H
tRNA^{phe}	894~896	960~962	67			H
12S rRNA	961~963	1917~1919	957			H
tRNA^{Val}	1918~1920	1984~1986	67			H
16S rRNA	1985~1987	3554~3556	1570			H
tRNA^{Leu}	3556~3558	3630~3632	75			H
ND1	3633~3635	4589~4591	957	ATG	TAA	H
tRNA^{Ile}	4589~4591	4657~4659	69			H
tRNA^{Gln}	4655~4657	4726~4728	72			L
tRNA^{Met}	4729~4731	4797~4799	69			H
ND2	4798~4800	5841~5843	1044	ATA	TAG	H
tRNA^{Trp}	5840~5842	5906~5908	67			H
tRNA^{Ala}	5908~5910	5976~5978	69			L
tRNA^{Asn}	5978~5980	6051~6053	74			L
OL	6054~6056	6084~6086	31			H
tRNA^{Cys}	6084~6084	6150~6152	67			L
tRNA^{Tyr}	6151~6153	6218~6220	68			L
COX1	6220~62222	7764~7766	1545	ATG	TAA	H
tRNA^{Ser}	7762~7764	7830~7832	69			L
tRNA^{Asp}	7838~7840	7905~7907	68			H
COX2	7907~7909	8590~8592	684	ATG	TAA	H
tRNA^{Lys}	8594~8590	8660~8662	67			H
ATP8	8662~8664	8862~8864	201	ATG	TAA	H
ATP6	8823~8825	9503~9505	681	ATG	TAA	H
COX3	9503~9505	10287~10289	785	ATG	TAA	H
tRNA^{Gly}	10287~10289	10355~10357	69			H
ND3	10365~10367	10712~10714	348	ATA	TAG	H
tRNA^{Arg}	10703~10705	10771~10773	69			H
ND4L	10772~10774	11068~11070	297	ATG	TAA	H
ND4	11062~11064	12439~12441	1378	ATG	TAA	H
tRNA^{His}	12440~12442	12509~12511	70			H
tRNA^{Ser}	12510~12512	12569~12571	60			H
tRNA^{Leu}	12571~12573	12640~12642	70			H
ND5	12632~12634	14461~14463	1830	ATA	TAA	H
ND6	14445~14447	14972~14974	528	ATG	TAA	L
tRNA^{Glu}	14973~14975	15041~15043	69			L
Cytb	15046~15048	16185~16187	1140	ATG	AGA	H
tRNA^{Thr}	16189~16191	16258~16260	70			H
tRNA^{Pro}	16258~16260	16323~16325	66			L

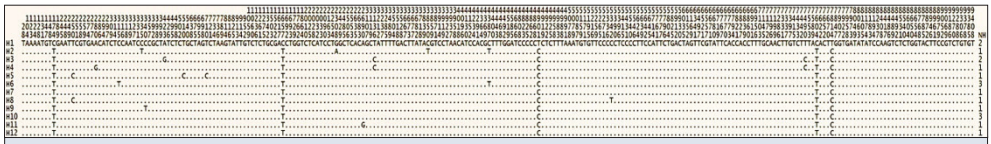

Fig 3. Frequencies of 12 haplotypes based on mitogenome sequence variations of Nangqian grey yak. NH on the right represents the number of individuals (sequences) that share each haplotype

Table 2. Comparison of genetic diversity indexes among Nangqian grey yak and other six domestic or wild yak breeds/populations in China

Breed/Population	Number of Sequences	Haplotype Diversity	Nucleotide Diversity	Reference
Huanhu yak	21~39	0.905~0.939	0.002	Wang XD et al.[8] Li GZ et al. [9]
Xueduo yak	23~30	0.992~0.989	0.002	Wang XD et al. [8] Li GZ et al. [9]
Yushu yak	20~32	0.963~0.976	0.003	Wang XD et al. [8] Li GZ et al. [9]
Qinghai Plateau yak	39	0.973	0.003	Li GZ et al. [9]
Qilian yak	22	0.948	0.003	Wang XD et al. [8]
Pamir yak	25	0.990	0.003	Wang XD et al. [8]
Wild yak	21~24	0.991~0.993	0.003~0.004	Li GZ et al. [9] Wang ZF et al. [10] Ma ZJ et al. [11]
Nangqian grey yak	18	0.948	0.001	This study

and 1.570 bp (16*S rRNA)* in length and were separated by *tRNA*^{Val}. 13 protein-coding genes ranged from 201 bp *(ATP8)* to 1.830 bp *(ND5),* and 22 tRNA genes ranged from 60 bp *(tRNA*^{ser}*)* to 75 bp *(tRNA*^{Leu}*)*. The nucleotide composition of mitogenomes was. A: 33.71%, T: 27.27%, C: 25.81%, G: 13.21%. The A+T content was 60.98% and the G+C content was 39.02%, showing a clear bias in nucleotide composition. All of the mitochondrial genes in Nangqian grey yak are encoded in the heavy chain except for eight tRNAs *(Gln*, *Ala*, *Asn, Cys*, *Tyr*, *Ser*, *Glu* and *Pro)* and *ND6* genes in the light chain. Duplication of ATPase genes appears to be common in the mitochondrial genomes of most vertebrates [16,17]. Here, there are 4 overlaps in the 13 protein-coding genes, respectively. For instance, *ATP6* and *ATP8* overlap by 40bp, *COX3* and *ATP6* overlap by 1bp, *ND4* and *ND4L* overlap by 7bp, *ND6* and *ND5* overlap by 17bp in the Nangqian grey yak mitogenome. Among the protein-coding genes, ATA is the starting codon of *ND2*, *ND3* and *ND5*, and ATG is the starting codon of *ND1*, *COX1*, *COX2*, *COX3*, *ATP6*, *ATP8*, *ND4*, *ND4L*, *ND6* and *Cytb*. Three complete stop codons are labeled, i.e. TAG *(ND2 and ND3),* AGA (*Cytb*) and TAA (*ND1*, *COX1*, *COX2*, *COX3*, *ATP8*, *ATP6*, *ND4*, *ND4L*, *ND5* and *ND6*) *(Fig. 2, Table 1).*

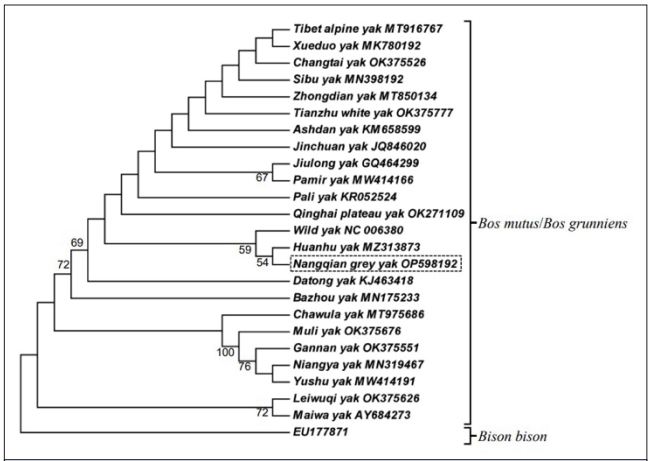

Fig 4. Phylogenetic relationship between Nangqian grey yak and 23 other yak breeds/populations in China based on mitogenome sequence variations. The support values (>50) next to the nodes are based on 1000 bootstrap replicates

After excluding four InDel sites, a total of 29 polymorphic sites were detected in the 18 mitogenomes alignment analysis, including 12 single polymorphic sites and 17 parsimony information sites. Totally, 12 haplotypes were identified in this study *(Fig. 3),* with the haplotype diversity and nucleotide diversity of Nangqian grey yak were 0.948±0.033 and 0.001±0.001, respectively *(Table 2).* The phylogenetic tree showed that Nangqian grey yak was most closely related to Tibet alpine, Xueduo, Changtai, Sibu, Zhongdian, Tianzhu white, Ashdan, Jinchuan, Jiulong, Pamir, Pali, Qinghai plateau, Huanhu, Datong, Bazhou and wild yak breeds/populations, closer to Chawula, Muli, Gannan, Niangya and Yushu. However, distant genetic relationships were found between Nangqian grey yak and the rest of domestic yak breeds (i.e. Leiwuqi and Maiwa yak) *(Fig. 4).*

Discussion

In this study, the mitogenome characterization and

maternal genetic diversity of Nangqian grey yak were analyzed for the first time. Our preliminary analysis showed that the mitogenome of Nangqian grey yak composed of noncoding control region (D-loop), two rRNA subunit genes, 22 tRNA genes and 13 protein coding genes, which indicated that the gene composition, structure and arrangement of Nangqian grey yak mitogenome are similar to that of most of other mammals [18-23].

Haplotype diversity was served as one of important indicators of maternal genetic diversity in animal populations. Compared with the genetic diversity index of the reported wild yak and six Chinese domestic yak breeds/populations [8-11], the Nangqian grey yak had a higher haplotype diversity, indicating rich maternal genetic diversity *(Table 2)*. In this study, the phylogenetic tree showed that Nangqian grey yak exhibited a mostly close genetic relationship with a majority of the yak breeds/populations (i.e. Tibet alpine, Xueduo, Changtai, Sibu, Zhongdian, Tianzhu white, Ashdan, Jinchuan, Jiulong, Pamir, Pali, Qinghai plateau, Huanhu, Datong, Bazhou and wild yak), closer relationship with the Chawula, Muli, Gannan, Niangya and Yushu breeds, but far away from other a few of yak breeds (i.e. Leiwuqi and Maiwa yak). To certain extent, the above result basically showed the clustering relationship and differentiation degree among them. However, to further thoroughly elucidate the genetic differences between Nangqian grey yak and other yak breeds/populations, a further extensive study of yak at whole-genome level is warranted in the future.

To sum up, the mitogenome of Nangqian grey yak was composed of noncoding control region (D-loop), two rRNA subunit genes, 22 tRNA genes and 13 protein coding genes. It owned rich maternal genetic diversity. Nangqian grey yak was most closely related to Tibet alpine, Xueduo, Changtai, Sibu, Zhongdian, Tianzhu white, Ashdan, Jinchuan, Jiulong, Pamir, Pali, Qinghai plateau, Huanhu, Datong, Bazhou and wild yak breeds/populations, closer to Chawula, Muli, Gannan, Niangya and Yushu, but far away from other yak breeds (i.e. Leiwuqi and Maiwa yak).

Availability of Data and Materials

The data that support the findings of this study are openly available in GenBank of NCBI at https://www.ncbi.nlm.nih.gov, accession number OP598192, OR085996-OR086006.

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Competing Interests

All authors reported no potential conflicts of interest.

Author Contribution Statement

ZJ participated in the conception and design; CP, MY performed the experiment and involved in data analysis; CP drafted the original manuscript; ZJ helped to revise the manuscript; CP, YH, LJ and ZJ carried out sampling; all authors critically reviewed and approved the final manuscript.

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The authors are responsible for conducting experimental and clinical studies on animal experiments within the framework of existing international legislation on animal rights. Authors must also obtain permission from the Animal Experiment Ethics Committees and provide relevant information in the Material and Method section to experiment with animals. In clinical studies, as well as the approval of the ethics committee, an "informed consent form" should be obtained from the animal owners and the information related to it should be declared in the Material and Method section. Declaration of "informed consent form" is sufficient for the articles in the "Case report" and "Letter to the Editor" category.

Ethics committee permission taken for a study can only be used in one article. It is unacceptable to use the same ethics committee approval number in articles with different names and contents. The editor/subject editors can request from the corresponding author, if necessary, to send a copy of the ethics committee approval form to the journal (electronically or by post).

In cases of violation ethical rules, the article is not taken into consideration or if it is in the evaluation stage, the procedure is terminated and the article is rejected.

• Conflicts of Interest/Competing Interests

The editor-in-chief pays attention to whether there is a conflict of interest or union of interest between editors, reviewers and author (s) for an objective and unbiased evaluation of the article. In addition, the authors should disclose any financial interests or links or any conditions that may raise the bias issue in research and article under the above heading.

• Copyright

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The authors must fill in the "Copyright Agreement Form" and sign it with a wet signature. Authors who submit articles from abroad should scan the signed form and send it to the editor via the system or by e-mail. Original forms that are wet signed for articles sent domestically should be submitted to the journal via mail or cargo. The works of the authors who do not submit the Copyright Agreement Form on time are not published.

• Withdrawal of a Submission

In case of if the authors detect a significant error or deficiency in their article under review or if this error is reported to them by the editor/ subject editor/referees they can contact immediately to the editor-in-chief and ask the request to withdraw the article by stating the reason. The decision on this issue is up to the editorial board.

• Erratum

After an article has been published, the corresponding author may request the editor to publish "erratum" for any errors or inaccuracies noticed by the authors, editors or readers. In collaboration with the authors, the editor prepares and publishes the Erratum article in the first upcoming issue. These articles, like other publications, should contain the publication tag and DOI number.

• Retraction

If any ethical problem is detected about the article that cannot be compensated and cannot be eliminated with erratum after the article is published, the editor-in-chief and associate editors prepare a justification about the article and apply the retraction procedure to the article. The text file on the web page of a retracted article is blocked and the reason for retraction is added to the system as a file, ensuring that it is constantly in the archive.

• Advertising

Kafkas Universitesi Veteriner Fakultesi Dergisi do not accept advertising and sponsorships that are believed to create a potential conflict of interest. If the article sent to Kafkas Universitesi Veteriner Fakultesi Dergisi is for the promotion of a commercial product and/or the work carried out is directly supported by a company, it is rejected without consideration.

OPEN ACCESS STATEMENT

Kafkas Universitesi Veteriner Fakultesi Dergisi is an open access publication. The journal's publication model is based on Budapest Open Access Initiative (BOAI) declaration. Articles published in Kafkas Universitesi Veteriner Fakultesi Dergisi are available online, free of charge at https://vetdergikafkas.org/archive.php.

Except for commercial purposes, users are allowed to read, download, copy, print, search, or link to the full texts of the articles in this journal without asking prior permission from the publisher or the author. The open access articles in the journal are licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC 4.0) licence.

ARTICLE EVALUATION AND PUBLICATION PROCESS

• Initial Evaluation Process

Articles submitted to Kafkas Universitesi Veteriner Fakultesi Dergisi are primarily evaluated by the editors and associate editors. At this stage, articles not having suitable scope and aims, with low original research value, containing scientific and ethically important errors, having low potential to contribute to science and the journal, and having poor language and narration are rejected by the editor without peer-review process. Initial evaluation process takes up to most 2 weeks.

• **Preliminary Evaluation Process**

Articles that are deemed appropriate for editorial evaluation are sent to the subject editor related to the category of articles to be examined in terms of scientific competence and to the statistics editor for evaluation in terms of statistical methods. The suject editors examine the article in all aspects and report their decisions (rejection, revision or peer-review) to the chief editor. This stage takes about 1 month.

• **Peer-review Process**

Double-blind peer-review is applied to the articles that have completed preliminary evaluation process. Suggestions of subject editors are primarily considered in referee assignment. In addition, reviews can be requested from the referees registered in the journal's referee pool. At least 2 referees are assigned for peer-review. Opinion of more referees can be required depending on the evaluation process. At this stage, referees send their decision (reject, revision or accept) about the article to the editor-in-chief. If the rejection decision given by a referee reflects sufficient examination and evidence-based negativities or ethical problems about the scientific content and accuracy of the article, this decision is checked by the editor-in-chief and associate editors and submitted to the authors regardless of the other referees' decisions. The time given to referees to evaluate an article is ~4 weeks.

• **Publication Process of an Article**

Total evaluation period of an article, which is completed in the peer-review phase after completing the initial and preliminary evaluation process, takes 4-6 months. The articles that have completed the subject editorial and peer-review evaluation stages and accepted by the editorial are sent to the corresponding author for final checks and necessary final additions. After the acceptance, the article designed in the publication format of the journal is given an DOI number and published immediately on the Article in Press page. When it is time to publish the periodic edition of the journal, a selection is made from the articles kept on the Article in Press page, taking into account the submission date. The time it takes for the article to be published by taking the page number is 6-12 months.

NO PUBLICATION FEE

Processing and publication are free of charge with the journal. There is no article processing char-ges, submission fees or any other fees for any submitted or accepted articles.

RESPONSIBILITIES OF THE PUBLISHER, EDITORS AND ASSOCIATE EDITORS

The publisher (Dean of the Faculty of Veterinary Medicine of Kafkas University) contributes to the execution of the journal's routine processes such as printing, archiving, and mailing, in line with requests from the editor.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and assistants in the article evaluation process and decisions.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and associate editors in the article evaluation process and decisions.

Editor-in-chief/editors/associate editors of Kafkas Universitesi Veteriner Fakultesi Dergisi evaluate the articles submitted to the journal regardless of their race, gender, religious belief, ethnicity, citizenship or political views. In addition, it undertakes not to give any information about the article except for the authors, subject editors and referees.

Kafkas Universitesi Veteriner Fakultesi Dergisi follows internationally accepted principles and criteria and takes the necessary decisions to apply in the journal.

Editor-in-chief/editors/associate editors conduct the evaluation and decision process in the journal in coordination within the principles of confidentiality and have independent decision-making authority and responsibility without being affected by any internal or external factors.

Editor-in-chief/editors/associate editors make and implement all kinds of planning for the development of the journal and its international recognition. They also follow national and international meetings or events on the development of journals and article evaluation, and ensures that the journal is represented on these platforms.

The editor-in-chief/editors/associate editors make every effort to ensure that the journal's subject editors and referee pool have international qualifications. Likewise, it makes the necessary attempts to strengthen the author's profile.

Editor-in-chief/editors/associate editors make plans to improve the quality of the articles published in the journal and carry out the necessary process.

Editor-in-chief/editors/associate editors regularly conduct and control the initial evaluation, preliminary evaluation, peer review and acceptance-rejection decisions of articles submitted to the journal. While carrying out these procedures, features such as the suitability of the study for the aims and scope of the journal, its originality, the up-to-date and reliability of the scientific methods used, and the potential it will contribute to the development of the journal as well as its benefit to science/practice are taken into consideration.

Editor-in-chief/editors/associate editors systematically review, inspect and make decisions about the articles submitted to the journal in terms of features such as author rights, conflict of interest, observance and protection of animal rights, and compliance with research and publication ethics.

The editor-in-chief conducts the evaluation/revision process between the authors and subject editors and referees, and ensures that it is completed within the prescribed time.

ARCHIVE POLICY

The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

Even if the journal ceases to be published for any reason, the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) will continue to protect the journal content in the long term and provide convenient access to users. Electronic services of Kafkas University Information Technologies Department will be used for the journal to maintain this responsibility.

RESPONSIBILITIES OF SUBJECT EDITORS

Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

Subject editors thoroughly examine the sections of the introduction, materials and methods, results, discussion and conclusion, in terms of journal publication policies, scope, originality and research ethics. Subject editor submits its decision (rejection, revision or peer-review) after evaluation to the chief editor in a reasoned report.

Subject editor may request additional information and documents related to the study from the authors, when necessary.

In multidisciplinary studies, the article can be submitted for the evaluation of multiple subject editors.

RESPONSIBILITIES OF REFEREES

Double-blinded peer-review procedure is applied in Kafkas Universitesi Veteriner Fakultesi Dergisi in order to evaluate the articles submitted to the journal in accordance with the principle of impartiality and in objective criteria; that is, referees and writers do not know about each other.

The referees submit their opinions and reports to the editor-in-chief to ensure the control and suitability of a submitted article, its scientific content, scientific consistency and compliance with the principles of the journal. When a referee makes a decision "reject" about an article, he/she prepares the reasons for the decision in accordance with the scientific norms and presents it to the editor.

The referee(s) also gives the authors the opportunity to improve the content of the article. Accordingly, the revisions requested from the authors should be of a quality that explains/questions specific issues rather than general statements.

Referees appointed for the evaluation of the articles agree that the articles are confidential documents and will not share any information about these documents with third parties, except for the editors participating in the evaluation.

Referees should place their criticism on scientific infrastructure and write their explanations based on scientific evidence. All comments made by the referees to improve the articles should be clear and direct, and should be written away from disturbing the feelings of the author. Insulting and derogatory statements should be avoided.

If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

The editor-in-chief can share the comments and reports from the referees with the editors/associate editors and the relevant subject editor, as necessary, to ensure that the decision on the article is optimal. If necessary, the editor may share the critical decision and its grounds that a referee has sent about the article with the other referee(s) and present them to their attention.

Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

RESPONSIBILITIES OF AUTHOR(S)

It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Universitesi Veteriner Fakultesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

It is essential that the articles to be sent to Kafkas Universitesi Veteriner Fakultesi Dergisi include studies that have up-to-date, original and important clinical/practical results and prepared in accordance with the journal's writing rules.

Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

The authors are obliged to revise the article in line with the issues conveyed to them during the initial evaluation, preliminary evaluation and peer-review phases of the article and to explain the changes they made/did not make sequentially in the "response to editor" and "response to reviewer comments" sections.

If information, documents or data regarding to the study are requested during the evaluation process, the corresponding author is obliged to submit them to the editorial.

Authors should know and take into account the issues listed in the "General Ethical Principles" section regarding scientific research and authors.

The authors do not have the right to simultaneously submit multiple articles to Kafkas Universitesi Veteriner Fakultesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

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INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (ISSN: 1300-6045 and e-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given on the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal includes all aspects of veterinary medicine and animal science.

Kafkas Universitesi Veteriner Fakultesi Dergisi is an Open Access journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

The official language of our journal is English. Additionally, all the manuscripts must also have Turkish title, keywords, and abstract (translation will be provided by our journal office for foreign authors).

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing, and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and supplementary files (figure etc.) should be submitted by using online manuscript submission system at the address of http://vetdergi.kafkas.edu.tr/

During the submission process, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Agreement Form signed by all the authors should be sent to the editorial office.

3- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that "informed consent" was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

4- Authors should know and take into account the issues listed in the "Ethical Principles and Publication Policy" section regarding scientific research and authors.

5- Types of Manuscripts

Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. Abstract should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but the abstract should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case reports describe rare significant findings encountered in the application, clinic, and laboratory of related fields. The title and abstract of these articles should be written in the format of full-length original articles (but the abstract should not exceed 100 words) and the remaining sections should be followed by the Introduction, Case History, Discussion and References. The reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages in total. The page limit does not include tables and illustrations.

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 3 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

"Invited review" articles requested from authors who have experience and recognition in international publishing in a particular field are primarily published in our journal.

Review articles submitted to our journal must be prepared in accordance with any of the three categories listed below.

Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

Systematic reviews include the search for original studies published in that field on a specific topic, the evaluation of validity, synthesis and interpretation within a systematic methodology.

Meta-analysis is a method of evaluating the results of many studies on a subject with the methods defined in this category and statistical analysis of the obtained findings.

6- The necessary descriptive information (thesis, projects, financial supports, etc.) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of the title.

7- At least 30% of the references of any submitted manuscript (for all article categories) should include references published in the last five years.

References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: Yang L, Liu B, Yan X, Zhang L, Gao F, Liu Z: Expression of ISG15 in bone marrow during early pregnancy in ewes. *Kafkas Univ Vet Fak Derg*, 23 (5): 767-772, 2017. DOI: 10.9775/kvfd.2017.17726

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in a book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of the book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: Mcllwraith CW: Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply with the other references. Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

Follow the link below for EndNote Style of Kafkas Universitesi Veteriner Fakultesi Dergisi;
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8- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

9- The editorial board has the right to perform necessary modifications and a reduction in the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

10- All responsibilities from published articles merely belong to the authors. According to the ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checking by plagiarism checker software, which compares the content of the manuscript with a broad database of academic publications.

11- There is no copyright fee for the authors.

12- The authors are charged a fee on acceptance of the manuscript to cover printing costs and other expenses. This payment information can be found at http://vetdergi.kafkas.edu.tr/

SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

- Cover letter

• Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction).

• Other information has been added that should be known by the editorial board (e.g.; the manuscript or any part of it has not been published previously or is not under consideration for publication elsewhere.

- Title page

• Title, running title (should be a brief version of the title of your paper, no exceed 50 characters)

• The author's name, institutional affiliation, Open Researcher and Contributor ID (ORCID)

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

- Manuscript

• Title, abstract, keywords and main text

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print

- Availability of Data and Materials

- Acknowledgements

- Funding Support

- Competing Interests

- Authors' Contributions

Further considerations

• Journal policies detailed in this guide have been reviewed

• The manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Statement of Author Contributions added to the text

• Acknowledgment and conflicts of interest statement provided