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RESEARCH ARTICLE

Determination of Seminal Characteristics in Turkish Aseel Roosters

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Abstract: Ornamental poultry is a hobby that has been of interest for centuries. The history of ornamental poultry associations in Europe dates back to the 19th century and to the Ottoman period in Turkiye. One of the most popular ornamental poultry species is Aseel roosters. Aseel roosters are indigenous of Pakistan and India, and they have been bred for competition during the Ottoman period. This study aims to determine the spermatological characteristics of Turkish Aseel roosters. In the study, 10 Aseel roosters were used, and semen was collected by the abdominal massage method twice a week. The sperm motility was estimated by a hot plate phase-contrast microscope under 400´ magnification. The sperm concentration of each ejaculate was determined by hemocytometer and percentages of viable, dead, and abnormal spermatozoa was calculated using eosin-nigrosine staining. Acrosome membrane integrity of rooster spermatozoa were assessed using fluorescein isothiocyanate-conjugated peanut agglutin (FITC-PNA). Spermatozoa membrane functionality was assessed with the hypoosmotic (HOS) test. The spermatological data obtained as a result of the experiment are as follows; ejaculate volume average 308.49±12.14 µL, spermatozoa motility 89.66±0.47%, spermatozoa concentration 2.39±0.10x10^9/mL, The general total morphological defect rate 17.19±0.75%, viability 85.45±0.88%, acrosome integrity rates 98.26±0.09%, and pH 7.81±0.02.

Keywords: *Aseel rooster, Ornamental poultry, Seminal characteristic*

Türk Aseel Horozlarında Seminal Özelliklerin Belirlenmesi

Öz: Süs kümes hayvanları yüzyıllardır ilgi gören bir hobidir. Avrupa'daki süs kanatlı birliklerinin tarihi 19. yüzyıla, Türkiye'de ise Osmanlı dönemine kadar uzanmaktadır. En popular süs kanatlı türlerinden biri Aseel horozlarıdır. Aseel horozları Pakistan ve Hindistan'a özgüdür ve Osmanlı döneminde yarışmalar için yetiştirilmiştir. Bu çalışma, Türk Aseel horozlarının spermatolojik özelliklerini belirlemeyi amaçlamaktadır. Çalışmada 10 adet Aseel horozu kullanıldı ve haftada iki kez karın masajı yöntemiyle sperma alındı. Sperm motilitesi, 400´ büyütme altında ısıtma tablalı faz contrast mikroskobu ile değerlendirildi. Her ejakülatın sperm konsantrasyonu hemositometrik yöntem ile belirlendi ve canlı, ölü ve anormal sperm yüzdeleri eozin-nigrosin boyaması kullanılarak hesaplandı. Spermatozoaların akrozom membrane bütünlüğü, floresein izotiyosiyanat konjuge peanut aglutin (FITC-PNA) kullanılarak değerlendirildi. Spermatozoa membrane işlevselliği hipoozmotik (HOS) testi ile değerlendirildi. Deney sonucunda elde edilen spermatolojik verilere göre ejakülat hacmi ortalaması 308.49±12.14 µL, spermatozoa motilitesi %89.66±0.47, sperm konsantrasyonu 2.39±0.10x10^9/mL, toplam morfolojik bozukluk oranı %17.19±0.75, canlılık %85.45±0.88, akrozom bütünlük oranları %98.26±0.09 ve pH 7.81±0.02 olarak bulunmuştur.

Anahtar sözcükler: *Aseel horozu, Spermatolojik özellikler, Süs kümes hayvanları*

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Introduction

Aseel roosters have been famous for their well-developed musculature, body formation, pugnacity, stamina and fight in Turkiye and all over the world. Aseel roosters are indigenous of Pakistan and India, and they have been bred for competition during the Ottoman period [1-3]. They are also preferred for their immunity, adaptation to harsh conditions and organic meat production [4,5]. The history of ornamental poultry associations in Europe dates back to the 19th century and to the Ottoman period in Turkey. However, organized and more scientific breeding started in Türkiye in 2016 [6]. Aseel roosters are one of the most popular ornamental poultry breeds in Turkiye. Producing the most valuable breeders is the common goal of all producers. However, this breed suffers from low egg production, poor hatching rates, and low fertilization [7]. Also, the quality of semen directly effects the fertility level. To get rid of the reproductive problems, it is necessary to select the Aseel individual with the best spermatological characteristics and high breeding value. Fertility in roosters depends on quality and quantity of semen, as well as the mating capacity [8-10]. Sperm fertilization is directly related to the ultrastructure of spermatozoa, motility, morphology, concentration, DNA fragmentation and genetic composition [11-15]. Semen quality in roosters is analyzed by using parameters such as motility, viability, membrane, and acrosome integrity [10,16].

It was aimed in this study to reveal the spermatological characteristics of Turkish Aseel roosters. Thus, reference values were obtained for the determination of quality breeders, cooling, and freezing of semen, and artificial insemination in Aseel roosters. These values were determined for motility, volume, concentration, pH, membrane functionality, acrosome integrity, morphological defects and viability.

Materials and Methods

Ethical Approval

The study was conducted with the permission of Istanbul University-Cerrahpasa Animal Experiments Local Ethics Committee (IUCHADYEK) with the approval number 2022/41

Animals and Semen Collection

The study was carried out at Istanbul University-Cerrahpasa Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination. Ten Assel breed roosters were used in the study at 2 years, weighing 2.1 to 3.4 kg with the phenotypic characteristics of the Aseel breed [3].

The roosters were kept separately (length C width C height) in individual cages measuring 1 m x 75 cm x 75 cm. and fed *ad libitum* with commercial chicken food. The roosters were trained in semen collection by abdominal massage. Semen was collected by abdominal massage twice a week for 5 weeks [17]. Semen samples were taken individually into 1.5 mL microcentrifuge tubes and then transported to the laboratory within 5 min.

Macroscopic Evaluation of Semen

The appearance of semen samples was scored by visual inspection on a scale of 1 to 5 [18]. Watery or clear semen was given 1 point, white smoky semen 2 points, medium white semen 3 points, dark, white semen 4 points, and very viscous, chalky white semen samples 5 points. The volume was measured with an automatic pipette and pH with pH strips without dilution [19,20].

Sperm Mass Activity

The sperm mass activity was estimated by a hot plate phase-contrast microscope under 10x magnification [21].

Sperm Motility Analysis

The sperm motility was estimated by a hot plate phase-contrast microscope under 400x magnification. Motility was expressed as the percentage of motile spermatozoa with moderate to rapid progressive movement. At least 3 microscopic fields were examined for each sample.

Sperm Concentration

The sperm concentration of each ejaculate was determined by hemocytometer in Formol (1%) saline solution ata1:400 ratio and expressed as billion (10^9) per mL [22].

Sperm Viability and Morphology

Percentages of viable, dead, and abnormal spermatozoa were calculated using eosin-nigrosine staining [23]. One drop of semen was placed on a clean, warm glass slide and mixed with a mixture of 1 drop of 5% eosin and 2 drops of 10% nigrosine stain. The slides were prepared and air-dried. One hundred spermatozoa in each preparation were examined under a fluorescent microscope (400´ magnification). Both fully and partially stained spermatozoa were counted as dead *(Fig. 1)*. Visible abnormalities in the head, neck, mid-piece and tail regions were used to estimate the percentage of abnormal spermatozoa by counting a total of 100 spermatozoa. *(Fig. 2, Fig. 3, Fig. 4)*.

Acrosome Integrity

Acrosome membrane integrity of rooster spermatozoa was assessed using fluorescein isothiocyanate-conjugated peanut agglutin (FITC-PNA). Principally, FITC-PNA labels the acrosome region of acrosome-reacted spermatozoa. The stock solution of FITC-PNA (1 mg/mL) was diluted (1:10) in PBS before staining. From all animals, 5 µL of

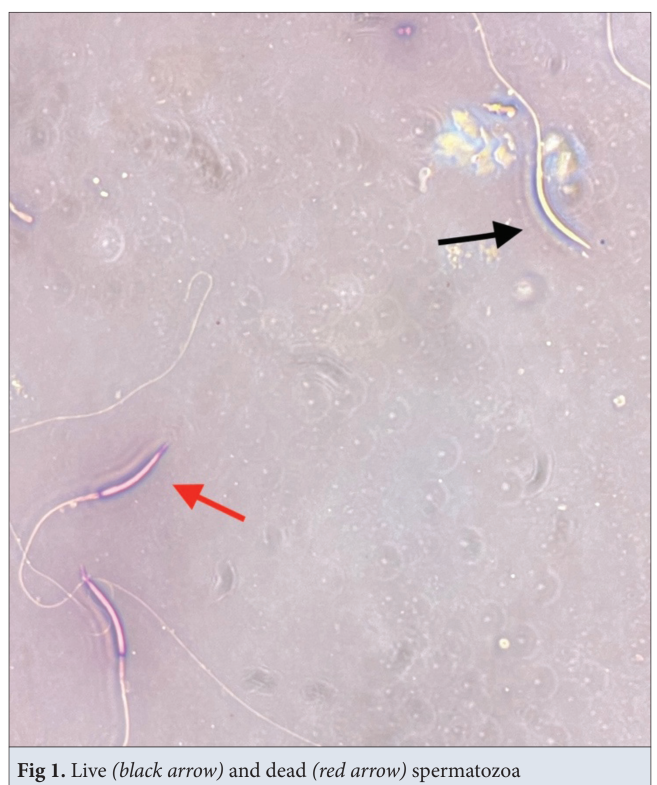

Fig 1. Live *(black arrow)* and dead *(red arrow)* spermatozoa

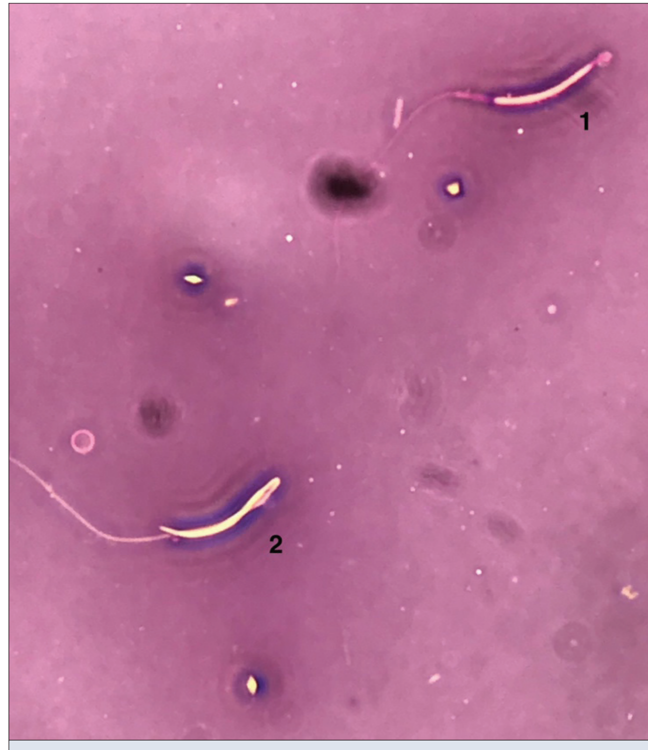

Fig 3. Acrosome swelling (1) and crooked beck (2) spermatazoa

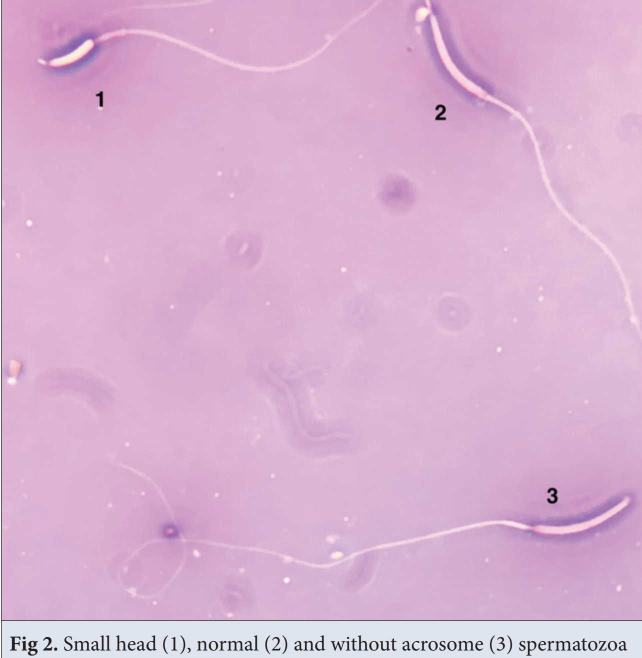

Fig 2. Small head (1), normal (2) and without acrosome (3) spermatozoa

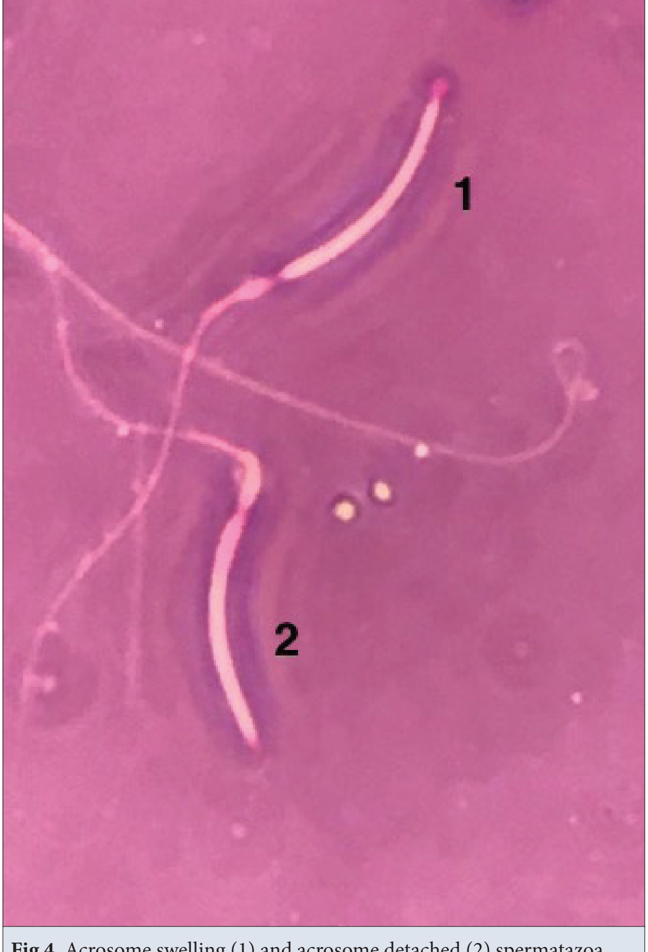

Fig 4. Acrosome swelling (1) and acrosome detached (2) spermatazoa

semen samples were diluted with 295 μL Tris-buffered media and 5 μL of FITC-PNA working solution were added to the diluted semen. Samples were loaded into a 96-well plate and incubated for 10 minutes at room temperature. Measurements were performed on a Guava® easyCyte™ (Luminex) flow cytometer using Guava® InCyte™ software. FITC-PNA positive (560 nm emission wavelength) and

negative spermatozoa were detected. Per sample, 10.000 events were acquired and gathered in diagrams *(Fig. 5)*.

Spermatozoa Membrane Functionality

Spermatozoa membrane functionality was assessed with the hypoosmotic (HOS) test, as described by Zhanget al.[24]. For the membrane test, 25 µL ofthe semen sample and 975 µL of HOST (100 mOsm /kg, 57.6 mM fructose and 19.2 mM sodium citrate) solution were mixed in Eppendorf tubes and incubated at 37°C for 30 min. 5 µL of the incubated solution was dropped onto a slide (37°C) and covered with a coverslip. 200 spermatozoa were counted under a phase contrast microscope at 100´ magnification with immersion oil. Sperm with a curved tail, swollen head, and spiral-like appearance were considered HOST test positive.

Statistical Analysis

Statistical evaluation of semen pH, viability, mass activity and HOST results was carried out by the "Kruskal-Wallis Test". "One-way Analysis of Variance" (ANOVA), followed by "Duncan's Multiple Range Test" was used to evaluate the data obtained from semen volume, concentration, subjective motility, total morphological defects and acrosomal integrity examinations. In addition, bivariate correlations between the semen variables (pH, semen volume, semen color, mass activity, subjective motility, viability, concentration, HOST+, total morphological defects and acrosomal integrity) were assessed by "Spearman's correlation coefficients *(r_s)*. Statistical analyzes were performed using the SPSS Version 22.0 for Windows (SPSS Inc., Chicago, IL, USA). The results were represented as mean ± standard error. Differences with values of P<0.05 were regarded as statistically significant.

Results

Average pH, volume and color scale results of semen obtained from 10 roosters are presented in *Table 1*. Semen pH values were similar in all roosters (C) (P>0.05). The general average sperm pH was 7.81±0.02. The lowest and the highest mean ejaculate volume were 188.46±24.95 µL and 469.64±42.66 µL respectively, and semen volume differed between individuals (P<0.05). It was determined that the average semen volume of 10 roosters was 308.49±12.14 µL Similarly, it was determined that the color of semen classified by the scale method between + and ++++, differed individually (P<0.05).

Microscopic examination results and acrosome integrity rates determined by flow cytometry are shown in *Table 2*. Mass activity (3.85±0.04), viability (85.45±0.88%), HOST positive (94.25±0.25%), and acrosome integrity rates (98.26±0.09%), were similar in all roosters (P>0.05). The lowest percentage of subjective motility was determined in the C7 rooster as 82.77±4.41%. The highest motility percentages were found in the cocks C1, C2 and C9, and

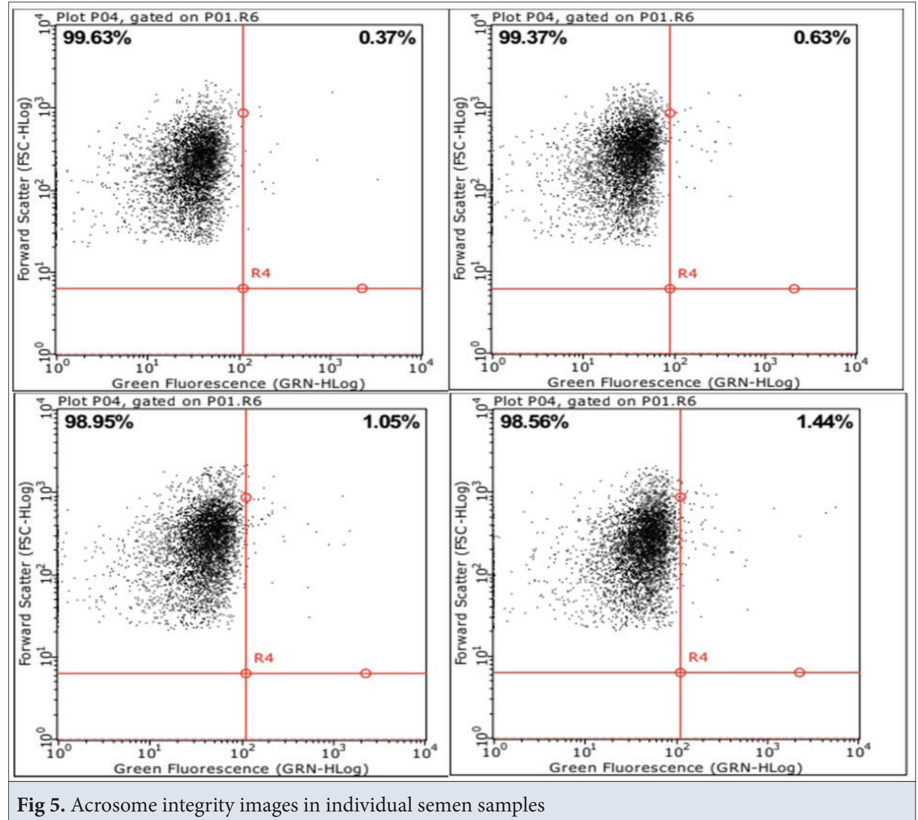

Fig 5. Acrosome integrity images in individual semen samples

Table 1. Some spermatological characteristics of Aseel cocks

Cock No	pH	Volume (µL)	Semen Color (1-5)
	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$
C1	7.84±0.06	214.64±20.62^{cd}	2.71±0.24^{a}
C2	7.80±0.07	306.42±36.03^{bc}	3.00±0.25^{a}
C3	7.69±0.12	188.46±24.95^{d}	2.53±0.18^{ab}
C4	7.85±0.06	260.41±23.31^{cd}	2.33±0.25^{ab}
C5	7.88±0.06	301.15±25.91^{bc}	2.53±0.24^{ab}
C6	7.92±0.04	469.64±42.66^{a}	2.76±0.20^{a}
C7	7.72±0.18	253.63±22.77^{cd}	1.90±0.21^{b}
C8	7.88±0.06	300.76±31.71^{bc}	2.53±0.24^{ab}
C9	7.83±0.07	383.33±25.68^{ab}	2.75±0.13^{a}
C10	7.65±0.13	421.00±43.82^{a}	2.80±0.24^{a}
General	7.81±0.02 (*n*:125)	308.49±12.14 (*n*:126)	2.60±0.07 (*n*:125)

^{abc} Values without common superscripts in the same column are statistically different, P<0.05

the motility percentages of these cocks were found to be higher than the cocks C3 and C7 (P<0.05). The general average of subjective motility was 89.66±0.47% (*n*: 119). Spermatozoa concentrations of C6 and C10 were higher in cocks compared to C3, C4, and C7 (P<0.05). The overall spermatozoon concentration was 2.39±0.10´ 10^9/mL. The total morphological defect rates in fresh rooster semen also differed individually (P<0.05). The general total morphological defect rate 17.19±0.75% (*n*:120) (Fig.5). With the lowest in C8, and the highest in C3 and C7. Also, the results show the correlation between spermatological examinations performed on fresh semen of Aseel roosters.

Discussion

Artificial insemination has a key role in overcoming reproductive problems. One of the most important factors of artificial insemination is the use of high fertility breeder roosters in industrial and ornamental poultry. Especially in ornamental poultry, it is imperative to determine the spermatological characteristics in selecting high-quality breeders and increase the success of artificial insemination.

In the present study, the lowest and highest seminal volume of total 125 ejaculates were 0.18846±0.02495 mL and 0.46964±0.04266 mL respectively with a mean volume of 0, 30849±12.14 mL. This result was similar to the results of Mavi et al.[10] (0.36±0.08) and lower than Keskin et al.[25]. The seminal volume for Leghorn and Gerze roosters has been reported almost similar to the result of the present study [10,26-28]. It is noteworthy that the mean seminal volume is reported as 0.7±0.01 mL in Denizli Roosters [27,28] which can be attributed to racial differences, nutrition, environmental factors, and hormone level differences.

Mavi et al.[10] found the volume in Aseel roosters. Alkan et al.[29] and Keskin et al.[25] found a mean ejaculate volume 0.27 mL and 0.6±0.1 mL for Erbro roosters, respectively. Chalov [26] 0.3 mL for Leghorn roosters and Tuncer et al.[27] 0.70±0.01 mL found for Denizli roosters and 0.37±0.006 mL for Gerze roosters. In this study similar results were obtained with Mavi et al.[10]. The different results obtained in other studies may be due to breed differences. However, various factors such as semen collection frequency, stress, nutrition, individual and species difference can affect the seminal volume.

Table 2. Average rate of mass activity, subjective motility, viability, concentration, HOST +, total morphological defects and acrosomal integrity in Aseel cocks

Cock No	Mass Activity (+, +++++)	Subjective Motility (%)	Viability (%)	Concentration (x10^9/mL)	HOST + (%)	Total Morphological Defects (%)	Acrosomal Integrity (%)
	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$
C1	3.92±0.07	91.15±1.15^{a}	83.78±3.27	2.57±0.38^{abc}	94.50±0.64	19.07±1.80^{ab}	98.26±0.23
C2	3.92±0.07	91.78±0.66^{a}	83.21±3.00	2.48±0.21^{abc}	93.60±0.83	19.14±2.26^{ab}	98.06±0.37
C3	3.76±0.16	86.66±1.12^{b}	90.38±0.87	1.72±0.28^{cd}	94.73±0.51	20.84±3.00^{b}	98.29±0.24
C4	3.83±0.20	90.45±0.81^{ab}	86.09±1.93	1.90±0.27^{bcd}	94.25±0.57	15.09±2.03^{ab}	98.12±0.36
C5	3.84±0.10	90.83±0.83^{ab}	86.76±2.67	2.61±0.28^{abc}	94.23±1.07	17.07±2.56^{ab}	98.13±0.31
C6	4.00±0.00	90.71±0.71^{ab}	81.76±4.04	3.07±0.32^{a}	95.23±0.70	13.92±1.60^{ab}	98.59±0.23
C7	3.20±0.35	82.77±4.41^{c}	85.20±2.79	1.22±0.22^{d}	91.05±1.48	28.00±2.49^{c}	98.41±0.27
C8	3.92±0.07	89.23±1.11^{ab}	89.30±2.87	2.45±0.26^{abc}	95.11±0.57	12.41±1.59^{a}	98.50±0.31
C9	4.00±0.00	91.25±0.65^{a}	82.66±1.93	2.72±0.39^{ab}	94.50±0.65	14.18±1.75^{ab}	98.02±0.39
C10	4.00±0.00	89.44±1.00^{ab}	85.50±2.17	3.08±0.36^{a}	94.75±0.38	14.50±1.93^{ab}	98.28±0.31
General	3.85±0.04 (n: 125)	89.66±0.47 (n: 119)	85.45±0.88 (n: 123)	2.39±0.10 (n: 126)	94.25±0.25 (n: 123)	17.19±0.75 (n: 120)	98.26±0.09 (n: 125)

^{abc} Values without common superscripts in the same column are statistically different, P<0.05

***Table 3.** Correlation values between spermatological examinations*

Parameters	Correlation	Statistical Data			Spermatological Examinations									
		n	$\bar{x}$	$S_{\bar{x}}$	SC	pH	VOL	MA	MOT	CON	VIA	HOST	AI	TMD
Spermatological examinations	SC	125	2.60	0.07	-									
	pH	125	7.81	0.02	.080	-								
	VOL	126	308.49	12.14	.210*	.187*	-							
	MA	125	3.85	0.04	.214*	.085	.220*	-						
	MOT	119	89.66	0.47	.268**	.099	.256**	.406**	-					
	CON	126	2.39	0.10	.471**	.101	.372**	.280**	.331**	-				
	VIA	123	85.45	0.88	.133	.198*	.018	.043	.020	.220*	-			
	HOST	123	94.25	0.25	.444**	.227*	.219*	.200**	.193*	.367**	.256*	-		
	AI	125	98.26	0,09	-.078	-.020	.070	.077	-.077	-.091	-.100	.091	-	
	TMD	120	17.19	0.75	-.099	-.069	-.123	-.228*	-.082	-.217*	-.102	-.005	-.192*	-

***SC:** Semen color, **VOL:** Volume, **MA:** Mass activity, **MOT:** Motility, **CON:** Concentration, **VIA:** Viability, **HOST:** Hypo-osmotic swelling test (+), **AI:** Acrosomal integrity, **TDM:** Total morphological defects, * P<0.05, ** P<0.01*

The color of semen was evaluated from 1 to 5 points from watery to creamy, with an average value of 2.60±0.07. According to Mavi et al.[10] color of Aseel roosters semen is creamy. In our study, color had an average score of 4 and more and, a high correlation was observed between semen color and semen concentration (P<0.01). Particular attention should be paid to this feature in the selection of breeders.

Spermatozoa motility is one of the most reliable parameters and gives information about the fertilization ability of semen. The higher is motility the better the fertilization results [30]. Alkan et al.[29] and Keskin et al.[25] determined spermatozoa motility as 85.83% and 79.4±11.5%, respectively. Tuncer et al.[27] and Tuncer et al.[28] determined spermatozoa motility 72.32±0.80% for Denizli roosters and 74.28±0.73% for Gerze roosters. In this study, the mean spermatozoa motility was 89.66±0.47%. which is higher than the values of several studies [25,27-29]. Especially, the motility result of the present study is higher than the result of another study with Aseel breed [10].

For Aseel roosters Mavi et al.[10] determined a spermatozoa motility of 75.87±5.73%, whilst, in our study, the mean overall sperm motility was 89.66±0.47%. The difference between the results of two studies with the same breed can be attributed to differences in nutrition, accommodation, and climatic differences.

Spermatozoa concentration directly affects the number of spermatozoa in sperm storage tubes and plays an important role in fertilization [31]. The mean spermatozoa concentration was 2.39±0.10 x10^9/mL in this study. The concentration of spermatozoa is affected by factors like individuality, breed, age, and season. Siudzinska and Lukaszewicz [32] reported that the mean sperm concentration in White Crested Black Polish cocks was 4.7 x 10^9/mL and in Black Minorcas breeds 4.2 x 10^9/mL. On the other hand, Tuncer et al. reported 2.4 x 10^9/mL and 2.38±0.03x10^9/mL in Gerze and Denizli roosters [27,28]. In the present study, a positive correlation was found between semen volume and concentration (P<0.01). It is recommended to consider these values when choosing a breeder.

Seminal pH has been associated with metabolic rate and spermatozoa motility. Turkey and rooster semen can tolerate a pH range of 6-8 [33], and the pH was found to be 7.81±0.02 (*n*: 125) in this study. Tuncer et al.[27] and Tuncer et al.[28] reported pH as 7.71 in Gerze roosters and 7.68±0.01in Denizli roosters. The results of these studies are all in the tolerance range for rooster semen.

Fertilization is affected more by morphological defects of spermatozoa than motility [19,33]. Many researchers state that acrosome defects of rooster semen are the most important effective trait of fertility [19]. In this study, total morphological defects (*Fig. 2, Fig. 3, Fig. 4*) were determined as 17.19±0.75% and total acrosome integrity (*Fig. 1*) as 98.26±0.09%. The rate of viable spermatozoa was 85.45%±0.88% and the total HOST was 94.25±0.25%. This study had resembling data to from other researchers [25,29,34,35]. However, Mavi et al.[10] achieved lower motility 75.50±7.99% and vitality 77.20±5.81% results. Various results of spermatological characteristics in other studies may be due to season, temperature, photoperiod, age, breed, ejaculation frequency, and individual differences. Testosterone has an important role in the formation and display of secondary sex characteristics and affects semen quality and production. High testosterone levels may explain the bellicose temperament

and original phenotype of Aseel roosters. Based on this, the relationship between testosterone levels and spermatological characteristics in Aseel roosters may be investigated.

This study can be considered the very preliminary study on the Turkish Aseel rooster's semen and aims to determine the spermatological characteristics of the species. The results will contribute to further research on Aseel cocks and artificial insemination with fresh, chilled, or frozen semen. It also provides up-to-date data for breeders of this interesting bird species.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (E. Günay). The data are not publicly available due to privacy or ethical restrictions.

Ethical Approval

The study was conducted with the permission of Istanbul University-Cerrahpasa Animal Experiments Local Ethics Committee (IUCHADYEK) with the approval number 2022/41

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Conflict of Interest Statement

The authors declare that there is no conflict of interest in publishing this article.

Author Contributions

SA and KD conceived and supervised this study. EG, RA and HŞ completed the main experimental content. RA and AE collected and analyzed data. EG 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

Evaluation of the Apoptotic Effect of Sinapic Acid in D17 Canine Osteosarcoma Cell Line

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Abstract: Sinapic acid (SA), one of the hydroxycinnamic acid derivatives, has powerful antioxidant and anti-inflammatory characteristics. Moreover, SA has also been shown to have an apoptotic effect against various cancer cells. Here, we investigated the cytotoxic and apoptotic effects of sinapic acid on D17 canine osteosarcoma cell line. We measured the properties of SA on cell viability with the XTT test and found its IC_{50} dose at 750 µmol at 72 h. We analyzed the effects on gene expression of apoptosis pathways by qRT-PCR. qRT-PCR results revealed significant increases in the mRNA level expressions of BAX, CASP3, CASP7, CASP8, CASP9, FAS and P53; whereas, there was a statistically insignificant downregulation in CYCS level and increase in BCL2 level. Our findings show that SA can induce apoptosis in the D17 cell line.

Keywords: *Apoptosis, Canine osteosarcoma, Sinapic acid*

Sinapik Asidin D17 Köpek Osteosarkom Hücre Hattında Apoptotik Etkisinin Değerlendirilmesi

Öz: Hidroksisinnamik asit türevlerinden biri olan sinapik asit (SA), güçlü antioksidan ve antienflamatuar özelliklere sahiptir. Ayrıca sinapik asidin çeşitli kanser hücrelerine karşı apoptotik bir etkiye sahip olduğu da bilinmektedir. Bu çalışmada, sinapik asidin D17 köpek osteosarkom hücre hattı üzerindeki sitotoksik ve apoptotik etkileri araştırılmıştır. SA'nın hücre canlılığı üzerindeki özellikleri XTT testi ile belirlenmiş ve IC_{50} dozu 72 saatte 750 µmol olarak bulunmuştur. qRT-PZR ile apoptoz yolağında bulunan genlerin ekspresyonu üzerindeki etkileri analiz edilmiştir. qRT-PZR sonuçları; BAX, CASP3, CASP7, CASP8, CASP9, FAS ve P53'ün mRNA düzeyinde ifadelerinde anlamlı artış gözlenmiştir. CYCS düzeyinde istatistiksel olarak anlamsız bir baskılanma ve BCL2 düzeyinde ise istatistiksel olarak anlamlı olmayan bir artış tespit edilmiştir. Bulgular, SA'nın D17 hücre hattında apoptozu indükleyebileceğini göstermektedir.

Anahtar sözcükler: *Apoptozis, Köpek osteosarkomu, Sinapik asit*

Introduction

Osteosarcoma (OS) is one of the most common bone malignancy in both dogs and children having tendency for local invasion and pulmonary metastasis and cause death. Dogs oftentimes develop indigenous OS with higher prevalence comparing human. Both dog and human OS have similar aspects including clinical, pathophysiological and therapeutic management. Despite the treatments applied, the survival times have not been improved enough and dogs diagnosed with OS often die due to metastasis with in the first year [1-3].

Phenolic compounds have many positive effects on health because of their antioxidative and antibacterial properties [4]. Previous literature showed that these compounds exhibit anti-allergic, anti-inflammatory, antidiabetic, anti-microbial, antipathogenic and antiviral effects [5,6]. They are often defined as inhibitors and potential antioxidants of harmful oxidative processes associated with diseases

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including inflammation and cancer [7,8]. Sinapic acid (SA; 3,5-dimethoxy-4-hydroxycinnamic acid) exists in free or esters form. Hydroxynamic esters present as esters of sugar (glycosides) or other various organic compounds [9]. SA, as a phytochemical substance, originates from various plants including spices, citrus fruits, vegetables, cereals, oilseed crops and strawberry [5,10].

Oxidative stress is crucial for aging and physiopathology of several diseases such as cancer, atherosclerosis, diabetes and neurodegenerative disorders. It was stated that dietary antioxidants can protect against these oxidative stress related diseases [11,12]. Previous *in vitro* studies indicated that SA plays a role in cancer therapy. These anti-carcinogenic effects of SA are mainly attributed to its ability to remove free radicals and stimulation of cytoprotective enzymes [6,13-15]. The objective of this study was to evaluate anti-cancerogenic properties of SA in D17 canine osteosarcoma cell. For this purpose, the cytotoxic effect of SA in OS cells was determined and the expression levels of apoptosis related genes were investigated.

Material and Methods

Ethical Statement

Since this is an *in vitro* study and only cell line was used, there is no need for ethical approval.

Cell Line

D17 (ATCC® CRL-8468) osteosarcoma cell line was purchased from ATCC and were cultured in EMEM media containing 10% FBS, 1% L-glutamine, 100 IU/mL penicillin and 10 mg/mL streptomycin in a cell culture. The proliferation, passages and follow-up of the cells were monitored with an inverted microscope and the cells were maintained at 37°C in humidified atmosphere containing 5% CO_2.

Cytotoxicity Assay

In order to evaluate IC_{50} dose and cytotoxic effect of SA, XTT assay was used as previously described [8]. Briefly, approximately 1000 D17 cell/well were seeded in 96-well plate and treated with different doses of SA (250-4000 μmol) for 24, 48 and 72 h. After addition of XTT [2,3-bis (2-Methoxy-4-nitro-5-sulfophenyl) 2H tetrazolium-5-carboxanilide] solution, cell viability was determined with at 450 nm using a microplate reader.

RNA Isolation, cDNA Synthesis and qRT-PCR Analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, USA), and Transcriptor First Strand cDNA Synthesis Kit (BIO-RAD) was used for cDNA synthesis. The primer sequences of apoptosis pathway and housekeeping genes were presented in *Table 1*. A 20 μL qRT-PCR reaction mix was set up including 10 μL 2X SyberGreen Supermix, 5

Table 1. *Primers sequences used for qRT-PCR analysis*

Gene	Primer Sequence	PCR Product Size (bp)
BAX	F:5-AGCAAACTGGTGCTCAAGG-3 R:5-GTGTCCCAAAGTAGGAGAGGA-3	151
*BCL2**	F:5-GTGGATGACTGAGTACCTGAAC-3 R:5-GAGACAGCCAGGAGAAATCAA-3	125
CASP3	F:5-CTCGGTCTGGTACAGATGTAGA-3 R:5-GCTTAGAAGCACGCAAACAAA-3	173
CASP7	F:5-TTTGTGCAGGCCCTGTG-3 R:5-CACATGGGATCTGCTTCTTCTC-3	150
CASP8	F:5-CTGACCTCTTACTTCACTGGTTC-3 R:5-GGACATCTTTCTCTTAGGCTCTG-3	296
CASP9	F:5-GGAAGCCCAAGCTCTTCTTTA-3 R:5-GGAGTGGGCAAACTAGACAC-3	184
CYCS	F:5-AAAGGGAGGCAAGCACAA-3 R:5-GGGATTCTCCAAATACTCCATCA-3	150
FAS	F:5-GCATGGCTTAGAAGTGGAAAG-3 R:5-CTCAAGGATTCCATGTTCACAC-3	136
P53	F:5-TGAGGAGGAGAATTTCCACAAG-3 R:5-TCAGCTCCAAGGCTTCATTC-3	140
*RPL32**	F:5-GGCACCAGTCAGACCGATATG-3 R:5-TGCGCACCCTATTGTCAATG-3	75
*YWHAZ**	F:5-TGTAGGAGCCCGTAGGTCATCT-3 R:5-TTCTCTCTGTATTCTCGAGCCATCT-3	102

**BCL2 [16], RPL32 [17] and YWHAZ [17] primers were obtained from previous literature. Other primers were designed in this study*

pmol of each primer and 2 μL cDNA. PCR amplifications were carried out with an initial denaturation at 95°C for 10 min, then 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. All resulting PCR products were evaluated using melting curve analysis and agarose gel electrophoresis.

Statistical Analysis

qRT-PCR data were normalized using Ct values of housekeeping genes (RPL32 and YWHAZ) and $2^{-\Delta\Delta Ct}$ values were calculated to compare fold change expression levels of control and treatment groups. Student *t*-test was used to assess statistically significant differences.

Results

Cytotoxic Effect of SA in D17 Cells

XTT assay was used to evaluate the ability of SA to inhibit the proliferation of D17 cells. *Fig. 1* illustrates that SA exhibited time- and concentration-dependent manner cytotoxic effect in osteosarcoma cells. The IC_{50} of SA in D17 cells was found to be 750 μmol for 72 h. Thus, dose of 750 μmol was used for in D17 cell in the following experiments.

Semiquantitative Real-Time PCR Analysis

Steady-state level expressions of apoptosis genes were evaluated using semiquantitative real time PCR in 750 μmol SA treated D17 cells and compared to the control. SA treatment in D17 cells resulted in the upregulated expressions of CASP3, CAS7, CASP8, CASP9, BAX, FAS and P53 (P<0.05). Increased BCL2 and downregulated CYCS expressions were observed but these differences were not statistically significant (*Fig. 2*, P>0.05).

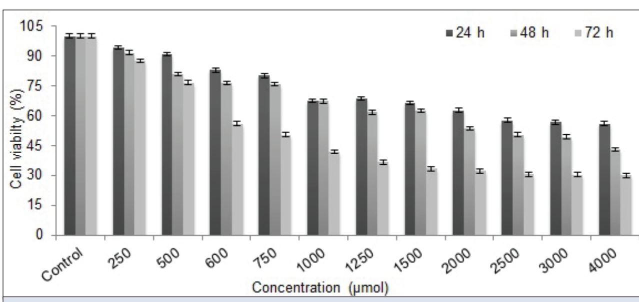

Fig 1. Effect of SA on the viability in the D17 cell. The cells were treated with SA and at different concentrations and time intervals and anti-proliferative effect was assessed by XTT assay. IC_{50} dose of SA in D17 cell line was found to be 750 μmol

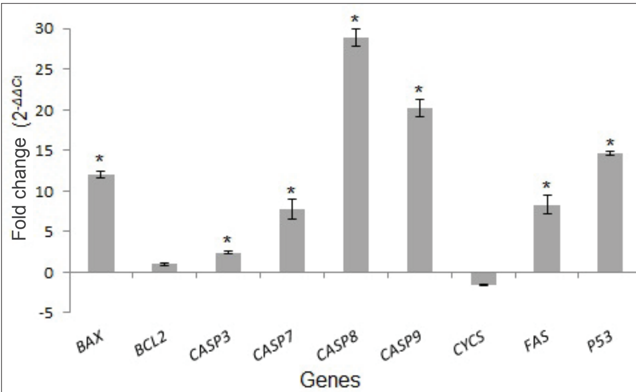

Fig 2. The changes in expression of genes that play an important role in apoptosis relative to the control group after SA treatment in D17 cell. * indicates statistically important (P<0.05) difference

Discussion

Using raw estimates of cancer incidence, 6 million new dog cancers cases are thought to be diagnosed each year [18] including lymphoma, adenocarcinoma, squamous cell carcinoma, osteosarcoma, mucosal melanoma, urothelial carcinoma and mast cell tumors [19,20]. These malignancies show strong similarities to human cancers, including histopathology, molecular mechanisms and therapy protocols [21]. According to the bone tumor database, OS is responsible for 98% of 1273 appendicular primary bone tumors in dogs [22]. Along with advancing science and technology, studies have focused on exploring complementary therapies as well as classical therapies in the fight against cancer. Regular consumption of whole grain products, which are natural antioxidant sources, reduces the risk of many chronic diseases such as diabetes, cardiovascular diseases and cancer [8].

In this study, the apoptotic effects of sinapic acid, which is known to have high antioxidative capacity and abundant in all cereal products, fruits and vegetables, in D17 canine osteosarcoma cell line were investigated. IC_{50} dose was determined by XTT test. Then qRT-PCR analysis of 9 different genes was performed to investigate the apoptotic effect of sinapic acid at the determined dose. Also, melting curve analysis was conducted and the resulting PCR products were electrophoresed using agarose gel.

In a previous study, the apoptotic effect of SA in PC-3 and LNCaP human prostate cancer cell lines was investigated. According to the XTT test results, the best half maximal inhibitory dose (IC_{50}) was 1000 μmol at 72 h [8]. In a previous literature, the effect of phenolic acids on LDL oxidation was investigated. It was reported that caffeic acid protects LDL against oxidation at 5 μmol (IC_{50} dose) and sinapic acid at 10 μmol (IC_{50} dose), preventing both hydroperoxide formation increasing apoprotein negative charge [23,24]. Liang et al.[25] examined the effect of gallic acid, which is defined as the main active fraction in herbal medicinal plants, on the growth inhibition of various cancer cells. According to the study, *in vitro* and *in vivo* anti-cancer properties of gallic acid on two human osteosarcoma cell lines were confirmed. It was stated that gallic acid induced apoptosis depending on the dose and time manner in human osteosarcoma cells. Walters et al.[26] investigated the anti-cancer effects of curcumin, another phenolic compound, against OS cells using vitality and apoptosis assays. It was reported that curcumin stimulates

apoptosis in OS cells [26,27]. It was showed that canola phenolic acid, orange peel extract (O-PMF) and O-PMF + Limonoid (1: 1) have the potential to inhibit OS growth in dogs [28]. In the present study, findings illustrated that SA can also inhibit D17 canine OS growth.

One of the most important tumor suppressor genes in the apoptosis pathway is P53. P53 plays a negative regulator role for cellular proliferation and suppresses cell growth and transformation [29]. Cell cycle and DNA replication were impaired in P53 loss of function. If this disruption cannot be repaired, it is resulted in increased BAX, FAS and APAF-1 expression and suppressed BCL2 and BCL-XL expression, thereby inducing apoptosis [30]. P53 mutations have involved pathogenesis and development of different cancers. Johnson et al.[31] reported that the P53 gene plays an important role in the generation of OS as a potential determinant of clinical prognosis, therapeutic response and therapy options. In the present study, it was observed that P53 level is elevated and promotes the cancerous cell to apoptosis.

Mainly, two molecular signal pathways lead to apoptotic cell death. The first, intrinsic pathway is associated with mitochondria. The second, extrinsic pathway involves binding of proapoptotic ligands to cell surface death receptors (DRs). Both initiates the enzymatic caspase cascade [32]. Wang and Youle, emphasized that levels of anti-apoptotic genes such as BCL2 should be decreased; however, proapoptotic genes for instance BAX should be increased in the mitochondrial membrane of cells undergoing apoptosis [33]. Eroğlu et al.[8] conducted qRT-PCR analysis to evaluate the effect of SA on the PC-3 and LNCaP human prostate cancer cell lines. According to the findings, it was stated that the upregulated CASP3, CASP7, BAX and CYCS expressions were statistically significant [8]. In the present study, the effects of sinapic acid on apoptotic and anti-apoptotic genes in D17 cells were evaluated at the mRNA level. Significant increases were observed in BAX (12-fold, P=0.000007), CASP3 (2.44-fold, P=0.0021), CASP7 (7.81-fold, P=0.0020), CASP8 (28.94-fold, P=0.0001), CASP9 (20.25-fold, P=0.00007), FAS (8.28-fold, P=0.0005) and P53 (14.62-fold, P=0.00001). Moreover, an increase in BCL2 (P=0.97) and a decrease in CYCS expression level (P=0.509) were found to be statistically insignificant.

In conclusion, it has been observed that SA, which is known to have strong antioxidative activity, shows apoptotic effect in D17 canine osteosarcoma cell line. Thus, SA have potential to act as an anti-cancer agent in canine osteosarcoma cells by showing anti-proliferative and apoptotic effects.

Availability of Data and Materials

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

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

ZB and HK designed the project. HK and CEG carried experiments. HK, CEG, MN, EK and ZB performed statistical analysis of data and wrote the article.

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RESEARCH ARTICLE

Protective Effects of 3-n-butylphthalide on Cerebral Infarction Induced by Local Ischemic Injury and Regulation Mechanism of the PI3K/Akt/GSK-3β Signaling Pathway

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Abstract: We aimed to study the protective effects of 3-n-butylphthalide (NBP) on cerebral infarction induced by local ischemic injury and regulation mechanism of the PI3K/Akt/GSK-3β signaling pathway. One hundred male Wistar rats aged 12-15 weeks were randomly divided into 5 groups (n=20). The middle cerebral artery occlusion (MCAO) model was established. NBP, P13K specific inhibitor LY294002 and NBP plus LY294002 groups were intraperitoneally administered on the first day after modeling, once a day for 7 days. Sham operation (Sham) and model groups were intraperitoneally given equal amounts of normal saline. Neuronal damage was detected by Nissl staining. Intact neurons were counted under light microscope. The protein expressions of Akt, P-Akt, GSK-3β and P-GSK-3β were detected by Western blotting. The mNS score of NBP group decreased significantly compared with that of model group ($P<0.05$). Compared with model group, the cerebral infarction volume of NBP group significantly reduced ($P<0.05$). Compared with model group, the number of intact neurons in NBP group significantly increased ($P<0.05$). Compared with model group, the phosphorylation levels of Akt and GSK-3β in NBP group significantly increased ($P<0.05$). By activating the PI3K/Akt/GSK-3β signaling pathway, NBP relieves neurological function damage and protects against cerebral infarction induced by local ischemic injury.

Keywords: *Butylphthalide, PI3K, Akt, GSK-3β, Apoptosis*

3-N-Butilftalidinin Lokal İskemik Hasarla İndüklenen Serebral Enfarktüs Üzerine Koruyucu Etkileri ve PI3K/Akt/GSK-3β Sinyal Yolunu Düzenleme Mekanizması

Öz: Bu çalışmada, 3-n-butilftalidinin (NBP) lokal iskemik hasarın neden olduğu serebral enfarktüs üzerine koruyucu etkileri ve PI3K/Akt/GSK-3β sinyal yolağını düzenlenme mekanizması incelenmiştir. 12-15 haftalık 100 erkek Wistar sıçan rastgele 5 gruba ayrıldı (n=20). Orta serebral arter oklüzyonu (MCAO) modeli oluşturuldu. NBP, P13K spesifik inhibitörü LY294002 ve NBP artı LY294002, modellemeden sonraki gün başlamak üzere ilgili gruplara 7 gün boyunca günde bir kez intraperitoneal olarak uygulandı. Sham ve model gruplarına intraperitoneal olarak eşit miktarda normal salin verildi. Nöronal hasar Nissl boyama ile tespit edildi. Sağlam nöronlar ışık mikroskobu altında sayıldı. Akt, P-Akt, GSK-3β ve P-GSK-3β protein ekspresyonları Western blotlama ile tespit edildi. NBP grubunun mNS skoru model grubuna kıyasla önemli ölçüde azalmıştı ($P<0.05$). Model grupla karşılaştırıldığında, NBP grubunun serebral enfarktüs hacmi önemli ölçüde azalmıştı ($P<0.05$). Model grupla karşılaştırıldığında, NBP grubundaki sağlam nöron sayısı önemli ölçüde artmıştı ($P<0.05$). Model grupla karşılaştırıldığında, NBP grubunda Akt ve GSK-3β fosforilasyon seviyeleri önemli ölçüde artmıştı ($P<0.05$). NBP, PI3K/Akt/GSK-3β sinyal yolunu aktive ederek nörolojik fonksiyon hasarını hafifletmekte ve lokal iskemik hasarın neden olduğu serebral enfarktüse karşı koruma sağlamaktadır.

Anahtar Sözcükler: *Butilftalid, PI3K, Akt, GSK-3β, Apoptozis*

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Introduction

Ischemic cerebral infarction is a clinically common central nervous system disease with high mortality and disability rates, which seriously threatens human health and quality of life [1]. With a complicated pathophysiological process, this disease is a blood flow disorder in the brain that is caused by various factors, leading to ischemia and hypoxia, apoptosis of neurons, functional damage of tissues and cells, as well as neurological dysfunction such as movement, language, sensation and memory [2]. At present, cerebral infarction is mostly treated by vascular recanalization in time, including thrombolysis and mechanical thrombectomy. Meanwhile, this therapy is usually combined with supportive treatments such as anticoagulation, reduction of intracranial pressure and nourishment of cranial nerves, which can relieve brain tissue damage and improve the prognosis. However, some cases still suffer from different degrees of hemiplegia, cognitive impairment, aphasia and swallowing dysfunction [3,4].

3-n-Butylphthalide (NBP) has significant therapeutic effects on the neurological function of patients with ischemic cerebral infarction [5]. It can enhance the blood circulation in the brain and prevent cerebral infarction caused by ischemia [6]. Meanwhile, NBP improves the energy metabolism in ischemic area and participates in the inhibition of neuronal apoptosis [7]. During cell apoptosis, the PI3K/Akt signaling pathway plays a key role in the regulation of related proteins, allowing membrane receptor signal to be transduced to cells for maintaining cell proliferation and inhibiting apoptosis [8]. As a substrate for Akt, GSK-3β is a key component regulating cell apoptosis [9]. The mechanism by which NBP protects against cerebral infarction induced by local ischemic injury by regulating the PI3K/Akt/GSK-3β signaling pathway remains unclear.

Therefore, we herein established a rat model of middle cerebral artery occlusion (MCAO) to assess the protective effects of NBP on cerebral infarction induced by local ischemic injury, and the regulation of the PI3K/Akt/GSK-3β signaling pathway. The findings provide valuable experimental evidence for clinical practice.

Material and Methods

Experimental Animals, Reagents and Apparatus

This study has been approved by the animal ethic committee of our hospital (approval No. TTH202001004), and great efforts have been made to minimize the animals' suffering. SPF-grade healthy Wistar male rats aged 12-15 weeks and weighing 200-220 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China). They were kept in an animal breeding room with constant temperature and humidity, and adaptive feeding was carried out with a 12 h/12 h light/dark cycle. All experiments were in line with the 3R principle.

NBP was bought from Shijiazhuang Pharmaceutical Group NBP Pharmaceutical Co., Ltd. (China). LY294002 was obtained from Sigma (USA). BCA protein concentration detection kit, DAB chemiluminescence kit, and antibodies against Akt, P-Akt, GSK-3β and P-GSK-3β were provided by Shanghai Renjie Biotechnology Co., Ltd. (China). Magnetic resonance imaging (MRI) scanner was purchased from Siemens (Germany). Gel imaging system was bought from Bio-Rad (USA). BXM-950 optical microscope was obtained from Shanghai Bingyu Optical Instrument Co., Ltd. (China).

Model Establishment and Grouping

One hundred rats were numbered and then randomly divided into 5 groups (n=20): sham operation (Sham) group, model group, NBP (10 mg/kg) group, P13K specific inhibitor LY294002 (LY, 10 mg/kg) group and NBP (10 mg/kg) plus LY294002 (10 mg/kg) (NBP + LY) group. Model establishment: Under anesthesia, a nylon thread with 0.285 mm diameter was used to occlude blood flow in the anterior segment of MCA and its lateral branch for 2 h, causing local MCA ischemia without affecting blood flow in the anterior cerebral artery [10]. After the nylon thread was withdrawn and the MCA blood flow was restored, the rat MCAO model was successfully established. For the Sham group, only vascular ligation or occlusion was not performed. Each group was intraperitoneally injected with corresponding drugs on the first day after modeling, once a day for 7 days. The same amounts of normal saline were intraperitoneally given to the Sham and model groups.

Scoring of Modified Neurological Severity (mNS)

Thirty minutes after administration on the 7th day, the rat behaviors were evaluated by mNS scoring according to the criteria below. 0 point: Rat crawls normally without asymmetric movement; 1 point: forelimb or hind limbs are bent when the tail is lifted vertically; 2 points: rat fails to walk straight based on the criterion for 1 point; 3 points: rat circles leftward while crawling; 4 points: rat falls down leftward during free movement; 5 points: rat drags its left forepaw backward based on the criterion for of 4 points; 6 points: rat fails to support its body or to crawl by itself [11].

Measurement of Cerebral Infarction Volume by MRI

After mNS scoring, 6 rats were randomly selected. The cerebral infarction volume was detected by MRI scanner. The rats were placed in the supine position. After the standard axial position was found, the coronal plane was subjected to three-layer scan based on cross-sectional T2-weighted imaging, with a layer thickness of 1.5 mm and a spacing of 0.2 mm. T2-weighted imaging was carried out to measure the volume of cerebral infarction. The infarct

area was pale white, and the normal brain tissue area was gray. Cerebral infarction volume (%) = (infarct volume/ volume of whole brain tissue) x 100%. The calculation was conducted using ImageJ software [12].

Detection of Neurons in Brain Tissue by Nissl Staining

After mNS scoring, 6 rats were randomly selected. Under anesthesia, the chest and abdomen were incised, and the exposed left ventricle was intubated. 4% Paraformaldehyde was perfused until the heart turned white. Then the brain tissue was taken out, placed on ice, immediately fixed with 4% paraformaldehyde, dehydrated under vacuum conditions with gradient concentrations of ethanol solutions, transparentized with xylene and paraffin-embedded. The treated brain tissue was thereafter sliced into 5 μm-thick coronal sections that were subjected to Nissl staining. Six different visual fields were observed under an optical microscope at the magnification of 400x, and images were processed by Image-Pro 6.2 software to count intact neurons in the cerebral cortex on the ischemic side [13].

Detection of Akt, P-Akt, GSK- 3β and P-GSK-3β Protein Expressions by Western Blotting

After mNS scoring, 6 rats were randomly selected. Under anesthesia, they were killed by cervical dislocation, from which the skull was rapidly disconnected to collect brain tissue on the ischemic side. Subsequently, the cerebral cortex was separated in an ice bath and stored in liquid nitrogen. The brain tissue was homogenized with RIPA lysis buffer, left still in ice bath for 5 min and centrifuged at 13000 rpm and 4°C for 10 min to collect the supernatant. Protein concentration in the supernatant was measured by BCA protein quantification kit. Afterwards, protein samples were resolved by 10% SDS-PAGE, and the product was electronically transferred onto a PVDF membrane. Then the membrane was blocked by 5% TBST at room temperature, incubated overnight with primary antibodies (1:1000 diluted) at 4°C, washed by TBST for 10 min, incubated with HRP-labeled goat anti-rabbit IgG secondary antibody (1:10000 diluted) at room temperature for 2 h, developed by DAB solution and observed by gel imaging analyzer. The relative expression levels of target proteins were detected by using GAPDH as the internal reference.

Statistical Analysis

All data were analyzed by SPSS16.0 software. The categorical data conforming to normal distribution were expressed as mean ± standard deviation. Multigroup comparisons were performed by one-way analysis of variance, and pairwise comparisons were conducted with the independent t test. P<0.05 was considered statistically significant.

Results

Compared with the Sham group, the mNS score of the model group was significantly higher (P<0.05). After NBP treatment, the mNS score of the NBP group decreased significantly compared with that of the model group (P<0.05), but the scores of LY and NBP + LY groups were similar to that of the model group (P>0.05) *(Fig. 1)*, indicating that LY294002 blocked NBP from improving the neurological function of MCAO rats.

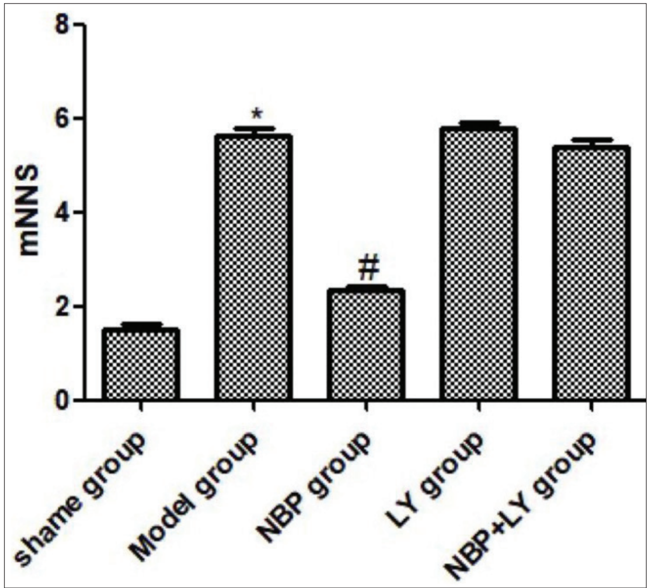

Fig 1. Effects of NBP on neurological function of MCAO rats. *Compared with Sham group, P<0.05; #compared with model group, P<0.05

No infarct area was found in the brain tissue of the Sham group. Compared with the Sham group, the cerebral infarction volume of the model group significantly increased (P<0.05). Compared with the model group, the cerebral infarction volume of the NBP group significantly reduced (P<0.05), whereas the volumes of LY and NBP + LY groups were not significantly different (P>0.05) *(Fig. 2)*, suggesting that LY294002 inhibited NBP from repairing ischemic cerebral injury.

The Sham group had normal neuron morphology. Compared with the Sham group, the model group had abnormal structures of neurons and significantly decreased number of intact neurons (P<0.05). Compared with the model group, the number of intact neurons in the brain tissue of the NBP group significantly increased (P<0.05), but the numbers of LY and NBP + LY groups were not significantly different (P>0.05) *(Fig. 3)*, indicating that LY294002 suppressed NBP from protecting the neurons undergoing ischemic brain injury.

Western blotting showed that compared with the Sham group, the phosphorylation levels of Akt and GSK-3β in the

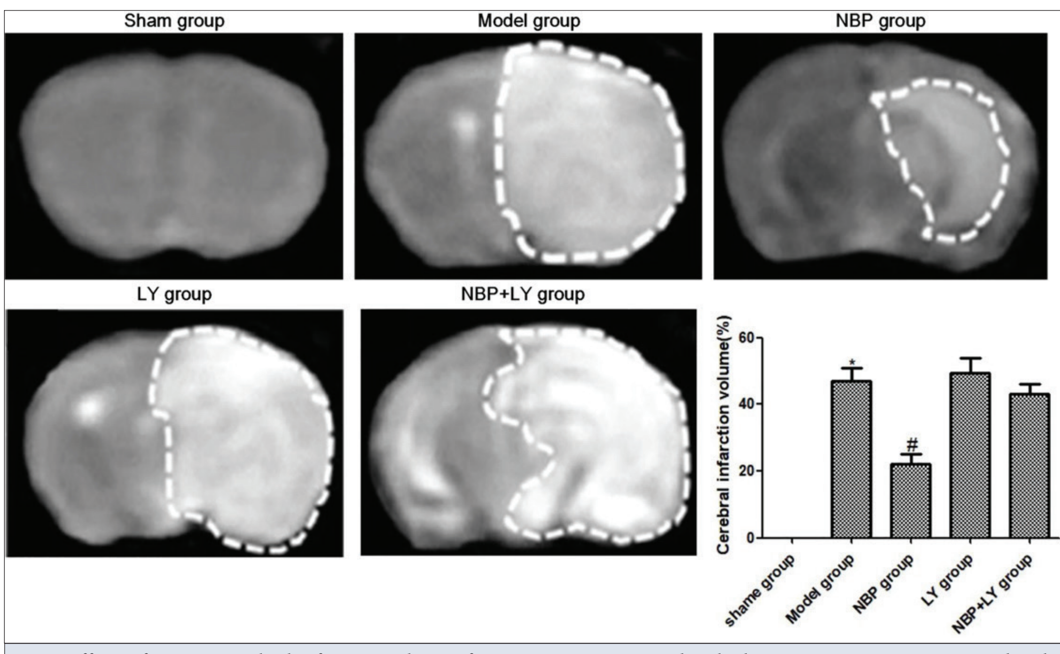

Fig 2. Effects of NBP on cerebral infarction volume of MCAO rats. *Compared with Sham group, P<0.05; #compared with model group, P<0.05

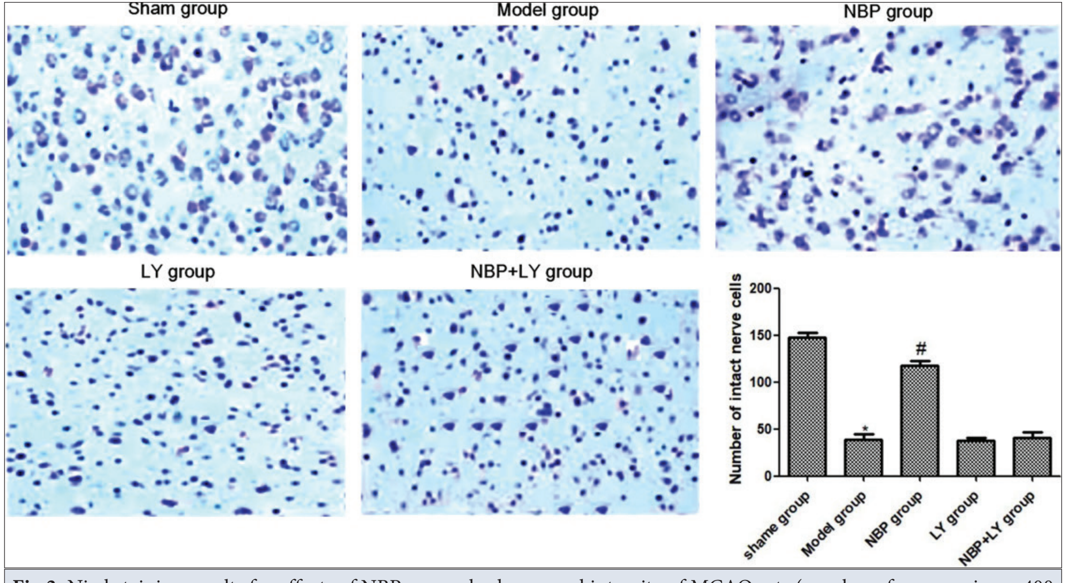

Fig 3. Nissl staining results for effects of NBP on cerebral neuronal integrity of MCAO rats (number of neurons in a ×400 visual field). *Compared with Sham group, P<0.05; #compared with model group, P<0.05

model group significantly decreased (P<0.05). Compared with the model group, such levels in the brain tissue of the NBP group significantly increased (P<0.05), whereas the levels of LY and NBP + LY groups were not significantly different (P>0.05) (*Fig. 4*), revealing that LY294002 counteracted the therapeutic effects of NBP by decreasing the levels of in brain tissues with ischemic injury.

Discussion

The impairment of brain function has serious adverse effects on physiological activities [14]. Particularly, ischemic cerebral infarction poses a great threat to human life [15]. NBP has been widely used as a therapeutic drug for ischemic cerebrovascular disease in clinical practice, with remarkable effects [16]. In this study, a MCAO model was first established, and then the concentration of butylphthalide was selected based on preliminary experiments. Compared with the model group, NBP-treated rats had significantly reduced volume of cerebral infarction, alleviated neurological damage and decreased number of apoptotic neurons, verifying that cerebral ischemic injury was alleviated. The results are consistent

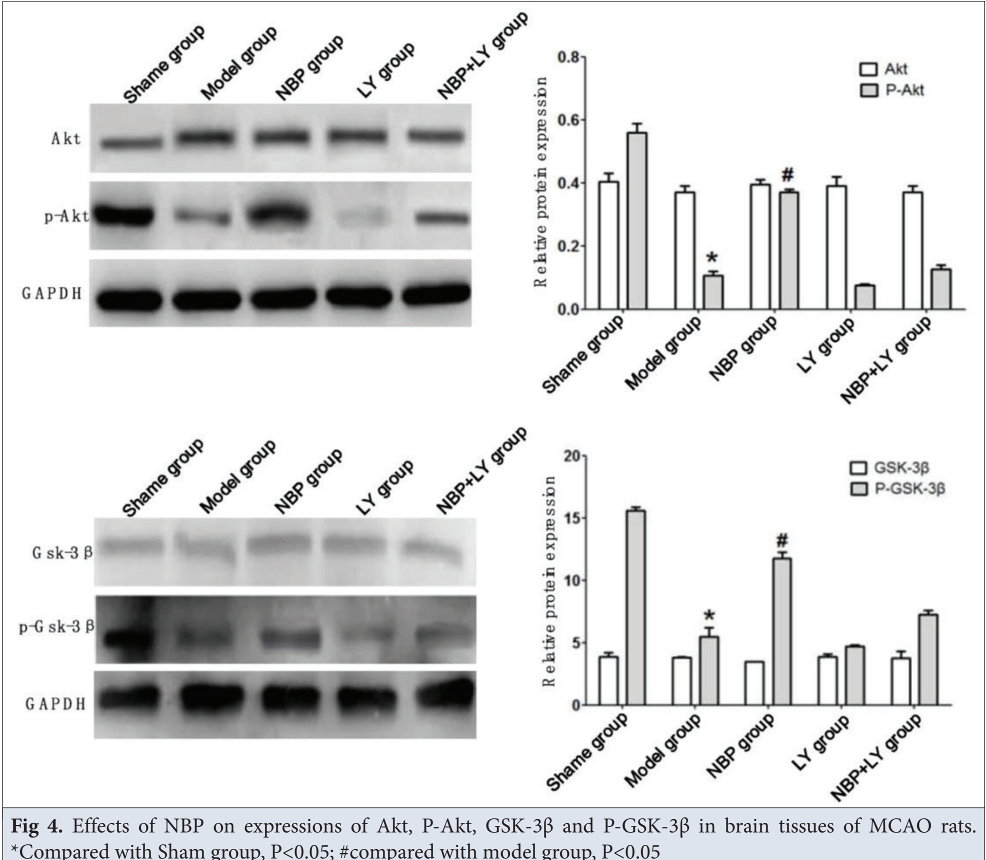

Fig 4. Effects of NBP on expressions of Akt, P-Akt, GSK-3β and P-GSK-3β in brain tissues of MCAO rats. *Compared with Sham group, P<0.05; #compared with model group, P<0.05

with a previous literature [17], confirming the therapeutic effects of NBP again.

The PI3K/Akt signaling pathway plays crucial roles in cell migration, mobilization, differentiation and apoptosis resistance [18]. As a key target protein, PI3K promotes the phosphorylation of GSK-3β in downstream pathways by phosphorylating Akt through PDK1 [19]. Subsequently, activated GSK-3β inhibits cellular oxidative stress and inflammatory response, and also predominantly resists apoptosis [20]. Many kinds of herbal and insect medicine have been reported to mitigate ischemic stroke-induced damage via the PI3K/Akt signaling pathway [21]. By enhancing the activity of mitochondrial ATPase, NBP protects mitochondria from ischemic damage, thereby suppressing cell apoptosis [22]. In addition, NBP may increase the expressions of VEGF and bFGF by acting on the ischemic site of the brain to protect against damage [23]. However, the mechanism remains largely unknown.

As a PI3K inhibitor, LY294002 specifically inhibits the activity of PI3K110 subunit and blocks PI3K-mediated signaling pathway [24]. In this study, LY294002 was used to clarify the mechanism by which NBP regulated the PI3K/Akt/GSK3β signaling pathway to protect against local brain injury. The phosphorylation levels of Akt and GSK-3β were significantly augmented in the NBP group compared with those of the model group, and the levels of the LY+ NBP group decreased significantly compared with those of the NBP group. Accordingly, LY294002 reversed NBP-mediated phosphorylation of Akt and GSK3β, indicating that NBP allowed Akt and GSK3β phosphorylation through PI3K [25].

It has previously been reported that the PI3K/Akt/GSK-3β signaling pathway inhibited cell apoptosis after reperfusion injury in the brain, and then participated in the repair process, playing a protective role [26]. Likewise, we herein found that compared with the NBP group, the cerebral infarction volume of the LY + NBP group was significantly enlarged, neurological damage was aggravated, and the number of apoptotic neurons was increased. Hence, NBP may protect against cerebral ischemic injury by regulating the PI3K/Akt/GSK-3β signaling pathway.

In summary, by activating the PI3K/Akt/GSK-3β signaling pathway, NBP relieves neurological function damage and protects against cerebral infarction induced by local ischemic injury. Nevertheless, whether other pathways are involved still needs further in-depth studies.

Availability of Data and Materials

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

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This study was not financially supported.

Conflict of Interest

There is no conflict of interest.

Authors' Contributions

KL designed this study, prepared this manuscript, analyzed experimental data; YC analyzed experimental data, manuscript writing.

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RESEARCH ARTICLE

Expression Profile of Prostaglandin Enzymes in Cystic Endometrial Hyperplasia in Dogs: The Results of a Hypothesis in Clinical Trial

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Abstract: The expressions of prostaglandin synthesis enzymes and estrogen, progesterone receptors in canine cystic endometrial hyperplasia (CEH) were reported in this manuscript. Uterine tissue samples were collected from bitches with CEH (n=5), CEH-P (Cystic endometrial hyperplasia-Pyometra) (n=5) and healthy-negative control group, CG (n=5). Immunohistochemistry was carried out for the estrogen (ER) and progesterone receptor (PR) detection. Shock-frozen samples were utilized in mRNA extraction and Real-Time PCR was performed. Gene expression of $PTGS_2/COX_2$, PTGES, PTGER4, PGFS, PTGFR and PGR were detected higher in the CEH group compared with CG. The PGFS and PTGFR (FP) mRNA expressions were significantly increased in CEH compared with other groups. Expression of progesterone receptor mRNA (PGR) was highest in CEH and statistically different from the CEH-P group ($P<0.05$). No PR immunostaining was observed. ER staining had been detected in endometrial glands, endometrial stoma and myometrium, however hyperplasic glands in propria mucosa had lower or no ER scores. Based on the results of this study, the high levels of prostaglandin enzymes and low ER scores in CEH could be a preliminary step for the next stages of severe differentiation of endometrium.

Keywords: *Canine, Cystic endometrial hyperplasia, Estrogen receptor, Prostaglandin*

Köpeklerde Kistik Endometriyal Hiperplazide Prostaglandin Enzimlerinin Ekspresyon Profili: Klinik Deneylerde Bir Hipotezin Sonuçları

Öz: Bu çalışmada köpek kistik endometriyal hiperplazi (KEH)'de prostaglandin sentez enzimleri, östrojen ve progesterone reseptörlerinin ekspresyonları rapor edilmiştir. Uterus örnekleri KEH (n=5) ve KEH-P (Kistik endomteriyal hiperplazi- pyometra) (n=5)'lı köpeklerden ve sağlıklı negatif kontrol köpeklerinden CG (n=5) toplanmıştır. Östrojen (ER) ve progesterone (PR) reseptörü belirlenmesi için immunohistokimya kullanılmıştır. Şok dondurulan doku örnekleri mRNA ekstraksiyonunda kullanılmış ve bu örneklerden Real Time PCR yapılmıştır. $PTGS_2/COX_2$, PTGES, PTGER4, PGFS, PTGFR ve PGR gen ekspresyonu KEH'de, kontrol grubuna göre yüksek bulunmuştur. PGFS ve PTGFR (FP) mRNA ekspresyonları KEH grubunda diğer gruplara göre istatistiksel olarak farklı bulunmuştur. Progesterone reseptor mRNA (PGR) ekspresyonu KEH'de en yüksek ve KEH-P grubuna göre farklı bulunmuştur ($P<0.05$). PR immun boyamada belirlenememiştir. ER boyanmaları ise endometriyal bezler, endometriyal stroma ve miyometriyumda gözlenirken, proprio mukozadaki hiperplastik bezlerde ya çok az boyanmış ya da hiç boyanma gözlenmemiştir. Bu çalışmanın sonuçlarına dayanarak, CEH'deki yüksek prostaglandin enzim seviyeleri ve düşük ER skorları, endometriumun şiddetli farklılaşmasının sonraki aşamaları için öncü bir adım olabilir.

Anahtar Sözcükler: *Köpek, Kistik endometriyal hiperplazi, Östrojen reseptör, Prostaglandin*

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Introduction

Cystic endometrial hyperplasia (CEH), is an important uterine pathology of non-castrated female dogs. The degenerative changes within the uterus could be life-threatening when CEH is accompanied by opportunistic pathogens [1]. The development of CEH has been considered the initial phase of pyometra and these morphological changes could be mostly diagnosed with pyometra [2]. Repeated endocrinological alterations, bacteriologic toxic factors (mainly *E. coli),* and the application of medroxy-progesterone acetate and tamoxifen which could be the consequence of altered receptor expressions in the uterus, are the underlying causes of the disease [1,3-5]. However, CEH develops in all older bitches some of which could progress to pyometra. As CEH does not inevitably progress to pyometra, CEH and pyometra could originate independently from each other [2,3].

The substantial changes resulting in CEH are widely accepted as the association of estrogen/progesterone imbalance on endometrium during estrus cycles in bitches [1]. However, various applications during diestrus have been reported to cause CEH such as intrauterine inoculation of *Escherichia coli* and intraluminal insertion of a wire [6,7]. Either way, the initiation of cellular changes is induced by infectious agents and/or foreign bodies in the uterus in diestrus. On the other hand, alterations in steroid hormone concentrations during estrus cycle could influence uterine immune surveillance in bitches [8,9]. The control of this process is achieved by prostaglandins [10,11]. Because, irreversible tissue differentiation is accepted that they are acting as wounds that fail to heal [12]. Apart from this information, a direct relationship between the levels of PG synthesis and the incidence of malignancy has been reported in human and animal models [13].

Cyclooxygenase (COX_2-PTGS2) is a rate-limiting enzyme that mediates the conversion of arachidonic acid to prostaglandins [14]. PGE_2 is an important COX protein product that is synthesized by specific synthases (PGESs). COX_2 and PGE_2 contribute not only to physiological processes but also to inflammation and oncogenesis [14-16]. PGE_2 exerts its cellular effects by interacting with EP receptors (EP1, EP2, EP3, or EP4), which are coupled to G proteins [17]. The interaction between PGE_2 and its EP receptors plays important roles in differentiation and progression in cells by complex regulation of signaling transduction [18]. Based on the close relationship between chronic inflammation and endometrial cancers, the central inflammatory pathway involved in carcinogenesis is known as cyclooxygenase 2-prostaglandin E_2-prostaglandin E_2 receptors (COX-2-PGE_2-EPs) [19].

In the light of the detailed information on the relation between malignancy and prostaglandin synthesis in humans [19], the possible transcription pattern of genes encoding prostaglandin synthesis in cystic endometrial hyperplasia has been studied. Endometrial hyperplasia is a precursor lesion for endometrial carcinoma in women [20]. Although cystic endometrial hyperplasia is widely not accepted as a malignancy of bitches, the non-infected CEH cases were evaluated in terms of prostaglandin synthesis. The hypothesis of this study is based on the determination of levels of genes encoding prostaglandin enzymes in cystic endometrial hyperplasia in the dog. The high levels of prostaglandins in non-infected CEH cases could be a precursor indicator for severe differentiation of cells. Although endometrial adenocarcinoma is a rare tumor in bitches and queens [21], a case of adenocarcinoma with cystic endometrial hyperplasia-pyometra complex in a dog and a cat were reported [22,23]. Owing to the information about the diagnosis of cystic endometrial hyperplasia, pyometra, and uterine neoplasia in a bitch, the results of the hypothesis of this study could provide new information on canine uterine disorders.

Material and Methods

Ethical Statement

Approval from the ethics committee of the Bursa Uludag University to use the animals was obtained (Approval no: 2019-09/01).

Animals

Fifteen bitches presented to the Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine in Bursa, were used in this study. All dogs had been brought to the clinic, either for purpose of spaying or with a history of pyometra. The clinic, ultrasound examinations were carried out and blood samples were collected for hematological and biochemical analysis before operations. Ovariohysterectomy was performed in bitches aged 2-10 years. They were allocated into groups after a histopathological examination of the uterus and diagnosis of canine CEH-P and CEH by two different pathologists. The classification of groups has been proposed according to Dow's morphology criteria, histological lesion and De Bosschere's histomorphological classification criteria *(Table 1)*.

No pus and/or mucus was observed either in macroscopic or histopathological examination of groups CG ad CEH. On the other hand, no signs of inflammation, including neutrophil leukocytes were observed during the histopathological examinations in these groups.

The patients were also evaluated clinically by means of the type of pyometra. The animals with open cervix pyometra were included in the study. Based on the information on last estrus of patients, obtained in anamnesis, all animals in three groups were in late diestrus [26]. Since

Table 1. Classification of groups

Groups	Dow's CEH Types Classification *	Endometrial Hyperplasia Index**	De Bosschere's Histomorphological Classification***
Cystic Endometrial Hyperplasia-Pyometra (CEH-P) Group (n=5; with mean age of 5.4±2.3 years)	Type IV Severe clinical signs of abdominal distention, damage in other abdominal organs with abnormal blood hematology and biochemistry	Grade 3 Severe hyperplastic and cystic changes with increased endometrial thickness of <2 times normal	Pyometra (hyperplastic)
Cystic Endometrial Hyperplasia (CEH) Group (n=5; with a mean age of 8.8±0.83)	Type I presenting signs of CEH without inflammatory process and no clinical signs	Grade 2 Hyperplastic and cystic change with an increased endometrial thickness of <2 times normal	Severe CEH
Control Group (n=5; with mean age of 3.0±0.70)	-	Grade 0 No uterine abnormalities and hyperplastic changes	Normal

*Type of CEH classification according to Dow [24]; ** endometrial hyperplasia index according to Munson et al.[25] and *** histomorphological classification according to De Bosschere et al.[3]

the animals in the CEH-P group were processed in late diestrus (52.00±15.68 days), the scheduled operations were submitted for the bitches in CEH and CG groups in the second stage of diestrus (49.80±16.22; 45.00±7.90 days, resp), as described by Veiga et al.[26]. The results of exfoliative vaginal cytology and the existence of CLs in CG and CEH groups were also recorded. CEH-P was diagnosed after the determination of leukocytosis, increased ALT, AST, urea and creatinine concentrations, including abnormal clinical signs such as inappetence, depression, polyuria and polydipsia. The fluid filled uterus was observed by transabdominal ultrasonography before operations.

Surgical Procedures

The bitches were premedicated with the administration of intramuscular xylazine HCl (1 mg/kg) (Alfazyn® 2%; Egevet/Turkey) and for induction 10 mg/kg ketamine HCl (Alfamin®, Egevet, Turkey) was applied intramuscularly. General anaesthesia was induced with isoflurane (ISO Forane®, Abbott, UK) in oxygen at a concentration of 2% and they were closely monitored during the surgery with electrocardiography. Analgesia was achieved with carprofen (3 mg/kg, i.m.) (Rimadyl®, Pfizer, İstanbul, Turkey). Laparotomy was performed by a suprapubic incision. The ovarian artery and vein were isolated by breaking down the mesovarium and ligated. Ligatures were placed on the ovarian pedicles and the body of the uterus adjacent to the cervix. Ovaries and uterus were removed carefully. The peritoneum, muscles and skin incision were closed with 1 absorbable and 1 non-absorbable suture materials, respectively.

Tissue Collection and Preservation

Immediately after operations, tissue samples from the middle part of cornu uteri showed cystic structures measuring 2x1x1 cm were excised and rinsed with wash buffer, and phosphate-buffered saline (1xPBS, pH 7.4). Specimens were fixed overnight in 10% neutral phosphate-buffered formalin for 24 h and subsequently dehydrated in a graded ethanol series. They were finally embedded in paraffin and used for routine histopathology and immunohistochemical staining. For total RNA isolation, small pieces of the uterus (1x1x1 cm) were snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Histopathological Examination

The histopathological examinations were completed with Haematoxylin and eosin (H&E) stained sections and they were classified according to Munson et al.[25] and De Bosschere et al.[3]

Total RNA Isolation

Deep-frozen (-80°C) uterine tissue samples were pulverized with a sterile mortar and pestle under liquid nitrogen. Total RNA was isolated from 15 mg of tissue powder using innuPREP RNA Mini Kit (Analytik Jena AG, Jena, Germany), including the elimination of genomic DNA, following the manufacturer's instructions. On ethidium bromide-stained 1% agarose gel, RNA integrity was tested by the presence of intact bands of 18S and 28S. mRNA purity and quantity were determined by optical density (OD) measurement (NanoDrop 2000c, Thermo Scientific, USA). The OD 260/280 ratio of all samples was between 1.8-2.0.

Real-Time PCR Measurements

Total RNA (adjusted to be 1.0 µg/11 µL with nuclease-free water) and 1 µL (100µM) Random Hexamer (RevertAid RT Reverse Transcription Kit, Thermo Fisher Scientific, USA) were incubated at 65°C for 5 min and snap cooled on ice. Twelve microliters of total RNA (1.0 µg) in 20 µL

final volume were then reverse transcribed with 4 μL 5xReaction Buffer, 1 μL RiboLock RNase Inhibitor (20 U/μL), 2 μL 10 mM dNTP Mix and 1 μL RevertAid RT (200U/μL) (all from RevertAid RT Reverse Transcription Kit, Thermo Fisher Scientific, USA). Real-time PCR was performed in a LightCycler® 480 II thermocycler (Roche Applied Science, Germany) by measuring each sample in triplicate. Reactions were performed in a final volume of 10 μL using 5 μL LightCycler® 480 SYBR Green I Master (Roche Applied Science, Germany), 10 μM of each primer, 1.9 μL PCR-graded water and 2.5 μL cDNA. Amplification conditions were the same for the target and the reference genes: initial denaturation for 5min at 95°C followed by 45 cycles at 95°C for 10s, 57°C for 10 s and 72°C for 10 s.

For each sample, amplification and melting curves were obtained using Absolute Quantitation/Second Derivative and Tm Calling analysis modes in the LightCycler® 480 II software. Melting curve analysis was used for each primer pair to confirm the gene-specific peak and the lack of primer dimer. Results were evaluated by E-method. To determine the relative expression of genes Ct value was normalized by β-Actin and GAPDH. The values in the negative control group (CG) were compared with CEH-P and CEH groups and fold change values were calculated (fold change>2= Target Upregulation; fold change<0.5= Target Downregulation). Primer pairs for the target genes $PTGS_2/COX_2$, PTGES, PTGER2, PTGER4, PGFS, PTGFR, PGR and the reference genes β-Actin and GAPDH were designed using NCBI *(https://www.ncbi.nlm.nih.gov/)* – Ensemble *(https://www.ensembl.org/index.html)* databases and purchased from Macrogen Inc. (Korea). *(Table 2)*.

Immunohistochemical Staining

Sections prepared from formalin-fixed paraffin-embedded uterine tissue samples were immunostained for the detection of the estrogen receptor protein (ER) and progesterone receptor (PR). Two μm tissue sections were incubated in a pre-treatment system (PT Link, DAKO, Denmark) at 97°C for 20 min for antigen retrieval before immunohistochemistry. To inactivate endogenous peroxidase, tissues were treated with 0.3% hydrogen peroxidase in methanol for 5 min at room temperature. Primary antibodies used were the following for 30 minutes: Monoclonal mouse anti-human progesterone receptor (1:250 dilution; Clone PgR, DAKO, Denmark) and monoclonal rabbit anti-human estrogen receptor α (ready-to-use; Clone EP1, DAKO; Denmark). Horseradish peroxidase streptavidin conjugate was used for 30 min and visualization was performed by using the substrate DAB chromogen mix for 10 min at room temperature.

Quantitative Evaluation

Sections were evaluated concerning ERα and PR localization semi-quantitatively using a light micro-

Table 2. *Primers used in Real-Time PCR analysis*

Target Gene	Gene ID	DNA Sequences of the Primers (5'-3')		Product Size (bp)
PTGS2/COX2	442942	Forward	CTGTACCCGAACAGGATTCTAC	120
		Reverse	CCCTTGAAGTGGGTAAGTATGT	
PTGES	480698	Forward	CAAGTGAGGCTTCGGAAGAA	102
		Reverse	GAGGCAGCGATCCACATC	
PTGER2	403797	Forward	CACCTCATTCTCCTGGCTATT	118
		Reverse	GAGCTTGGAGGTCCCATTT	
PTGER4	403589	Forward	CAGATGGTCATCCTGCTCAT	107
		Reverse	TTCACCAAACGTGGCTGATA	
PGFS	497070	Forward	GGACATCATCCTGACTGCATAC	101
		Reverse	CATTGAGAACTGGGTCCTTCAG	
PTGFR	479981	Forward	GCATTTGCTGGAGTCCATTTC	117
		Reverse	TTTGATTCCACGTTGCCATTC	
PGR	403621	Forward	TACCAGCCGTACCTCAACTA	132
		Reverse	GACACCATAATGACAGCCTGAT	
GAPDH	403755	Forward	GAACATCATCCCTGCTTCCA	129
		Reverse	CAGGTCAGATCCACAACTGATAC	
β-Actin	403580	Forward	GCCAACCGTGAGAAGATGA	97
		Reverse	CAGAGGCGTACAGGGACA	

scope. Three different fields, endometrial glands, endometrial stoma and myometrium, of each section per specimen at 40× magnification were evaluated for immunohistochemical staining. Values were recorded as percentages of positively stained target cells in each of the four intensity categories as follows: no staining; +, weak; ++, moderate; +++, strong staining. HSCORE value was calculated by summing the percentages of cells that stained at each intensity category and multiplying that value by the weighted intensity of the staining using the formula HSCORE = Σ(I X PC), where I is the intensity score and PC is the corresponding percentage of the cells.

Statistical Analysis

The statistical analysis of Real-Time PCR data was analyzed by means of Kruskal-Wallis variance analysis ($P<0.05$). Differences by Kruskal-Wallis Test were compared by the Mann-Whitney U test. Results were expressed as mean ± SE for each category. The Real-Time PCR data were subjected to statistical analysis using $2^{-\Delta\Delta Ct}$ values to determine statistically significant differences in all gene expressions. Expression values in CEH-P and CEH groups were checked against the values in the control group (CG).

The statistical analysis of immunohistochemical staining of the endometrial gland and myometrium samples were carried out using Kruskal-Wallis variance analysis, whereas endometrial stroma immunostaining scores were analyzed by ANOVA. Differences between Kruskal-Wallis Test and ANOVA were compared by Mann-Whitney U and Tukey test, respectively. All statistical analysis was performed with SPSS software (SPSS for Windows. Standard version release 11,5. Copyright SPSS Inc., 2002, the USA).

Results

Gene Expression of Prostaglandins

Gene transcription of $PTGS_2/COX_2$, PTGES, PTGER2, PTGFR and PGR were statistically different between groups [($P<0.05$); ($P=0.006$), ($P=0.038$), ($P=0.007$), ($P=0.002$), ($P=0.007$), respectively]. The highest expression of $PTGS_2/COX_2$ mRNA was detected in CEH-P compared with CG [($P<0.05$); ($P=0.008$)], whereas approximately 8-fold increased expression of $PTGS_2/COX_2$ mRNA in the CEH group was also statistically different compared with CG [($P<0.05$); ($P=0.008$)] *(Fig. 1)*. The only difference of PTGES mRNA expression was found between CEH and CG groups [($P<0.05$); ($P=0.008$)], although the expression of PTGES was 15- and 6.5 fold higher than

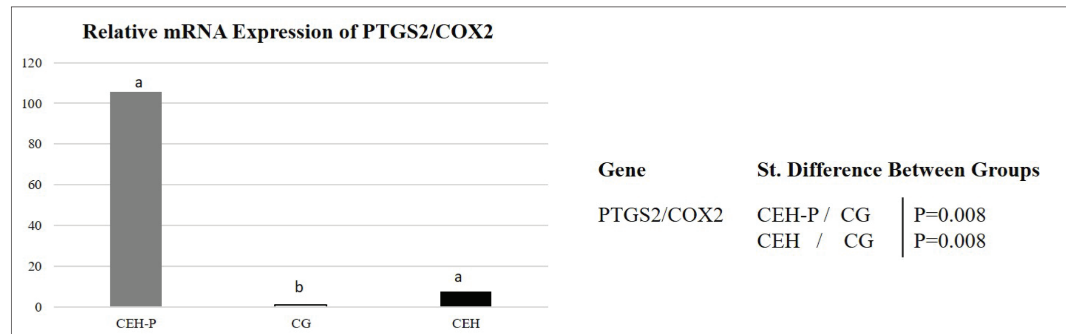

Fig 1. qPCR of PTGS2/COX2 in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown as fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group. a,b indicates the difference between groups, P<0.05

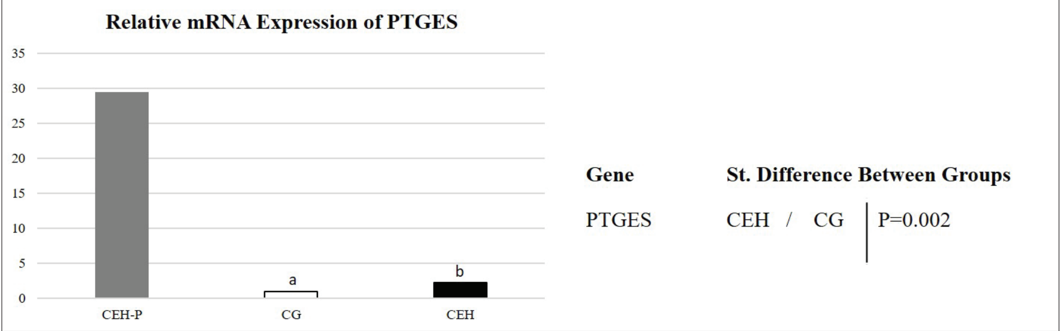

Fig 2. qPCR of PTGES in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown as fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group. a,b indicates the difference between groups, P<0.05

CG and CEH groups, respectively (*Fig. 2*). A higher transcriptional level of PTGER2 was observed in CEH-P than CEH and CG groups [(P<0.05); (P=0.008)], whereas no statistical difference was detected of PTGER4 expression between groups (P>0.05) (*Fig. 3, Fig. 4*). The PGFS and PTGFR (FP) mRNA expressions were significantly increased in CEH compared with other groups and the statistical difference in PTGFR mRNA was observed between groups (P<0.05) (*Fig. 5, Fig. 6*). Expression of progesterone receptor mRNA (PGR) was highest in CEH and statistically different from the CEH-P group [(P<0.05); (P=0.016)] (*Fig. 7*).

Immunohistochemical Localization of Estrogen and Progesterone Receptors

Immunohistochemical staining of ER receptor was

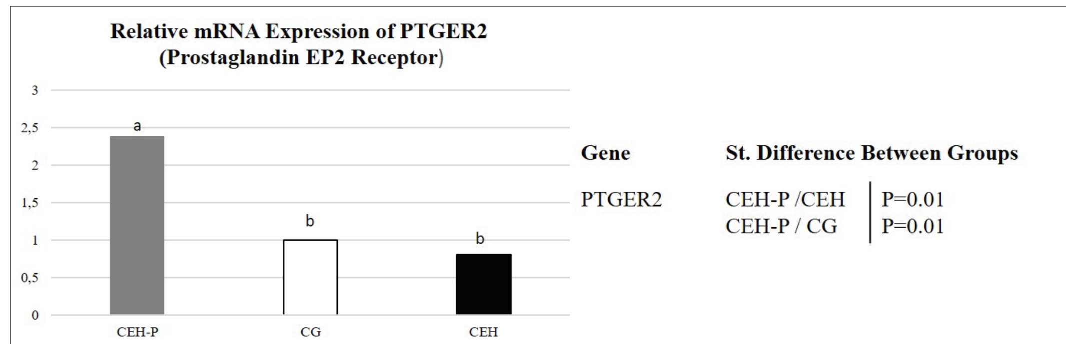

Fig 3. qPCR of PTGER2 in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group. a,b indicates the difference between groups, P<0.05

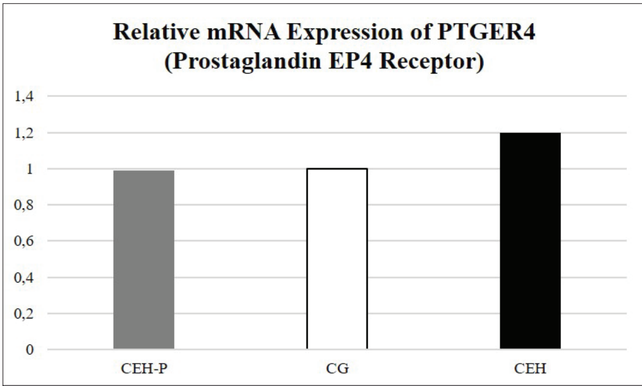

Fig 4. qPCR of PTGER4 in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown as fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group, P>0.05

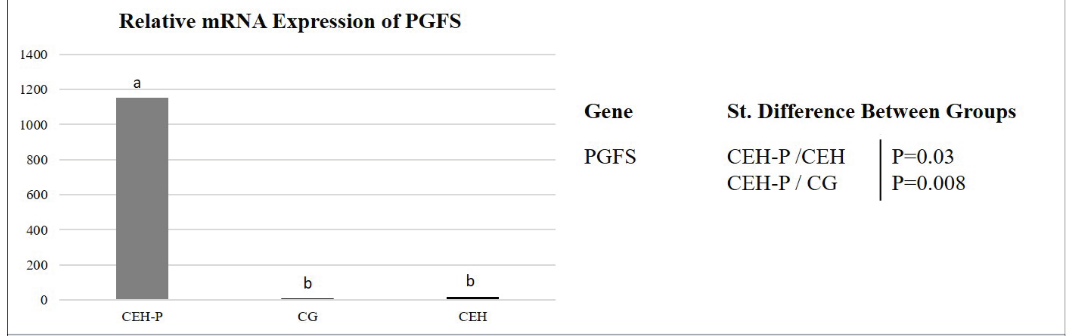

Fig 5. qPCR of PGFS in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown as fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group. a,b indicates the difference between groups, P<0.05

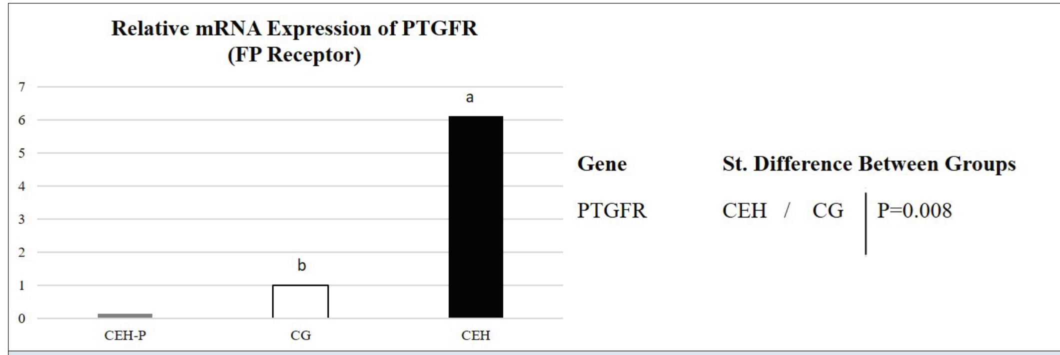

Fig 6. qPCR of PTGFR in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown as fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group. a,b indicates the difference between groups, P<0.05

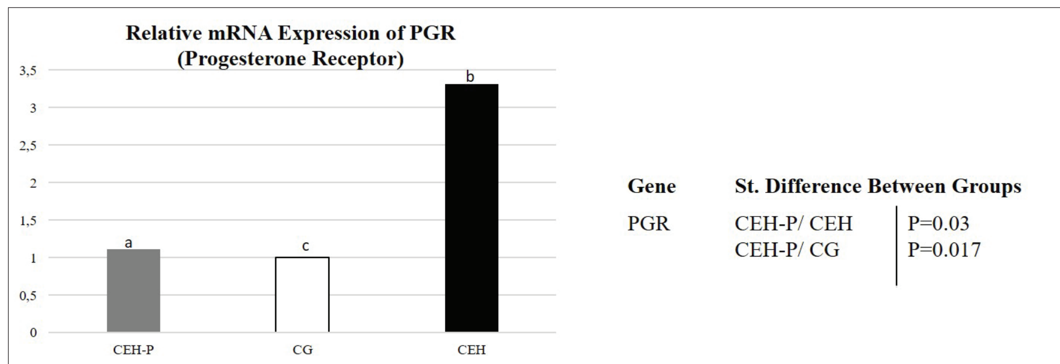

Fig 7. qPCR of PGR in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown as fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group. a,b,c indicates the difference between groups, P<0.05

statistically different among groups in endometrial glands, endometrial stoma and myometrium [(P<0.05); (P=0.030); (P=0.011); (P=0.011), respectively] *(Table 3)*. However, the only statistical difference in the immunostaining score of ER was detected between CEH-P and CG in all regions of the uterus (P=0.008) *(Fig. 8, Fig. 9, Fig. 10)*. Epithelial cells in the mucosal area and glands in propria mucosa had high estrogen receptor scores, whereas hyperplasic glands in propria mucosa had lower or no estrogen receptor scores *(Fig. 11)*. No PR immunostaining was observed in all groups.

Histopathological Examination Findings

In the sections examined in the CEH group, it was observed that the cuboidal cells in the lamina epithelialis protrude into the lumen (hyperplasia) by producing two-three-layer epithelial cells. In addition, similar formations were also found in the glandular epithelium of the lamina propria. It was also noted that the gland lumens filled with eosinophilic (proteinous) fluid and became cystic, and the cuboidal cells were flattened. In addition to lesions similar to the above, neutrophil leukocyte infiltrations were found in the gland lumens in CEH-P group *(Fig. 12)*.

Table 3. HSCOREs of ER and PR staining in endometrial gland, endometrial stroma and myometrium in CEH-P, CG and CEH groups. a, b: Different letters in superscript in the same line indicate statistically significant difference

Examined Microscopic Fields		CEH-P	CG	CEH	*P*
ER	Endometrial Gland	207.10±89.48^{a}	300^{b}	248±71.55^{a,b}	0.030
	Endometrial Stroma	133.20±93.11^{a}	300^{b}	230.60±95.37^{a,b}	0.011
	Myometrium	258.76±33.01^{a}	300^{b}	282±44.24^{a,b}	0.011
PR	Endometrial Gland	No staining	No staining	No staining	-
	Endometrial Stroma				
	Myometrium				

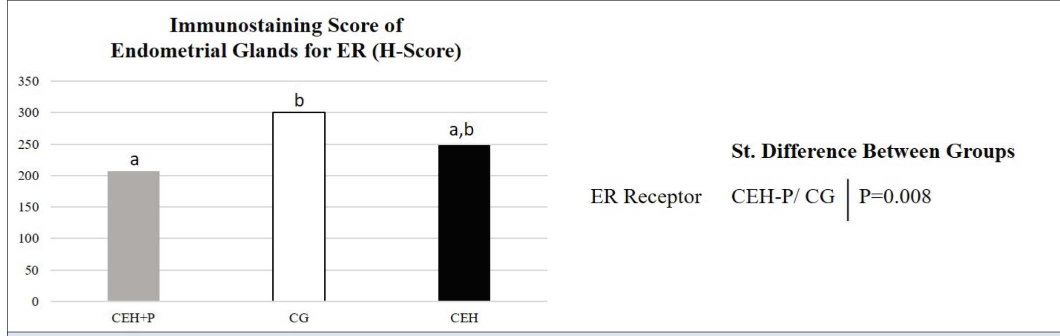

Fig 8. HSCOREs of ER reactions of endometrial glands in control group comparing with CEH-P and CEH groups. a,b indicates the difference between groups, P<0.05

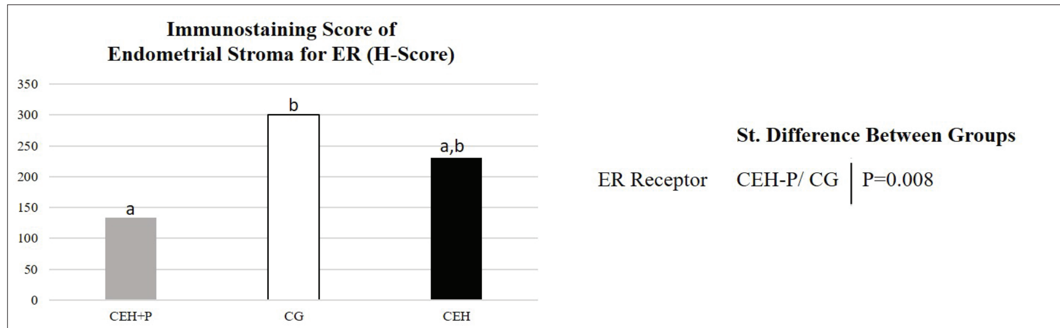

Fig 9. HSCOREs of ER reactions of endometrial stroma in control group comparing with CEH-P and CEH groups. a,b indicates the difference between groups, P<0.05

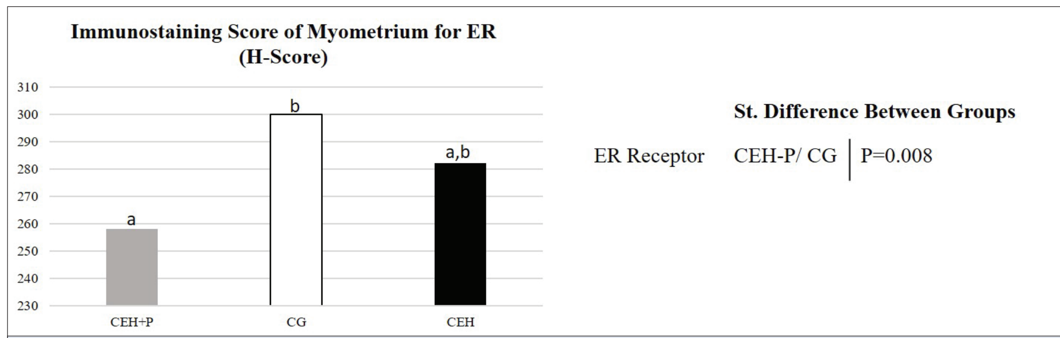

Fig 10. HSCOREs of ER reactions of myometrium in control group comparing with CEH-P and CEH groups. a,b indicates the difference between groups, P<0.05

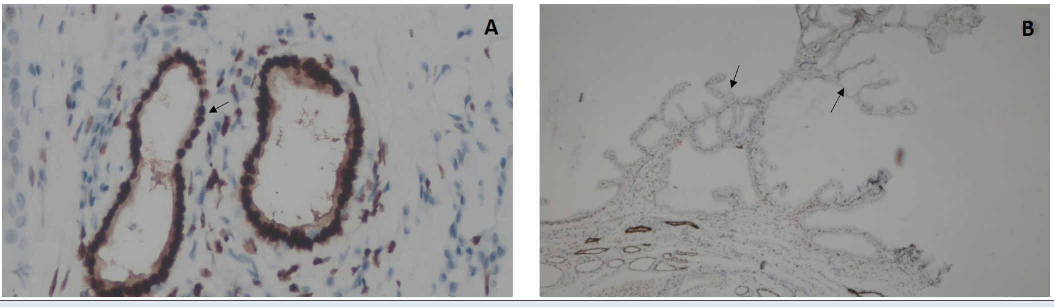

Fig 11. Immunpositive staining of endometrial gland epithelial cell nuclei (ER+) x40 magnification (A). Unstained cells with ER in hyperplastic areas (B) x20 magnification

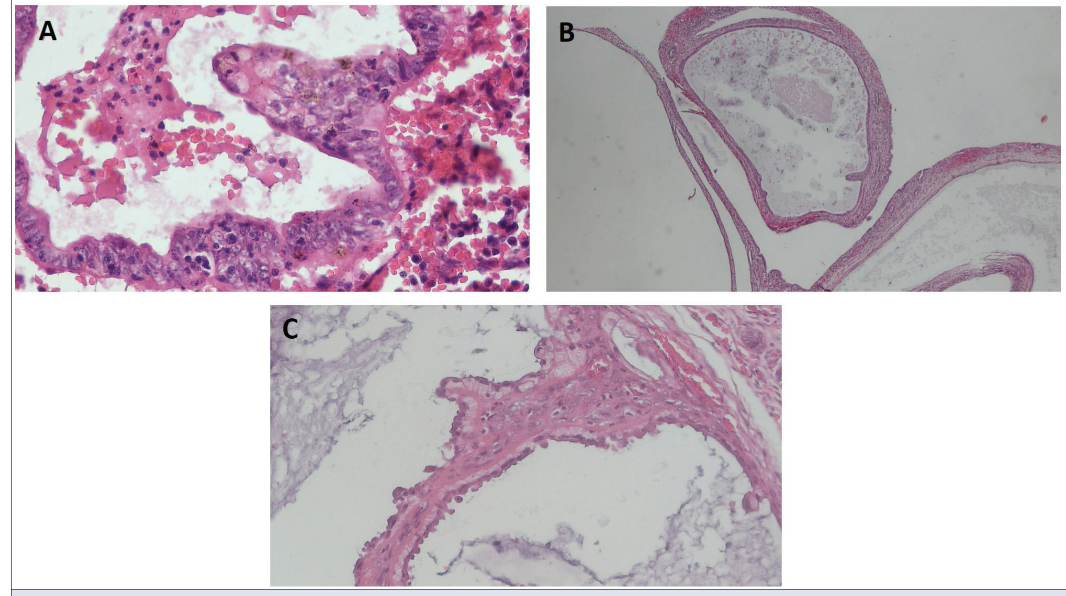

Fig 12. Haematoxylin and eosin (H&E) stained sections demonstrating (A) cystic endometrial hyperplasia-pyometra (CEH-P) (x20 magnification) and (B&C) cystic endometrial hyperplasia (CEH) (x4 magnification; x20 magnification)

Discussion

This paper reports, the regulation of the genes related to prostaglandin synthesis and its receptors in spontaneously occurring CEH cases by comparing CEH-P and CG groups. The expressional profiles of the genes are measured in CEH without any uterine accumulation and/or infection. All genes and receptors in prostaglandin synthesis were evaluated in terms of whether the reason could be leading to severe differential changing of endometrium, for the first time.

The important roles of cyclooxygenases and prostaglandins have been revealed in animal cancers. The overexpression of COX_2 has been thoroughly examined in many cancer types in animals, however, $PTGS_2/COX_2$ is known as an important enzyme, playing role in inflammatory diseases [11,14]. In the present study, extremely high $PTGS_2/COX_2$ gene transcription has been measured in CEH-P cases in dogs, which is in accordance with previous studies [14]. Further to that, 8 times higher $PTGS_2/COX_2$ transcription is being detected in spontaneously occurring CEH cases without infectious. On the other hand, a similar expression pattern was observed in PGES gene transcription. The higher PGES content was measured in bitches with CEH-P, possibly due to infection, whereas the endometrium in the CEH group had significant upregulation of the PGES gene.

Via cell surface G protein-coupled receptors (EP1-EP4), PGE_2 influences many intracellular signaling pathways, which contribute to various stages and different types of cancer [27]. The importance of EP subtypes in animal tissue differentiation is a rather new field, however, elevated levels of EP2 and EP4 biosynthesis have already been detected in response to PGE_2 in human endometrial carcinoma [27]. PTGER4 (EP4) expression was higher in the CEH group, whereas PTGER2 (EP2) expression was downregulated compared with CG. The low expression of PTGER2 in CEH cases is unknown. Other EP receptors should be taken into consideration; as various EPs could contribute to different pathologies by inducing different pathways. The upregulation of PTGER4 and downregulation of PTGER2 might follow a different pathway in CEH development, as this pathology is not been accepted as a canine cancer type.

In relation to infectious, high expression levels of PGFS and PTGFR could be the consequence of increased local inflammatory response in CEH-P cases [11,28,29]. $PGF_{2\alpha}$ acts through PTGFR and PGFS, leading to neutrophil infiltration [30]. The evidence of the high capacity of PGFS and PTGFR in CEH cases without infection compared to CG production could be a new debate in controlling endometrial changes in bitches. Though CEH is not a malignancy in canine uterine disorders, the regulation pattern of prostaglandin enzymes is different from CEH-P and healthy bitches.

CEH-P develops through the influence of sequential progestational stimulations in diestrus [31]. The strong immunoreaction for estrogen receptors (ER) and moderate reaction for progesterone receptors (PR) have been revealed in CEH with infection cases [32]. No immunostaining was observed in the immunostaining of PR in this study, whereas strong PGR gene expression was detected in CEH cases compared with CEH-P and CG. Downregulated PGR gene expression was an interesting

finding in bitches with CEH-P, which is not in accordance with previous results.

The detection of active gene expression of progesterone receptors by quantitative PCR but no immunostaining on protein level was also reported in human endometrial and breast cancers and mammary tumors in dogs [33,34]. The mismatch could be related with the isoforms of progesterone receptors or/and non-coding of truncated receptor variants by alternatively spliced transcripts [33,35,36]. The genes coding for progesterone receptors could be alternatively spliced by exon-skipping or exon duplication [33]. The defined two isoforms of progesterone receptor; isoform A (PRA) and isoform B (PRB) were transcribed from a single gene under the control of different promoters. The unexpected results are possibly related to alternative splicing as it's known as not every splice variant is translated into protein in malignant cases [33]. The different immunostaining patterns or no staining in canine mammary tumors had been revealed, suggesting that the regulation from gene to protein could be lost during malignant transformation. Further studies are needed by DNA sequence analysis.

On the other hand, distinct staining for ER was localized to endometrial glands, endometrial stroma and myometrium. Besides, decreased or no ER staining was another important finding in bitches with CEH. Long-term follow-up studies have shown that a lower percentage of ER staining has a poor prognosis with higher metastatic potent in human breast cancer [37]. On the other hand, malign tumors had decreased ER expression in canine mammary tumors [38]. The expressional alterations of ER in the hyperplastic field of the uterus should be evaluated whether they are preliminary differentiation of endometrium in further studies.

Canine adenocarcinomas are rare uterine neoplasms [21]. The dog was reported with cystic endometrial hyperplasia-pyometra complex with adenocarcinoma by Janowski et al.[23]. A similar case of multiple pathologies of the feline uterus was also available [22]. The relationship between CEH and adenocarcinoma is still unknown in bitches and queens as they are rare cases in these species. However, mild or simple hyperplasia is known as a risk of becoming endometrial cancer in women. Until now CEH cases are not accepted as neoplasia in dogs. The two pathologies in a bitch might bring new questions although no studies on the molecular level had been carried out. The high levels of prostaglandin gene expression without infectious could be related to the first step of preliminary differentiation of endometrium. CEH and pyometra cases are mostly treated by elective ovariohysterectomy operations when they are diagnosed. If it could be possible to follow these patients for a longer time, the results could easily answer the questions of the relationship between two endometrial changes in dogs and the high expressional levels of prostaglandins in CEH cases.

In summary, the high levels of prostaglandin enzymes and low ER scores in CEH could be a preliminary step for the next stages of severe differentiation of endometrium.

Availability of Data and Materials

The data sets during and/or analyzed during the current study are available from the corresponding author (G. R. Özalp) upon reasonable request.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contribution

YK: Experimental design, collection of the data, writing manuscript. ÖY: contribution to experimental design, histopathological examination, editing manuscript. AA: statistical analyses, editing manuscript. BB: Real-Time PCR analysis. MÖÖ: contribution to experimental design, histopathological examination, editing manuscript. RGÖ: Experimental design, writing manuscript & editing, supervision. All authors read, revised, and approved the final manuscript.

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Research Article

Evaluation of the Repellent Activity of 13 *Achillea* L. Species from Türkiye Against the Virus Vector *Aedes aegypti* (L.) Mosquitoes

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Abstract: Mosquitoes serve as vectors of numerous dangerous animal diseases and some human diseases including malaria, filariasis, yellow fever, dengue, and other viral zoonotic infections in both tropical and temperate countries. Many synthetic chemicals have insecticidal and/or repellent effects that are used to control vectors and protect populations from vector-borne diseases. Since some synthetic chemicals have undesirable effects when used as repellents, attention has turned to developing biodegradable and non-toxic products, including essential oils, extracts, and secondary metabolites of various plants. In this study, to find new and alternative agents to control mosquitoes from natural sources, *n*-hexane, chloroform, and methanol extracts obtained from 13 *Achillea* L. species, including 4 taxa endemic to Türkiye, were evaluated for their insect-repellent activity against an important vector, *Aedes aegypti* (L.) mosquitoes by a cloth-patch assay. Among the tested samples, *n*-hexane extracts of *A. multifida, A. crithmifolia, A. setacea,* and *A. teretifolia* (MED: 0.344, 0.375, 0.409 and 0.437 mg/cm^2, respectively) showed higher repellency. These results indicate that the lipophilic components make a major contribution to repellency and that *Achillea* species can be used as a natural source for insect-repellents.

Keywords: *Achillea, Aedes aegypti, Insect repellent, Vector-borne disease*

Türkiye'den 13 *Achillea* L. Türünün Virüs Vektörü *Aedes aegypti* (L.) Sivrisineklerine Karşı Kovucu Aktivitesinin Değerlendirilmesi

Öz: Sivrisinekler, hem tropikal hem de ılıman ülkelerde çok sayıda tehlikeli hayvan hastalığının ve sıtma, filaryaz, sarı humma, dang humması ve diğer viral zoonotik enfeksiyonlar dahil olmak üzere bazı insan hastalıklarının vektörleri olarak görev yapmaktadırlar. Esas olarak böcek öldürücü ve/veya kovucu etkileri olan birçok sentetik kimyasal, vektörleri kontrol etmek ve halkı vektör kaynaklı hastalıklardan korumak için kullanılmaktadır. Bazı sentetik kimyasalların kovucu olarak kullanıldıklarında istenmeyen etkilerinin olması nedeniyle, dikkatler biyolojik olarak parçalanabilen ve toksik olmayan, bitkisel kaynaklı çeşitli uçucu yağlar, ekstreler ve sekonder metabolitler içeren ürünlerin geliştirilmesine çevrilmiştir. Bu çalışmada, sivrisinekleri kontrol etmek için doğal kaynaklı, yeni ve alternatif ajanlar bulmak amacıyla, 4 taksonu Türkiye'de endemik olmak üzere, 13 *Achillea* L. türünden elde edilen *n*-hekzan, kloroform ve metanol ekstreler, önemli bir vektör olan, *Aedes aegypti* sivrisineklerine karşı böcek kovucu aktiviteleri açısından bez-yama testi ile incelenmiştir. İncelenen örnekler arasında, *A. multifida, A. crithmifolia, A. setacea* ve *A. teretifolia*'dan elde edilen *n*-hekzan ekstreleri daha yüksek kovucu etki göstermişlerdir (sırasıyla MED: 0.344, 0.375, 0.409 ve 0.437 mg/cm^2). Elde edilen sonuçlar, lipofilik bileşenlerin kovucu etkiye büyük katkı sağladığını ve *Achillea* türlerinin böcek kovucular için doğal bir kaynak olarak kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Achillea, Aedes aegypti, Böcek kovucu, Vektörel hastalık*

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Introduction

Mosquitoes live in a wide variety of environments worldwide, and colonize almost every aquatic habitat. Their blood feeding abilities make them the vectors of large variety of pathogens, and they are the most important group of arthropods from the standpoint of public and veterinary health [1]. Mosquitoes are important vectors that can transmit various pathogens that infect humans and animals, and generally, *Anopheles*, *Aedes,* and *Culex* species play an important role in mosquito-borne diseases [2]. *Aedes* mosquitoes are responsible for yellow fever, dengue haemorrhagic fever, and lymphatic filariasis in humans. They also transfer eastern equine encephalitis and setariasis between horses; rift valley fever and lumpy skin disease between cattle; avian malaria between poultry; dirofilariasis (Heartworm) between canines; and tularemia between birds, sheep, horses and pigs [1,3].

The mosquito vector *Aedes aegypti* L. (Diptera: Culicidae) has adapted to the urban environment and is the main cause of human transmission of many mosquito-borne arboviruses, which makes it one of the greatest global public health challenges in the world, and this mosquito continues transmitting diseases like dengue fever, yellow fever, chikungunya and Zika. *Ae. aegypti* mosquitoes are the primary arthropods that serve as vectors for dengue virus, and it is estimated that around 4 billion people live in areas at risk of dengue transmission [4]. Dengue and yellow fever vaccines are the only available vaccines, and no commercial vaccines are available for chikungunya and Zika. Integrated mosquito control strategies, including repellents, attractants, larvicides, adulticides, *Wolbachia*-infected or genetically modified mosquitoes may contribute to control of mosquito-borne diseases [5]. Therefore, reducing the mosquito population by using insecticides, and limiting the biting activities of mosquitoes by repellent compounds are the main control strategies for mosquito-borne diseases. However, new and safer insecticides or topical repellents are needed to provide protection against mosquito bites, as inappropriate and excessive application of these synthetic chemicals have undesirable effects on non-target organisms, on the environment, and on human health [6-8]. Frequent use of any single insecticide class, such as pyrethroids, can lead to non-target effects and the development of insecticide resistance, as numerous studies have shown that *Ae. aegypti* has developed resistance to conventional insecticides. As a solution on these challenges, research on novel mosquitocides with different modes of action such as larvicides, adulticides, pupicides, ovicides, repellents and growth inhibitors are continuing intensively, and plant derived natural products make a major contribution to this area [9-12].

Ethnobotanical and ethnoveterinary studies have reported the use of various plants for protection against vector-borne diseases. In rural regions, people use screens in windows and doors, proper clothing, bed nets, and natural repellents to protect themselves [13]. Hanging or sprinkling plants around the house, spraying plant juices in the house or livestock shelters, rubbing plants or applying plant juices onto skin or fur, and application of smoke by burning of plant parts are the most common practices used to repel mosquitoes and insects [13-15]. Citronella essential oils *(Cymbopogon nardus* and *C. winterianus)* are the most widely used as natural repellents in many repellent products today [16,17]. The synthetic repellent *N*,*N*-diethyl-3-methylbenzamide (DEET) is considered as the gold standard of insect repellents; however, the presence of insecticide-resistant strains and concerns with safety of DEET have prompted the search efforts in medicinal and aromatic plants to develop natural and eco-friendly products, if used in appropriate doses according to the list recommended in The Centers for Disease Control and Prevention (CDC) and the Environmental Protection Agency (EPA) [8,9,18,19].

The genus *Achillea* L. (Asteraceae) is commonly known as yarrow. Yarrow is an important medicinal and aromatic plant in traditional medicine and is generally used for bleeding, gastro-intestinal complaints, and menstrual spasm as an infusion internally, or as a poultice externally [20-22]. The genus is represented by about 115 taxa in the world, and *A. millefolium* L. is among the most widespread species [20]. The genus is represented by 61 taxa, of which 33 are endemic to Türkiye [23]. In the traditional medicine of Türkiye, the genus is generally known as “civanperçemi”, “binbiryaprak” or “ayvadana”, and herbal teas prepared from various *Achillea* spp. have been used as diuretics, emmenagogues, for abdominal pain, against diarrhea and flatulence [24,25]. Besides having a variety of activities, insecticidal, insect-repellent, and larvicidal activities of essential oils, different extracts and secondary metabolites from various *Achillea* spp. against different insects have been reported [26-29]. A wide variety of biological and pharmacological activities have been mainly attributed to essential oils, proazulenes, flavonoids, guaianolides, polyacetylenes, sesquiterpene lactones, dicaffeoylquinic acids, and alkamides [20-22,30].

Therefore, since there are scientific studies reporting insect-repellent properties of different *Achillea* species, thus, we investigated *n*-hexane, chloroform, and methanol extracts of various *Achillea* species, that were collected from different regions of Türkiye, against *Ae. aegypti* mosquitoes by using a human-based cloth patch repellent bioassay.

Material and Methods

Ethical Statement

This study was approved by the University of Florida Human Use Institutional Review Board (IRB-01, Protocol no: 2005-636).

Plant Material and Extraction

Thirteen *Achillea* taxa were collected during the flowering period from different regions of Türkiye and voucher specimens are deposited in the IZEF Herbarium of Ege University Faculty of Pharmacy, Dept. of Pharmaceutical Botany. The local names and collection sites are given in Table 1.

Table 1. *Scientific, and local names, voucher numbers, collection sites, and yields of extracts of Achillea taxa*

#	Plant Material	Local Names*	Voucher Numbers	Collection Sites	Solvents	Yield of Extracts (% of dry weight)
1	*A. multifida* (DC.) Griseb. (Endemic)	Ebulmuluk	(IZEF5598)	Bursa, Uludag	*n*-Hex	1.37
					$CHCl_3$	4.86
					MeOH	4.81
2	*A. teretifolia* Willd. (Endemic)	Beyaz civanpercemi	(IZEF5497)	Nigde, Altunhisar	*n*-Hex	1.08
					$CHCl_3$	4.50
					MeOH	5.22
3	*A. schischkinii* Sosn. (Endemic)	Deli civanpercemi	(IZEF5503)	Sivas, Karacaoren	*n*-Hex	1.37
					$CHCl_3$	2.46
					MeOH	3.08
4	*A. setacea* Waldst. & Kit.	Ayvabala	(IZEF5476)	Kirklareli, Saray	*n*-Hex	1.15
					$CHCl_3$	2.94
					MeOH	3.82
5	*A. crithmifolia* Waldst. & Kit.	Guzel namusotu	(IZEF5477)	Kirklareli, Kiyikoy	*n*-Hex	2.09
					$CHCl_3$	2.61
					MeOH	4.75
6	*A. falcata* L.	Sırcanotu	(IZEF5509)	Burdur, Elmaliyurt	*n*-Hex	0.59
					$CHCl_3$	1.60
					MeOH	2.73
7	*A. arabica* Kotschy	Hanzabel	(IZEF5501)	Konya, Aksaray	*n*-Hex	0.93
					$CHCl_3$	1.44
					MeOH	5.66
8	*A. coarctata* Poir.	Kirpit	(IZEF5473)	Tekirdag, Ganos Mountain	*n*-Hex	1.37
					$CHCl_3$	2.08
					MeOH	5.16
9	*A. pannonica* Scheele	Kurpotu	(IZEF5481)	Kirklareli, Igneada	*n*-Hex	1.78
					$CHCl_3$	2.62
					MeOH	3.39
10	*A. clypeolata* Sibth. & Sm.	Yilancicegi	(IZEF5479)	Kirklareli, Vize	*n*-Hex	0.79
					$CHCl_3$	1.69
					MeOH	2.88
11	*A. kotschyi* Boiss. subsp. *kotschyi*	Ayvadana	(IZEF5505)	Erzurum, Oltu	*n*-Hex	1.36
					$CHCl_3$	1.80
					MeOH	1.97
12	*A. phyrigia* Boiss. & Bal. (Endemic)	Ozge civanpercemi	(IZEF5498)	Kirsehir, Mucur	*n*-Hex	0.84
					$CHCl_3$	2.55
					MeOH	3.48
13	*A. nobilis* L.subsp. *neilreichii* (A. Kern.) Formánek	Binbir yaprak	(IZEF5510)	Burdur, Elmaliyurt	*n*-Hex	1.19
					$CHCl_3$	4.16
					MeOH	4.98

** Local names were provided from the reference [23]*

Dried flower heads of plants (50 g) were extracted using an orbital shaker (150 rpm) sequentially using *n*-hexane (*n*-hex), chloroform ($CHCl_3$), and methanol (MeOH) for 16 h (500 mL for each; all solvents were analytical grade) at room temperature. The extracts were separately filtered and concentrated under reduced pressure at 40°C with a rotary-evaporator and stored at -20°C for further experiments.

Mosquitoes

Aedes aegypti (Orlando strain, 1952) was used in this study. The mosquito colony was maintained at the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE-USDA-ARS) in Gainesville, FL. Pupae were obtained from the colony, and nulliparous female mosquitoes aged 6-10 days were maintained on 10% sugar water and kept in laboratory cages at an ambient temperature of 28±1°C and relative humidity of 35-60%. Nulliparous female mosquitoes were preselected from stock cages using a hand-draw box and trapped in a collection trap. After 500 (±10%) females were collected in the trap, they were transferred to a test cage (dimensions 45x37.5x35 cm, ≈59.000 cm^3) and allowed to acclimatize for 17.5 (±2.5) min before testing was initiated [31].

Repellent Bioassay

Repellency was determined as the minimum effective dosage (MED, mg/cm^2) of *Achillea* extracts using human-based cloth patch assay *(Fig. 1)*. The samples were dissolved in *n*-hex, or $CHCl_3$, or MeOH, and each sample was tested by application of a suitable amount to a cloth to produce successive serial dilutions of 1.500, 0.750, 0.375, and 0.187 mg/cm^2. Each concentration was applied to a cloth to determine the point where the repellent failed for each of the volunteers. The test was conducted by having each volunteer affix the treated cloth onto a plastic sleeve to cover a 32 cm^2 window previously cut into the sleeve. Each of the volunteers wore this sleeve/cloth assembly above a nylon stocking that covered the arm, and protected the hand with a glove. The arm with the sleeve/cloth assembly was inserted into a cage where approximately 500 female *Ae. aegypti* mosquitoes (aged 6-10 days) had been preselected as host-seeking using a draw box. Failure of the repellent treatment is predetermined to be 1% bite through, i.e. the volunteer receives 5 bites through the cloth over the sleeve window in the 1 minute assay, and three repetitions were conducted for all samples [32]. During the experiment, solvents were used as negative controls, and DEET (97%, Sigma) was used as a positive control.

Statistical Analysis

All calculations [Analysis of variance (ANOVA), followed by mean separation with the Tukey-Kramer method (α=0.05) and followed by Dunnett's test (α=0.05)] were determined using a standart statistical software (JMP Pro.16.0 software; SAS Institute Inc. Cary, NC, USA).

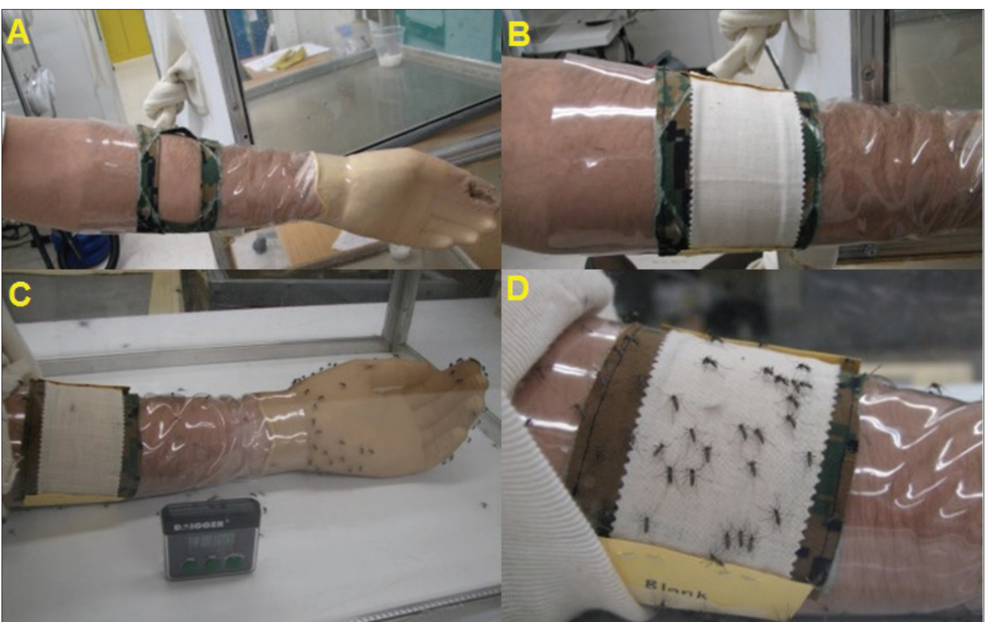

Fig 1. Cloth patch assay (Photo by Greg Allen, Natasha M. Agramonte, Ulrich R. Bernier, USDA, ARS, CMAVE). **A-** The plastic sleeve that has a 32 cm^2 window previously cut, **B-** The arm with the sleeve/cloth assembly. The sample to be tested is applied to the cloth, **C-** The arm with the sleeve/cloth assembly is inserted into a cage containing approximately 500 female *Ae. aegypti* mosquitoes, and waited for 1 min, **D-** Failure of repellent treatment is determined by 1% bites (i.e. the volunteer receives 5 bites, for 500 mosquitoes) through the cloth on the sleeve window within 1 min

Results

Results of Extraction

In this study the *n*-hexane, $CHCl_3$, and MeOH extracts from 13 *Achillea* sp., of which 4 are endemic to Türkiye were screened for the first time by a cloth-patch assay against *Ae. aegypti* to find new and alternative agents to control mosquitoes from natural sources. Scientific, and local names, voucher numbers, collection sites, and yields of extracts of investigated *Achillea* taxa are given in *Table 1*.

Results of Repellent Bioassay

Minimum Effective Dosage (MED) values of tested *Achillea* samples against *Ae. aegypti* are presented in *Table 2*. The obtained results showed that the hexane extracts were the most active when compared to the chloroform, and to the methanol extracts. Among the tested samples, *n*-hexane extracts of *A. multifida* (endemic), *A. crithmifolia, A. setacea,* and *A. teretifolia* (endemic) showed higher repellency (MED: 0.344, 0.375, 0.409 and 0.437 mg/cm^2, respectively). Additionally, MED values of *Achillea n*-hexane extract samples were compared with previously reported data in our original article [33], including four essential oils obtained from *Tanacetum annuum* (BTEO), *Anthemis scorbicularis* (ASEO), *Caryopteris* x *clandonensis* (CCEO) and *Prangos platychlaena* (PPEO). One-way ANOVA and Tukey's tests (α=0.05) showed no treatment differences among 78 comparisons. Dunnett's test revealed that MED values of *A. clypeolata* (ACLHex), *A. kotschyi* (AKHex), and *A. schischkini* (ASCHex) hexane extracts were different from the mean of control (DEET) but *A. multifida* (AMHex), and *A. crithmifolia* (ACRHex) were close to the ASEO, CCEO, and PPEO (*Fig. 2*). Among the $CHCl_3$ extracts repellent activity of *A. nobilis* and *A. setacea* (MED: 0.312 and 0.375 mg/cm^2 respectively) were notable and the other endemic plant *A. phyrigia* was effective at 500 mg/cm^2.

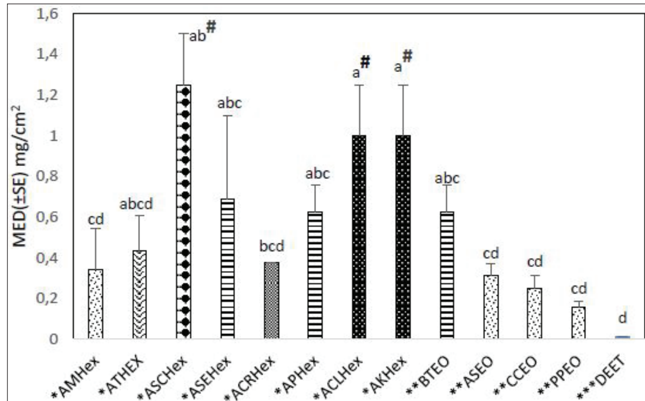

Fig 2. Comparison of minimum effective dosage (MED mg/cm^2 ± SE) values of *n*-hexane extracts of **Achillea* taxa. *A. multifida* (AMHex), *A. teretifolia* (ATHex), *A. schischkini* (ASCHex), *A. setacea* (ASEHex), *A. crithmifolia* (ACRHex), *A. pannonica* (APHex), *A. clypeolata* (ACLHex), and *A. kotschyi* (AKHex) samples were tested on human volunteers against *Ae. aegypti* mosquitoes. Responses were compared with response to known MED values of blue tansy (*Tanacetum annuum*) essential oil (**BTEO), *Anthemis scorbicularis* essential oil (**ASEO), *Caryopteris×clandonensis* essential oil (**CCEO), *Prangos platychlaena* essential oil (**PPEO), and to the standard insect repellent ***DEET (*N,N*-diethyl-3-methylbenzamide). Means followed by the same letter are not significantly different based on the Tukey-Kramer method; α=0.05, with human subjects, *n*=3. Samples with # (ACLHex, AKHex and ASCHex) significantly different from the positive control (DEET) by analysis of Dunnett's test (α=0.05)

Table 2. *Minimum Effective Dosage (MED) values of tested Achillea samples against Ae. aegypti*

#	Plant Material	*MED (± SE) mg/cm^2		
		***n*-Hex**	**$CHCl_3$**	**MeOH**
1	*A. multifida*	0.344±0.204	1.000±0.250	0.750±0
2	*A. teretifolia*	0.437±0.165	1.500±0	0.750±0
3	*A. schischkinii*	1.250±0.250	1.000±0.250	1.250±0.250
4	*A. setacea*	0.687±0.409	0.375±0	1.500±0
5	*A. crithmifolia*	0.375±0	0.750±0	1.500±0
6	*A. falcata*	NT**	0.625±0.125	1.250±0.250
7	*A. arabica*	NT**	0.675±0.125	0.750±0
8	*A. coarctata*	NT**	0.750±0	0.750±0
9	*A. pannonica*	0.625±0.125	0.750±0	1.500±0
10	*A. clypeolata*	1.000±0.250	0.750±0	1.500±0
11	*A. kotschyi*	1.000±0.250	0.750±0	1.500±0
12	*A. phrygia*	NT**	0.500±0.125	1.500±0
13	*A. nobilis*	NT**	0.312±0.062	1.500±0
	DEET (positive control)	0.011±0.001	0.011±0.001	0.011±0.001

**MED: Minimum Effective Dosage; SE: Standard errors; **NT: Not Tested; Those samples had not enough quantity to test*

Discussion

Mosquitoes act as vectors of various dangerous zoonotic diseases such as malaria, filariasis, yellow fever, dengue, and other viral infections that threaten public health and the health of animals including livestock, domestic, and wild animals [7,34,35]. Since the synthetic chemicals have undesirable effects on the environment, and human health, interest in medicinal plants has increased to develop natural insecticides and insect-repellents for medicinal and also veterinary purposes [6,27,29].

Plants are a rich source of bioactive chemicals, and in many studies insecticidal, insect-repellent, and larvicidal activities of essential oils, different extracts, and secondary metabolites from various *Achillea* spp. against different insects have been reported. In previous studies, it was reported that the essential oil of *A. millefolium* has toxic and/or repellent effect on several agricultural pest species such as *Tetranychus urticae* [36], *Sitophilus zeamais* [6], *Myzus persicae*, *Plodia interpunctella* [37], *Aegorhinus nodipennis* [38], *Leptinotarsa decemlineata* [39] and *Varroa destructor* [40]. In addition, essential oils of *A. biebersteinii* (currently *A. arabica*), *A. santolina*, and *A. mellifolium* were tested against the *Trogoderma granarium* [26]. Beside essential oil studies, MeOH extract of *A. damascene*, and aqueous extract of *A. biebersteinii* were determined to have insecticidal/repellent activity against *Bemisia tabaci* [41]. Although the ethanol extract of *A. millefolium* provided a good level of repellency against *Acanthoscelides obtectus* [28].

In terms of vector-borne diseases, the repellent activity of the essential oil of *A. wilhelmsii* was evaluated on human subjects against field collected fleas (*Pulex irritans*) and ED_{50}, and ED_{90} values were reported as 0.457 and 2.22 mg/cm^2, respectively [27]. In another study, essential oil of *A. santolina* showed insecticidal and insect repellent activities on both domestic flies and honeybees, while ethanolic extract had no activity at 500 ppm [42].

In various studies, several *Achillea* species were reported to be effective against *Aedes* mosquitoes. Ethyl acetate extract of *A. millefolium* (1%, in acetone) was reported to reduce biting by mosquitoes (including *Ae. communis, Ae. cantans, Ae. cinereus and Ae. diantaeus*) by field studies. The major metabolites in the extract with known repellent and/or insecticide activities were reported as (-)-germacrene D (49%), β-pinene (27%), sabinene (22%), α-pinene (14.1%), 1,8-cineole (10.9%), camphor (6%), β-caryophyllene (5.1%), and *p*-cymene (4.4%) [29]. In another study, field tests revealed that the extracts and oils exhibited promising activity against *Ae. aegypti*, *Ae. communis* and *Ae. cinereus* [43]. Studies performed on ethanolic extracts of *A. millefolium* resulted in a high antifeedant and repellent effect on *Ae. aegypti* [44,45]. Insecticidal activity of *A. millefolium* essential oil was tested against the larvae of the *Ae. albopictus*, and results showed that the essential oil had insecticidal activity at 300 ppm with the mortality rates ranging from 98.3% to 100% [7].

In this study, *n*-hexane extracts of *A. multifida* (endemic), *A. crithmifolia, A. setacea* and *A. teretifolia* (endemic) (MED: 0.344, 0.375, 0.409 and 0.437 mg/cm^2, respectively) showed higher repellency. Among the $CHCl_3$ extracts repellent activity of *A. nobilis* and *A. setacea* (MED: 0.312 and 0.375 mg/cm^2 respectively) were notable and the other endemic plant *A. phyrigia* was effective at 500 mg/cm^2 (*Table 2*). In our previous studies composition of the essential oils from various *Achillea* spp. were investigated, and major components were determined as 1,8-cineole (38.2%), camphor (11.5%), and borneol (9.2%) in *A. setacea* [46]; ascaridol (27.2%), and camphor (18.8%) in *A. crithmifolia*; 1,8-cineole (20.8%) in *A. kotschyi* subsp. *kotschyi* [47]; and piperitone (16.3%), linalool (14.1%), and 1,8-cineole (12.6%) in *A. nobilis* subsp. *neilreichii* [48]. These major components are known to have repellent and/or insecticide activities [29]. Studies comparing the components of essential oils and *n*-hexane extracts from various *Achillea* spp. reported that hexane extracts also include volatile components in varying amounts [49,50]. The amount of the major components were similar in the essential oils and hexane extracts of *A. gypsicola* (camphor: 40 and 25%, and 1,8-cineole: 22 and 16%, in EO and extract, respectively) and *A. biebersteinii*, (camphor: 23 and 18%, and 1,8-cineole: 38 and 15%, in EO and extract, respectively) [49]. In another study, similar results were reported for *A. wilhelmsii* (camphor: 46.6 and 44.7%, and 1,8-cineole: 14.4 and 19.5%, in EO and extract, respectively) [50].

As a result, our findings indicate that the lipophilic components of *Achillea* species make a major contribution to the repellency, which is compatible with literature. Therefore, *n*-hexane extracts are promising to investigate their lipophilic composition. Our results suggest that the *Achillea* species growing in Türkiye would be an important source for biodegradable and non-toxic insect-repellent natural products, or would be a starting point for further studies to find new and alternative agents to control mosquitoes, and vector-borne diseases.

Availability of Data and Materials

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

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

C.K. and N.T. designed and leaded the study. C.K. and S.D. collected the plant materials and performed extraction. N.T., U.R.B, and K.J.L. performed repellent bioassay and data analysis. All authors participated in the study, and contributed manuscript preparation and reviewed the manuscript.

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Research Article

Molecular Detection of *Theileria annulata* Infection: An Emerging Disease of Pet Dogs in Pakistan

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Abstract: Tick-borne maladies of canines are increasing in sub-tropical and tropical areas around the globe. Theileriosis is an emerging issue in canines, especially dogs but the data regarding the disease prevalence in Asian countries is scarce. Therefore, the current study was designed to check the molecular prevalence of *Theileria* spp. in dogs of Lahore, Pakistan. A total of 102 dog blood samples were initially screened by microscopy and later on by PCR. The results revealed that 10.78% (11/102) and 6.86% (7/102) of pet dogs were found positive by PCR and microscopy, respectively. The phylogenetic analysis showed the sequences resemblance up to 98-99% with *cytb* gene fragments of *Theileria annulata* isolates from Iran, India, Turkey, Sudan, China, and Tunisia. Assumed risk factors revealed a significant (P<0.05) association of house hygiene, tick infestation, and tick prevention applications with the disease dynamics. The infected animals also showed a significant decrease (P<0.05) in values of red blood cells (RBCs), hemoglobin level, PCV, and platelet count. This is the first report regarding the molecular evidence of *T. annulata* infection in dogs of Pakistan. This study will help control the increasing tick-borne maladies of dog population in Pakistan.

Keywords: *Dog, Phylogenetic analysis, Theileria annulata, Risk factors, Hematology*

Theileria annulata Enfeksiyonunun Moleküler Tespiti: Pakistan'da Evcil Köpeklerde Ortaya Çıkan Bir Hastalık

Öz: Köpeklerde kene kaynaklı hastalıklar dünya genelinde sub-tropikal ve tropikal bölgelerde artış göstermektedir. Theileriosis, özellikle köpeklerde ortaya çıkan bir problemdir ancak Asya ülkelerindeki hastalık prevalansına ilişkin veriler yetersizdir. Bu nedenle, bu çalışma Pakistan'ın Lahor kentindeki köpeklerde *Theileria* spp.'nin moleküler prevalansını belirlemek için planlanmıştır. Toplam 102 köpek kan örneği önce mikroskopi ile daha sonra PCR ile analiz edilmiştir. Sonuçlar, evcil köpeklerin sırasıyla %10,78'inin (11/102) PCR ve %6,86'sının (7/102) mikroskopi ile pozitif olduğunu ortaya koymuştur. Filogenetik analiz, sekans sonuçlarının İran, Hindistan, Türkiye, Sudan, Çin ve Tunus'tan izole edilen *Theileria annulata* izolatlarının *cytb* gen fragmanlarıyla %98-99'a varan benzerlik gösterdiğini ortaya koymuştur. Varsayılan risk faktörleri, ev hijyeni, kene istilası ve kene önleme uygulamaları ile hastalık dinamikleri arasında anlamlı bir ilişki olduğunu ortaya koymuştur (P<0.05). Enfekte hayvanlar ayrıca eritrosit (RBC), hemoglobin seviyesi, PCV ve trombosit sayısı değerlerinde önemli bir düşüş göstermiştir (P<0.05). Bu çalışma, Pakistan'daki köpeklerde *T. annulata* enfeksiyonunun moleküler tanısına yönelik ilk rapordur.

Anahtar sözcükler: *Köpek, Filogenetik analiz, Theileria annulata, Risk faktörleri, Hematoloji*

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Introduction

Canine theileriosis is a globally emergent vector-borne malady, transmitted by ticks [1,2]. Various species of *Theileria* (phylum Apicomplexa) have been reported to cause the disease in dogs [3]. In the infective stage of the pathogen, sporozoites infect leukocytes and mature into merozoites which finally infect erythrocytes of vertebrate hosts to form piroplasms [4]. The first reported *Theileria* spp. in dogs was *Theileria annae* [5,6] which was later on reported in various countries including Germany [5], Croatia [7], Spain [8-11], USA [12], Barcelona [13], Sweden [14], Portugal [15], Serbia [16], France [17] and Southern Europe [18]. However, *Theileria annulata* has been identified and reported by Bigdeli et al.[19] and Aktas et al.[20] in Southern Iran and Turkey, respectively. Furthermore, in diseased dogs of Paraguay, France, South Africa, and Nigeria, *Theileria equi* has also been confirmed [3,21-23]. Though, the molecular occurrence of *Theileria* spp. in pet dogs of Pakistan is not yet reported.

Clinically the diseased dogs present a wide range of signs including anorexia, lethargy, anemia, pale mucous membranes, jaundice, tachypnea, splenomegaly, tachycardia, and yellowish-orange to brownish colored urine [24]. Furthermore, the severe manifestation of similar clinical signs in other animals has also been reported [25]. The routine diagnosis of the disease is based on the history of tick infestation, clinical findings, and hematological alterations. Microscopic examination of blood smears can also be used for the detection of the responsible pathogen [26]. The serological methods used for the disease diagnosis include IFAT and ELISA [27] but molecular technique like Polymerase Chain Reaction (PCR) is considered a more sensitive, expedient, and reliable technique for diagnosis [28,29].

Mainly *T. annulata* is considered to be involved in persistent infection of cattle. However, the pathogen is reported to cause severe maladies among dogs across the globe, but the data regarding the molecular detection of *T. annulata* in pet dogs is limited in our country. In Pakistan, theileriosis has been reported in bovines, small ruminants, and equines [30-35], but the presence of *T. annulata* infection in pet animals especially dogs is not reported. Therefore, the current study proves to be the first molecular evidence of *T. annulata* infection in dogs of Pakistan. Assessment of risk factors and alterations in hematology values associated with the *Theileria* infection was also a part of the current study.

Material And Methods

Ethical Statement

The current study was approved by the ethical committee of advanced studies research board vide approval number DAS/8438 in the University of Veterinary and Animal Sciences, Lahore. No animal was harmed during the sampling and all the standard operating procedures were followed throughout lab activities.

Study Design

The study was conducted at different private and government veterinary hospitals of district Lahore, Pakistan from March to September 2020 *(Fig. 1)*. A total of 102 pet dogs (n=102 dogs) having tick infestation or

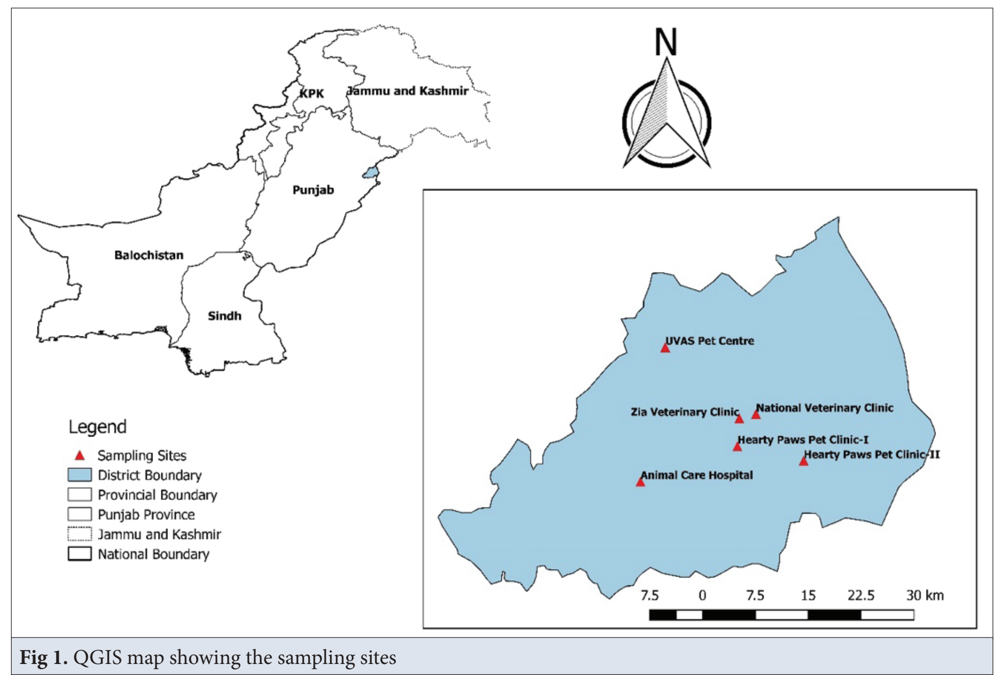

Fig 1. QGIS map showing the sampling sites

showing signs like pyrexia, pale mucous membrane, jaundice, anemia, and anorexia were involved in the study regardless of breed, sex, and age of animals. Consent was obtained from officials before sampling.

Blood sampling was done from study animals in two ways. For microscopic examination, blood smears were made in triplets from ear vein puncture and air-dried on spot. Later on, samples were evaluated through microscopy by performing Giemsa staining of all slides. After that, for molecular analysis, 3 mL of blood was drained aseptically through a cephalic vein from each dog and transferred to Medicine Research Laboratory, Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore. The association of the disease with various risk factors was elaborated by collecting information about various environmental and animal management factors on a predesigned questionnaire.

DNA Extraction

The DNA from each 200 µL blood sample was extracted by using a DNA extraction kit (GeneAll® Exgene™ Blood SV mini 105-101) following the manufacturer's directions. The testing of purity and concentration of all extracted genomic DNA samples was performed using Nano-drop at 260/280 nm. For further processing, DNA samples were kept at -20°C.

PCR Amplification

The amplification of *cytb* gene of *Theileria* species was executed using primers consisting of forward primer as F=GGGAGCTACAGTCATAGGTGGT and reverse primer as R=TCCTGCCATTGCCAAAAGTC and the conditions used for running the PCR reaction were as mentioned by Zaheer et al.[36]. PCR products were visualized under UV light in a 1.5% ethidium bromide-stained agarose gel after gel electrophoresis.

Sequencing

The positive amplified bands were sliced from the gel under ultra-violet light using a sterile blade and subjected to purification by a gel extraction kit (GeneAll® Expin™ Gel SV (102-150). For sequencing, samples of purified genomic DNA were shipped to 1st Base biological technology, Singapore. The sequencing results were evaluated through the NCBI Blast and CLUSTAL W alignment method. A phylogenetic tree was constructed on sequence distance using the Maximum likelihood method of phylogeny testing on Mega X (Molecular Evolutionary Genetics Analysis version 10.0) software.

Hematological and Statistical Analysis

Various hematological parameters including; WBCs count, RBC count, hemoglobin (Hb), and packed cell volume (PCV) were analyzed using a hematology analyzer. The statistical analysis was performed on the data regarding risk factors and the hematological parameters using SPSS version 20.00. The association of risk factors like age, sex, breed, previous tick history, type of acaricide, tick control status, interval of acaricide application, house hygiene, tick infestation, and housing type, was analyzed statistically using logistic regression analysis and Chi-square test. An independent sample t-test was used to evaluate the changes in blood parameters with a confidence interval of 95%. The variables with P-value <0.05 and odd ratio >1.00 were assumed to be significantly associated with disease occurrence.

Results

Epidemiology of *Theileria* spp. in Pet Dogs

The study showed a 10.78% (11/102) molecular-based prevalence of canine theileriosis in dogs from Lahore, Pakistan. The sequencing of samples declared the gene sequence to be of *T. annulata*. However, the microscopic-based examination showed a 6.86% prevalence of *Theileria* spp. infection in studied animals.

Assessment of risk factors associated with canine theileriosis revealed tick infestation to be significantly associated (P=0.014) with the disease occurrence. Tick control status was also a significantly proven (P=0.016) risk factor. The other risk factor significant (P=0.036) associated with the disease incidence was house hygiene as dogs raised in good hygiene conditions showed less disease prevalence as compared to dogs raised in poor hygiene conditions. Factors like age, breed, sex, previous tick history, acaricide type, and interval of acaricide application were also statistically analyzed and found non-significant risk factors for the occurrence of the disease. Housing type was also proved a non-significant determinant. However, dogs raised in cages revealed more disease prevalence (14.3%) as compared to the dogs reared in houses made of concrete (8.3%) *(Table 1)*.

Theileria cytb Gene Analysis

This is the first molecular-based evidence for the existence of *Theileria* spp. in dogs of Lahore, Pakistan. The sequencing of PCR products of study samples was done for *cytb* gene of Theileria and was analyzed later on using Bioinformatics software including CLUSTALW and BLAST alignment. Various *cytb* sequences of *Theileria* were obtained from the Genbank database to compare them with study isolates. Phylogenetic analysis was performed using maximum likelihood method *(Fig. 2)*. The study isolates showed a high similarity with the *T. annulata* sequences from the GenBank database. The phylogenetic analysis revealed the present study sequences of *T. annulata* show the highest resemblance with the gene sequence of *T. annulata* from Pakistan which is isolated

***Table 1.** Results of Chi-square test on various assumed risk factors associated with theileriosis*

Study Variable	Category	*T. annulata* Positive (%)	*T. annulata* Negative (%)	P Value
Gender	Male	4 (7.7)	48 (92.3)	0.305
	Female	7 (14.0)	43 (86.0)	
Age	≤ 1Years	5 (10.0)	45 (90.0)	0.802
	> 1 Year	6 (11.5)	46 (88.5)	
Breed	German Shepherd	4 (13.3)	26 (86.7)	0.363
	Labrador Retriever	1 (5.9)	16 (94.1)	
	Kohati Gultair	1 (7.7)	12 (92.3)	
	Mix / Non-descript	5 (20.8)	19 (79.2)	
	Pit bull	0 (0.0)	7 (100.0)	
	Pug	0 (0.0)	11 (100.0)	
Tick Infestation	Yes	9 (18.8)	39 (81.2)	0.014*
	No	2 (3.7)	52 (96.3)	
Previous Tick History	Yes	8 (17.0)	39 (83.0)	0.060
	No	3 (5.5)	52 (94.5)	
Tick prevention applications	Yes	1 (2.3)	43 (97.7)	0.016*
	No	10 (17.2)	48 (82.8)	
Route of Acaricide application	Topical	1 (2.9)	33 (97.1)	0.052
	Parenteral	0 (0.0)	10 (100.0)	
	NA	10 (17.2)	48 (82.8)	
Interval of Acaricide application	< 2 Months	1 (2.9)	33 (97.1)	0.052
	> 2 Months	0 (0.0)	10 (100.0)	
	NA	10 (17.2)	48 (82.8)	
Housing Type	Concrete	5 (8.3)	55 (91.7)	0.340
	Iron	6 (14.3)	36 (85.7)	
House Hygiene	Good	3 (5.2)	55 (94.8)	0.036*
	Poor	8 (18.2)	36 (81.8)	

** Indicates risk factors significantly associated with theileriosis in dogs*

from cattle (Accession no. OL420757). Our study isolates showed more resemblance with each other than the others. Furthermore, the current study isolates showed high similarity with the gene sequences from India (Accession no. MN044040, MN044047). However, the gene sequences from other countries like Sudan (LC431534, LC431528), Spain (DQ287958), Turkey (MK693128, MK693130), Iran (MN422305, MT812969), China (KP731977), and Tunisia (KF732026, KF732025) resembled our study isolates of *T. annulata*. Moreover, the isolate of dog from France (Accession no. JX454779) showed a significant variation with our study isolates, hence making an out-group.

Effect of *Theileria* on Hematological Parameters of Dogs

A comparative study on hematological parameters of healthy and diseased dogs was done. The results indicated a non-significant (P=0.475) decrease in the WBCs count of diseased animals as compared to healthy ones. Among WBCs the decreasing pattern in lymphocytes and monocytes was non-significant (P>0.05) while the results of granulocytes were significant (P<0.05). Other hematological parameters like red blood cells (RBCs), hemoglobin level, PCV, and platelet count were found to have a significant (P<0.05) association with the disease incidence. A significant reduction in average values of the parameters was observed in diseased animals *(Table 2)*.

Discussion

The prevalence of tick-borne pathogens in dogs is increasing around the globe and is of significant importance in both veterinary and human health [37,38]. Canine theileriosis is an emerging vector-borne disease

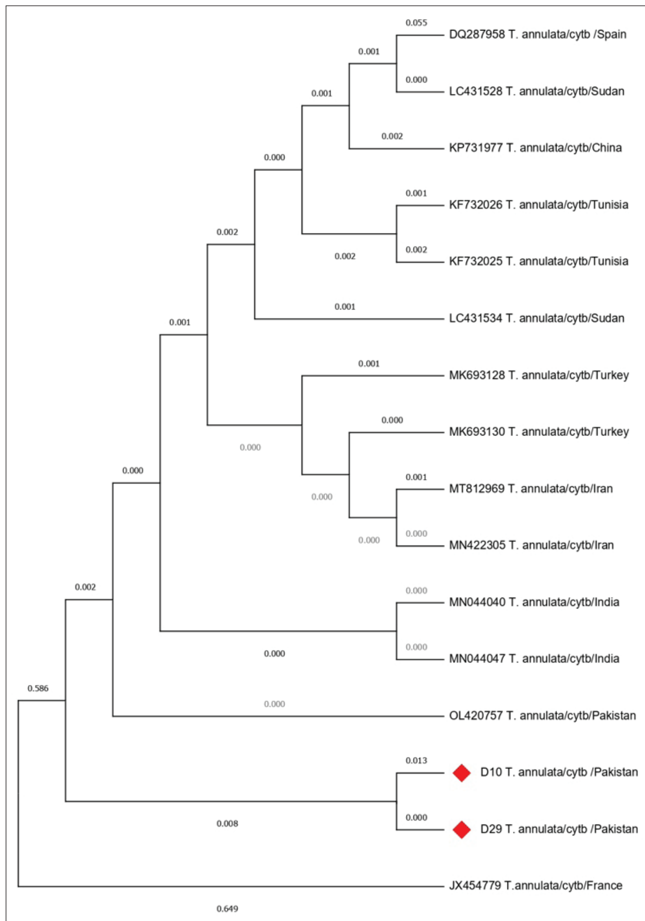

Fig 2. Phylogenetic analysis of *cytb* gene of study isolates with NCBI Genbank reported sequences by using maximum likelihood method and 1000 bootstrap technique

that is distributed and reported globally [1]. The variation in the prevalence of the disease in various countries can be related to alterations in environmental conditions and epidemiological factors like age, sex, breed, habitat of dogs, and management practices [39]. The diagnosis of canine tick-borne pathogens by morphological identification in blood smear through the use of microscopy is not reliable. Therefore, a molecular technique like PCR is required for chronic and subclinical infection in carrier dogs [40]. In Pakistan, vector-borne diseases of dogs like babesiosis [41] and ehrlichiosis [42] have been reported on the molecular level, but till now, no data is available on the molecular characterization of canine theileriosis in dogs. The present study is the first report of canine theileriosis in dogs concerning the molecular diagnosis, phylogenetic analysis of the isolated sequences, and the relationship of presumed risk factors with disease dynamics in pet dogs of Lahore, Pakistan.

In the current study, overall microscopy and PCR-based prevalence of 6.86% (7/102) and 10.78% (11/102) were reported in dogs of district Lahore, respectively. Similar to the finding of the current study, Bigdeli et al.[19] and Aktas et al.[20] have also confirmed and reported the presence of *T. annulata* in southern Iran and Turkey, respectively. The molecular findings of the present study agreed with the outcomes of Cicuttin et al.[43] who described a 10.1% prevalence of theileriosis in dogs from non-investigated areas of Serbia. The findings also matched to results of Niu et al.[44] and Adamu et al.[23] who reported 6.4% and 4% prevalence of *Theileria spp.* in pet dogs of China and Nigeria, respectively. Similarly, a slightly higher prevalence (13%) of *Theileria* spp. was also reported by Gholami et al.[45] in sheepdogs from Iran. However, conflicting with the present study conclusions, Miro et al.[11] reported a higher molecular-based prevalence (62.5%) of *T. annae* in Spain. Also, Matjila et al.[40] described (41.1%) molecular prevalence of *Theileria* spp. from South Africa. The results of the current study were also different from the outcome of Rosa et al.[22] who reported a higher prevalence of *Theileria* spp. (66.6%).

The current study report was also contrary to the observations of [9,12,13] who reported 1.9%, 0.7%, and 0.6%

Table 2. *Effect of canine theileriosis on various hematological parameters of dogs based on independent sample t-test*

Parameter	Unit	*Theileria* Negative	*Theileria* Positive	F Value	Mean Difference	Confidence Interval	P Value
		Mean ± SD	Mean ± SD				
WBCs	x 10^3/µL	8.74±1.64^a	7.4±3.38^a	1.044	1.26	-5.139-2.619	0.475
Granulocytes	%	77.32±6.21^a	57.44±15.69^b	1.915	19.88	-2.469-37.290	0.03*
Monocytes	%	5.68 ±1.97^a	4.52±1.92^a	0.013	1.16	1.681-4.001	0.374
Lymphocytes	%	20.98±4.25^a	15.60±9.03^a	1.057	5.38	4.919-15.679	0.263
RBCs	x 10^6/µL	6.62±0.952^a	3.61±0.974^b	0.003	3.01	1.596-4.407	0.001*
Hemoglobin	g/dL	14.72±2.09^a	7.72±2.70^b	0.097	7.00	3.475-10.524	0.002*
PCV	%	41.64±3.69^a	24.64±5.32^b	1.164	17.00	10.312- 23.687	<0.001*
Platelets	x 10^3/µL	239.40±48.41^a	71.60±26.16^b	5.068	167.80	111.04-224.55	<0.001*

*^{aa} indicates a non-significant difference between healthy and diseased animals; ^{ab} indicates a significant difference between healthy and diseased animals; * indicates the significant association of blood parameters with the disease*

molecular prevalence of *T. annae* in Spain, Mississippi (USA), and Barcelona, respectively. While, Inacio et al.[3] reported a lower prevalence (0.3%) of *T. equi* in dogs from Paraguay, and Xu et al.[46] also reported the lowest prevalence (0.1%) of *T. orientalis* in dogs from China. The difference between *Theileria* distributions may be due to different geographical regions and variable numbers of prevailed vectors in each region, however, management practices and hygienic conditions also confer the difference in the prevalence of *Theileria* in every region.

The current report indicated a higher prevalence of the disease in female dogs (14.0%) compared to male dogs (7.7%) which was as per the outcomes of Miro et al.[11] who recognized a higher infection rate in females dogs (67.2%) comparative to male dogs (65.5%) of Spain. The results were also supported by the study of Aktas et al.[20] who reported a slightly higher infection rate in female dogs (5.5%) as compared to male dogs (5.3%) from Turkey. In the present study, the higher disease incidence in female dogs could be attributed to the fact that female dogs suffer extra stress during pregnancy and lactation making them more prone to infections. The results revealed a higher prevalence (11.5%) in adult dogs (>1 year), compared to younger ones (≤1 year) (10.0%) which was in agreement with the outcomes of Aktas et al.[20]. However, current study findings were contrary to the observations of Miro et al.[11] who found higher disease prevalence in younger dogs (≤3 years) (81.54%) as compared to older ones (>3 years) (46.81%). The higher prevalence of the disease among old animals might be due to an increased opportunity for exposure to ticks over time as described by Hegab et al.[47].

In this study, higher disease prevalence was observed in mixed/non-descriptive breeds (20.8%) as compared to pure breeds such as German shepherd (13.3%), Labrador retriever (5.9%), and Kohati Gultair (7.7%). Similar findings were also documented by Miro et al.[11] who described a high prevalence of the disease in Cross-breed/Mixed (c/m) (66.6%) as compared to pure breeds (61.6%). The lower disease incidence in pure breeds was probably due to the reason they spend more time in the gardens and indoor. Additionally, inspection and cleaning practices like bathing and brushing are regularly followed for pure-breed dogs making these dogs less prone to tick-borne infections.

Tick infestation and previous tick history were found to be significant risk factors for disease incidence. In the current study, it is observed that dogs having tick infestation or previous tick history were at greater risk of developing the disease compared to dogs that neither have previous tick history nor current tick infestation. These findings were compiled with the observation of Miro et al.[11] and Ahmed et al.[48] who reported the association of tick infestation with tick-borne maladies in pet animals.

To control vector-borne diseases like babesiosis and theileriosis in dogs, the control of ticks by acaricides or pesticides is considered an effective way. In this report, the dogs without ticks prevention applications revealed a higher prevalence of disease (17.2%). This factor was statistically analyzed and its P-value was noted (0.016) which is a significant ($P<0.05$) contributing factor towards disease dynamics and that could be clarified by the fact that ticks act as a biological vector to spread the *Theileria* infection from diseased dogs to healthy ones. So, the dogs that have a higher exposure to ticks or inadequate tick control approaches will have more chances of disease occurrence.

This study provides the first insight into the genetic characterization of *Theileria* spp. targeting the *cytb* gene in dogs from district Lahore, Pakistan. The local study isolates showed similarity with the *T. annulata* isolates from India, Iran, China, Turkey, Sudan, and Tunisia isolates. The transboundary movement of animals through shared borders with Iran, India, and China and import of various breeds of pet dogs from these countries could be a reason for the similarity of the current study isolates with *T. annulata* isolates of Iran, Turkey, India, and China.

In the current study, the values of RBCs, PCV, Hb, and thrombocytes (platelets) were found significantly decreased in infected dogs. WBCs showed a non-significant decrease in infected animals. Decreased RBCs, PCV, and Hb are considered important indicators of anemia. The current study outcomes were supported by Rosa et al.[22] who reported anemia with thrombocytopenia in 5 out of 6 dogs from South Africa. Thrombocytopenia is the most frequent and typical finding of canine theileriosis and was described by Falkenö et al.[14]. Additionally, Garcia [10] also described anemia along with thrombocytopenia as a common manifestation and leukocytosis as an uncommon finding in diseased dogs of northern Spain. In the present study, the differential WBCs counts indicated a general tendency towards neutrophilia and eosinopenia which was in agreement with the outcomes of Garcia et al.[10]. Additional to these reports, the findings of several other studies including Simes et al.[15] and Keefe et al.[39] were also following the outcomes of the current study. Rosa et al.[22] documented that the thrombocytopenia was probable to be secondary to immune-mediated devastation, platelet consumption, and splenic sequestration in *Theileria*-affected dogs from South Africa. Leukocytosis in addition to thrombocytopenia was also observed in diseased dogs in several other reports [10,14,15,22,40].

This study provides molecular-based evidence of *Theileria* spp. in dogs of Lahore, Pakistan. The amplification of the *cytb* gene fragment by PCR revealed an overall *Theileria* prevalence of 10.78%. Assessment of risk factors revealed that house hygiene, tick control status, and tick infestation

were proven significant determinants for disease dynamics. Moreover, hematological parameters of infected animals showed a significant reduction in the RBCs, PCV, Hb, and platelet count. The current study will be helpful to plan control measures for the *T. annulata* infection in pet dogs of Pakistan.

Availability of Data and Materials

The information regarding the datasets analyzed during the present study is available from the corresponding author (M. Ijaz) upon request.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Y.N., M.I.: Conceptualization, methodology, data curation, investigation, resources, project administration, funding acquisition, writing, review, and editing; N.Z.G., A.A., R.M.A.: Methodology, data curation, investigation, software, visualization, validation; M.U.J., A.A.: Writing of original draft; I.M.: Writing, review, and editing. All authors read and approved the final manuscript.

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Research Article

The Quinolone Resistance Genes in the Bacteriophage DNA Fractions in the Healthy Calf Stool Samples Via qPCR

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Abstract: The One Health approach shows that people, animals, plants, and environmental factors can affect each other. Phages are one of the mobile genetic elements. Quinolones are a critical group of antibiotics for both human and animal health and monitoring their antimicrobial resistance is very important. The aim of the study is to determine the frequency of the quinolone resistance gene in bacteriophage DNA fractions obtained from healthy calf stool samples. In our study, 50 samples from 6-9 months old calves, which were found to be healthy and not treated with any group of antibiotics in Sanlıurfa province, were included. DNA isolation was made from phage lysates of stool samples and specific primers were used *qnrA*, *qnrB* and *qnrS* genes. qPCR was performed on LightCycler480. Despite not receiving any antibiotic treatment, *qnrB* was the most detected gene among the phage DNA fractions detected in 11 calves. While *qnrA*, *qnrB* and *qnrS* quinolone resistance genes were detected together in one sample, *qnrB* and *qnrS* resistance genes were found together in two samples. Our data, obtained from the study in Türkiye to search for antimicrobial resistance genes in phage fractions, showed the importance of the One Health approach and determined that it was highly effective in quinolone resistance gene shedding in healthy calves that had never been treated with antibiotics. It has been concluded that in empirical treatment with quinolone, attention should be paid to all living things and unconscious antibiotic use may cause the spread of resistance genes more than expected.

Keywords: *Antibiotic resistance genes, Bacteriophage, qPCR, Quinolone*

Sağlıklı Buzağı Dışkı Örneklerindeki Bakteriyofaj DNA Fraksiyonlarındaki Kinolon Direnç Genlerinin qPCR ile Belirlenmesi

Öz: "Tek Sağlık" yaklaşımı, insanların, hayvanların, bitkilerin ve çevresel faktörlerin birbirini etkileyebileceğini gösterir. Fajlar, hareketli genetik elemanlardan biridir. Kinolonlar hem insan hem de hayvan sağlığı için kritik bir antibiyotik grubudur ve antimikrobiyal dirençlerinin izlenmesi çok önemlidir. Bu nedenle çalışmamızın amacı, sağlıklı buzağı dışkı örneklerinden elde edilen bakteriyofaj DNA fraksiyonlarında kinolon direnç gen belirteçlerinin *(qnrA*, *qnrB* ve *qnrS* genleri) sıklığını belirlemektir. Çalışmamıza Şanlıurfa ilinde bulunan mandıralardan alınan 6-9 aylık buzağılardan sağlıklı olduğu tespit edilen ve herhangi bir grup antibiyotik ile tedavi edilmeyen 50 dışkı örneği dahil edildi. Dışkı numunelerinin faj lizatlarından DNA izolasyonu yapılmış ve *qnrA*, *qnrB* ve *qnrS* genleri için spesifik primerler kullanılmıştır. qPCR, LightCycler480'de gerçekleştirilmiştir. Hiçbir antibiyotik tedavisi görmemesine rağmen 11 buzağı dışkısında tespit edilen faj DNA fraksiyonları arasında en çok tespit edilen gen *qnrB* idi. Bir örnekte *qnrA*, *qnrB* ve *qnrS* kinolon direnç gen belirteçleri birlikte saptanırken, iki örnekte *qnrB* ve *qnrS* direnç gen belirteçleri birlikte bulundu. Türkiye'de faj fraksiyonlarında antimikrobiyal direnç geni araştırması yapan çalışma ile elde edilen verilerimiz, "Tek Sağlık" yaklaşımının önemini göstermiş, ayrıca sağlıklı, antibiyotikle hiç tedavi edilmemiş buzağılarında kinolon direnç geni saçılımında oldukça etkili olduğu belirlenmiştir Kinolon ile ampirik tedavide tüm canlılara dikkat edilmesi gerektiği ve bilinçsiz antibiyotik kullanımın tahmin edilenden fazla direnç genlerinin yayılmasına neden olabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Antibiyotik direnç genleri, Bakteriyofaj, qPCR, Kinolon*

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Introduction

Bacterial antimicrobial resistance (AMR), which reduces the effect of drugs used to treat bacterial infections, threatens the whole world as one of the leading public health problems of the 21st century. According to the reports of the World Health Organization (WHO), it is estimated that 10 million people will die in 2050 due to antimicrobial resistance [1]. The One Health approach indicates that factors originating from humans, animals, plants and the environment can have effects on each other. Therefore, it is thought that the development of antimicrobial resistance may be related to the misuse of antimicrobials in these resources and contribute to the spread of antimicrobial resistant bacteria and antimicrobial resistance markers throughout the world within or between these sectors. It is a well-known fact that many similar classes of antimicrobials used to treat bacterial infections in humans are also frequently used in animals [2]. Transduction by bacteriophages (phages) is one of the many horizontal gene transfer mechanisms and it has been demonstrated that phage-mediated transduction makes an important contribution to the spread of antimicrobial resistance genes [3,4].

Bacteriophages are known as bacterial viruses that invade the cells of Gram-positive and Gram-negative bacteria [5]. According to their genomic and morphological structures, there is a wide variety among phages. The size of phage genomes can range from a number of to 100 kb [6]. It is known that ARGs are transferred to the environment from antibiotic-resistant bacteria when bacteriophages invade these bacterial cells [7,8]. Phages are one of the mobile genetic elements (MGEs), and antimicrobial resistance genes (ARGs) can be acquired and transferred between bacteria via these MGEs such as phages, conjugative plasmids, insertion sequences, integrons, and transposons [9]. Metagenomic studies have shown that due to the bacterial diversity in the gut microbiota, bacteriophages can also be found extensively in the human and animal gut microbiota, and that the gut is an excellent ecological environment for these phages, which can probably proliferate by infecting bacterial communities of the gut. In addition to this, ARGs of Gram-negative and Gram-positive bacteria can be carried and transferred within these phage DNA fractions [10,11]. Quinolones and fluoroquinolones have been classified as critical antibiotics for human health by the WHO. Resistance to these compounds is widespread in Europe, and due to this rapid spread, monitoring of antimicrobial resistance to quinolones is crucial for both human and animal health [12]. The most common disease treated in cattle is the neonatal calf diarrhea. According to the recommendations and depending on the results of antimicrobial susceptibility test, the use of quinolones in the treatment of this disease should be in a limited amount and should be used as a last choice in the treatment in cases of diarrhea due to *E. coli* and *Salmonella* spp. infections [13]. However, some 3rd generation fluoroquinolones, such as enrofloxacin, can be used empirically for the treatment of several diseases of animals, which may lead to the development of resistance genes against quinolones [14].

In our study, we aimed to determine the frequency of quinolone resistance gene markers *(qnrA*, *qnrB* and *qnrS* genes) by qPCR, which are critical for human and animal health, in bacteriophage DNA fractions obtained from healthy calf stool samples.

Material and Methods

Ethical Statement

This study was approved by the Veterinary Control Central Research Institute Local Ethics Committee (Approval no: 2022/24).

Sampling and DNA Isolation

In our study, random sampling was used to select stool samples. 50 stool samples taken from 6-9 months old Holstein calves (25 male and 25 female) from the dairy farms in Sanliurfa, that were found to be healthy and not treated with any kind of antibiotics, were included. Stool samples were taken into sterile containers and delivered to the laboratory under appropriate conditions. All stool samples were checked for the *Bovine Coronavirus* (BcoV), *Bovine Rotavirus* (BRV) *group A*, *Escherichia coli* K99+, *Cryptosporidium parvum* and *Giardia* by using the Anigen Rapid BoviD-5 Ag rapid test kit (Bionote, Inc. Korea) according to the instructions of manufacturer. No pathogen was detected in these 50 stool samples which were included in the study.

Standard PCR Procedures

Stool samples were diluted with a 1:5 (weight/volume) ratio in PBS solution and homogenized with magnetic stirrer for 15 min (2 g stool sample were homogenized in 10 mL of PBS). The homogenate was centrifuged at 3.000 x g and the phage lysate or the homogenate was concentrated by purification. DNase (100 U/mL) was added in order to eliminate free DNA outside the phage particles in the suspension [11]. The phage DNA fraction was extracted from the 200 µL homogenate suspension by using the QIAamp DNA stool minikit (Qiagen GmbH, Hilden, Germany) according to the instructions of manufacturer [15].

qPCR Procedures

Quinolone resistance gene markers *(qnrA*, *qnrB* and *qnrS* genes) were analyzed by using the qPCR method in the LightCycler 480 system according to the instructions of manufacturer. Specific primers for *qnrA*, *qnrB* and

qnrS genes and the qPCR procedure were used for the detection of antimicrobial resistance gene markers [16]. qPCR reactions were performed in accordance with the instructions of manufacturer by using the specific primers, LightCycler 480 Sybr Green I Master kit. 5 µL template DNA and 15 µL PCR master mix (3 µL sterile water, 1 µL forward primer [10 mmol/L], 1 µL reverse primers [10 mmol/L] and 10 µL master mix) were added in 96-wells. qPCR melting analysis was performed for both internal control of DNA presence and specific *qnr* determination.

Results

Antimicrobial resistance gene markers of *qnrA, qnrB* and *qnrS* genes detected in phage DNA fractions obtained from 50 stool samples which were included in the study are shown in *Table 1*. Quinolone resistance gene markers were detected in the phage DNA fractions which were detected in a total of 11 samples (22%), whereas quinolone resistance gene markers were not detected in remaining 39 samples (78%). Among the quinolone resistance genes, the most commonly detected one was *qnrB* gene which was found in 7 (14%) samples.

Table 1. *Distribution of quinolone resistance gene markers in phage DNA fractions*

Result	Positive	
	n	%
Total qnrA	4	8
Total qnrB	7	14
Total qnrS	6	12

While *qnrA, qnrB* and *qnrS* quinolone resistance gene markers were detected together in one sample (6%), *qnrB* and *qnrS* gene resistance gene markers were detected together in two samples (4%) and shown in *Table 2*.

Table 2. *Distribution of quinolone resistance gene markers in phage DNA fractions*

Result	Positive	
	n	%
qnrA only	1	2
qnrB only	3	6
qnrS only	2	4
qnrA + qnrB	1	2
qnrA + qnrS	1	2
qnrB + qnrS	2	4
qnrA + qnrB + qnrS	1	2

Discussion

Antibiotic resistance is an important and expanding public health problem. For this reason, many studies are currently being conducted about the mechanisms and spread of antibiotic resistance. The contribution of bacteriophages to the mobilization of ARGs in the environment is known but this issue has not been extensively studied. However, recent studies suggest that phages play an important role in animal and human diseases [17-20]. Therefore, in our study, we focused on the detection of quinolone resistance gene markers in phage lysates in healthy calf stool samples.

It has been determined as a result of many studies that, phages have the potential to be a reservoir and vector for the acquisition of ARGs [20,21]. Furthermore, it has been shown in several studies that transfer of ARGs is done by phages through transduction in natural environments such as mud, wastewater, sediment, soil, animal and human stool [22-28].

In our study, ARG scanning was performed on healthy calf stool samples and the rate was determined as 22%. Although the mechanism of AMR spread is not known exactly, it is generally thought that it occurs as a result of unconscious antibiotic use in both humans and animals. It is known that there is a continuous flow of ARGs among humans, animals and the environment in which they form a triad on the ecosystem, and it is planned to carry out the necessary controls and applications at these 3 key points in order to prevent the flow of ARGs.

The contribution of phages to the spread of antibiotic resistance is not fully known. Some recent researches suggest that the role of ARG-bearing phages in the environment is much more important than previously thought [29]. For this reason, many studies have investigated the transport of ARGs in bacteriophage DNA fractions in samples of sludge, wastewater, sediment, soil, water, and animal and human stool. The key role played by phages in the construction of the bacterial microbiota of the human gut flora has been extensively investigated by Mills et al.[30], Scanlan [31], and Guerin and Hill [32]. Camarillo-Guerrero et al.[33] showed in their study that the gene flow produced by phages is not limited to a single bacterial species or genus, but they form gene flow networks among phylogenetically different bacteria. Phages dominate the viral fraction of the human gut microbiota [34,35]. Up to 1012 virus-like particles (VLP) per mL in human stool have been reported by Hoyles et al.[36]. Camarillo-Guerrero et al.[33] have identified more than 142,000 redundant viral genomes in the human gut, mostly belonging to phages. Dutilh et al.[37] and Edwards et al.[38] determined in their studies that crAssphage and crAss-like phages are quite common worldwide. CrAss-like phages are associated with Bacteroidetes, which is the most abundant bacteria phylum in the human gut microbiota [39].

In the study of Quirós et al.[11], within the stool of 80 healthy human, ARGs were detected in 70% of the samples. The

most detected genes in bacteriophage DNA fragments isolated in the study were *blaTEM, qnrA* and *blaCTX-M-1* genes. Brown-Jaque et al.[39], in their study, determined the rates of 9 ARGs *(blaTEM, blaCTX-M, blaCTX-M-9, blaOXA-48, qnrA, qnrS, mecA, sul1,* and *armA)* found in the bacteriophage DNA fragments obtained from 150 healthy human stool. 72% of the samples included in the study were positive for at least one ARG.

In several studies, fluoroquinolone resistance genes *(qnrA* and *qnrS)* were frequently detected in environmental samples [25-27]. Similarly, in our study, *qnrA* and *qnrS* genes were detected in DNA fractions of phages found in the healthy calf stool. Colomer-Llunch et al.[15] detected *qnrA* and *qnrS* in bacteriophage DNA fragments obtained from samples of urban wastewater, river water and animal stool and they suggested that *qnr*-encoding phages might be generalized transforming particles. It is thought that the presence of qnr-encoding phages is an important factor for the formation of quinolone resistant strains and the spread of ARGs [40].

To investigate the contribution of bacteriophages to the spread of resistance genes, in China, a large-scale screening for 32 ARGs was performed in pig stool from three different commercial farms. The most common gene detected as a result of this scan was the *qnrA* gene [41]. Transfer of ARGs to the environment is a critical issue for both human and animal health. In several other studies conducted about the bacteriophage DNA fractions, a large number of ARGs have been similarly detected in waters contaminated with human and animal stool [42,43]. Although the fact that our study is single-centred and performed with a low number of samples in which these seem to be our limitations, it is still valuable in terms of presenting a preliminary data on this situation in our country.

As a result, our data, which is the study on the antimicrobial resistance genes in phage fractions in our country, showed that a One Health approach is very important, because it has been found that bacteriophage fractions can be detected in the stool of healthy calves and quinolone resistance genes can be carried in these fractions. It has been concluded that antibiotics, quinolone groups particularly, which are frequently used in different areas, can be transported through these phages and so, the antibiotic applications should be done carefully regardless of the type of living organism, whether it is animal or human.

Availability of Data and Materials

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

Financial Support

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Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Statement

The study was conducted with the permission of the Veterinary Control Central Research Institute Local Ethics Committee (Approval no: 2022/24).

Author Contributions

SE, MD, DD and AY conceived and executed the idea, designed experiments, analyzed results and a deep revision of the manuscript. SE, AY, MD collected samples, performed experiments, contributed to tand implementation of the research. All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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Research Article

Factors Affecting Elective Course Preferences of Veterinary School Students in Türkiye

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Abstract: It is aimed to investigate the factors affecting the elective course preferences of veterinary students in Türkiye and to evaluate these factors according to specific socio-demographic criteria. "Elective Course Preference Attitude Scale" was applied to 765 students from 18 veterinary faculties. Data were analyzed with SPSS 25.0. Female students made up 52.4% of the participants. It was determined that the personal score was lower in men than in women. The environmental score was low in the 1st and 2nd grades, and in the 3rd and 4th grades, the structural score was high. While the additive score was low for the students studying in the Eastern, Central Anatolian and Black Sea regions, the environmental score was low in the Marmara Region. It was determined that the environmental score of the students studying in the faculties founded after 2007 was low. The fact that students need knowledge and experience in choosing elective courses reveals the effect of the friend factor while their expectation of contribution to personal and professional life reveals the effect of regional opportunities. The elective course pool to be prepared in line with the interests, wishes, and needs of the students will contribute to their motivation and self-efficacy perceptions. Choosing elective courses in line with the professional, academic and personal factors and the student's academic profiles will be beneficial in increasing learning and success levels.

Keywords: *Education, Elective course, Student, Türkiye, Veterinary medicine, Veterinary school*

Türkiye'de Veteriner Fakültesi Öğrencilerinin Seçmeli Ders Tercihlerine Etki Eden Faktörler

Öz: Türkiye'de veteriner fakültesi öğrencilerinin, seçmeli ders tercihlerine etki eden faktörlerin araştırılması ve tercihlerini etkileyen bu faktörlerin belirli sosyo-demografik kriterlere göre değerlendirilmesi amaçlandı. 18 veteriner fakültesinden 765 öğrenciye "Seçmeli Ders Tercihi Tutum Ölçeği" uygulandı. Veriler SPSS 25.0 ile analiz edildi. Katılımcıların %52.4'ünü kadın öğrenciler oluşturdu. Personel skorunun erkeklerde kadınlara göre düşük olduğu belirlendi. 1 ve 2. sınıflarda çevresel skor düşük, 3 ve 4. sınıflarda ise yapısal skor yüksek olarak belirlendi. Doğu, İç Anadolu ve Karadeniz Bölgesinde öğrenim gören öğrencilerde katkısal skor, Marmara Bölgesinde ise çevresel skor düşük olarak tespit edildi. 2007 yılından sonra kurulan fakültelerde öğrenim gören öğrencilerde çevresel skorun düşük olduğu belirlendi. Öğrencilerin seçmeli ders tercihinde bilgi ve tecrübeye ihtiyaç duymaları arkadaş faktörünün, kişisel ve mesleki yaşama katkı beklentileri ise bölge olanaklarının etkisini ortaya koymaktadır. Öğrencilerin ilgi, istek ve ihtiyaçları doğrultusunda hazırlanacak seçmeli ders havuzunun motivasyon ve öz yeterlik algılarına katkı sağlayacağı; mesleki, akademik ve kişisel faktörler ile öğrencilerin akademik profilleri doğrultusundaki seçmeli ders tercihinin öğrencilerin öğrenme ve başarı düzeylerini artırmada yararlı olacağı söylenebilir.

Anahtar sözcükler: *Eğitim, Öğrenci, Seçmeli ders, Türkiye, Veteriner fakültesi, Veteriner hekimliği*

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Introduction

Obtaining a veterinary school diploma in Türkiye requires the completion of a five-year (10 semesters) education in a veterinary school. Despite the different systems applied in veterinary schools, the curricula are developed in the context of the minimum requirements set by the European Association of Veterinary Education Organizations (EAEVE) and the World Veterinary Medical Association and implemented in all veterinary schools in Türkiye [1]. Veterinary schools in Türkiye are divided into divisions, and each division is divided into departments. Despite some minor differences in the organization of departments, there are five main divisions in veterinary schools. As a result of increasing contacts with EAEVE, coordination among veterinary schools is increased, and thus Turkish veterinary medicine schools adapt to the European education system. This alignment includes audit visits by EAEVE expert committees to assess the suitability of faculties with the European education system.

As the curriculum content of veterinary schools reaches significant proportions following advances in medical knowledge and biotechnology, it becomes impossible to ensure that all students at the beginning of their careers have such a broad knowledge base [2]. Therefore, the education curriculum should increase students' ability to adapt to changing conditions, instill the desire and ability to work in teams, and develop their life skills. Academic education and innovations are expected to awaken the intellectual curiosity and commitment to lifelong learning that graduates will need to maintain the confidence of the society where they will work in the future [3].

Problem-based learning amplifies life experiences to promote learning, integration of knowledge, and lifelong learning skills, which are essential for veterinary education. In addition to problem-based learning, there is a need to continuously evaluate the teaching strategies used in veterinary medicine schools to ensure effective learning [2].

Veterinary educators are responsible for preparing students for the profession through an intensive and professional training program. This program requires training in many areas so that students become proficient in the expected knowledge, skills, and abilities of veterinary medicine [4]. In this context, counselors can help students choose the courses that best meet their needs by encouraging them to consider their interests and motivations [5].

Elective courses (EC) are optional courses explicitly designed to increase and reinforce knowledge, facilitate academic study, and increase student participation in the scientific research process [6]. The choice of ECs is an essential factor in which the student will discover interests and abilities in own professional career, test and develop knowledge and experience as a result of own decisions, and prepare for the future [7,8]. This course format, common in higher education, offers students the chance to receive a "multi-dimensional" education outside a predetermined lesson plan [6]. It is also reported that it is a model that provides flexibility in choosing the course and maintains its popularity [9].

ECs not only help students accumulate enough credits to complete their undergraduate degrees but also provide knowledge beyond the core subject to increase achievements. For this reason, the importance of supporting knowledge must first be established in the student's mindset. Therefore, it is important to reveal the preferences of ECs in order to manage demand and supply. Understanding the patterns and determinants of demand, including understanding why one elective is preferred over another, should aid in planning ECs offerings and resources (e.g. facilities and instructors) [5].

In general, ECs aim to develop individuals socially and culturally. It is emphasized that there is a relationship between the success obtained from these courses and culture, art, sports, health activities, environmental awareness, and communication skills [10]. However, it is reported that in veterinary schools, where ECs are offered in addition to the compulsory course load, students' interest in ECs decreases due to the intensity of the courses [11]. For ECs to be successful, students' interests and wishes must be prioritized, and appropriate conditions must be provided [7]. For this reason, a flexible curriculum that provides a solid basic education, and sufficient time for relevant ECs, should be the cornerstone of veterinary education [11].

The associate and undergraduate education and training regulations of universities in Türkiye set out the courses that are planned to be taught and included in the ECs pool, and the procedures and principles to be followed for taking these courses [12-14]. According to the "European Veterinary Education Evaluation System Standard Operating Procedure Guide", each student should freely choose the course from the list of allowed courses due to the nature of the EC. In the guide, it is stated that the total number of courses that each student will take from various course groups, the selection procedures of the ECs, and the definition of the degree of freedom in their selection (the number of students required to open the ECs, etc.) should be made [15].

This study aimed to investigate the factors affecting the ECs preferences of the students studying at veterinary schools located in different geographical regions of Türkiye and to evaluate these factors according to specific socio-demographic criteria.

Material and Methods

Ethical Statement

This study was carried out with the approval of Selcuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee (SÜVDAMEK) dated 27.02.2020 and numbered 2020/24.

Data Collection

The study material consisted of data from 765 students studying at 18 different veterinary schools in Türkiye.

The "Elective Course Preference Attitude Scale" developed by Aslım et al.[16] was used. The data for this study was obtained anonymously, and the confidentiality of the participants was protected.

A sample size of 765 achieves 84.259% power to detect an effect size (W) of 0.1000 using 5 degrees of freedom Chi-Square Test with a significance level (alpha) of 0.05000.

Statistical Analysis

The scale was applied to the students between 26.03.2021 and 14.04.2021, and the data were obtained. SPPS 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) statistical package program was used to evaluate the data obtained in the study. Mean±standard deviation and Median (Maximum-Minimum) percentage and frequency values were used for the variables. Variables were evaluated with the Shapiro-Wilk and Levene Test after controlling for normality and homogeneity of variances. While performing data analysis, Independent two-group t-test (Student's t-test) for two-group comparison, Mann Whitney-U test when prerequisites are not met, One-Way Analysis of Variance (One-way ANOVA) for three or more group comparisons, and Tukey from multiple comparison tests. Kruskal Wallis test and Bonferroni-Dunn test, one of the multiple comparison tests, were used when the HSD test did not provide it. Categorical data were analyzed with Fisher's Exact Test and Chi-Square test. In cases where the expected frequencies were less than 20%, the Monte Carlo Simulation Method was used to include these frequencies in the analysis. The values of $p<0.05$ and $p<0.01$ were accepted for the significance level of the tests.

Results

A total of 765 students, 124 were from the Mediterranean Region, 98 were from the Eastern Anatolia Region, 113 were from the Aegean Region, 95 were from the Southeastern Anatolia Region, 110 were from the Central Anatolia Region, 84 were from the Black Sea Region and 141 were from the Marmara Region. The number of female students was 401 (52.4%) and 364 (47.6%) male. 146 (19.1%) of the participants were 1st-grade, 207 (27.1%) were 2nd-grade, 148 (19.3%) were 3rd-grade, 119 (15.6%) were 4th-grade, and 145 (19.0%) were of 5th-grade students. The findings regarding the factors affecting the choice of ECs according to the regions where the faculties are located, the date of foundation, the grade of the students, and their gender are presented in *Table 1*.

In the study, it was examined whether gender affected the attitude of choosing ECs. The additive score showed statistical similarity according to gender. Personal score is lower for men than for women. Structural, Instructor, and Environmental scores are not distributed differently by gender *(Table 1)*.

It was examined whether the students' attitudes towards ECs changed according to the geographical regions they studied. The additive scores of students studying in Eastern Anatolia, Black Sea, and Central Anatolia regions are statistically lower than in other regions. Personal, Structural, and Instructor scores are statistically similar according to the education regions. The Environmental score of the students studying in the Marmara region was statistically lower than those in other regions *(Table 1)*.

Considering the differences in the ECs preference attitudes of the universities according to the foundation date, Additive, Personal, Structural, and Instructor scores are statistically similar according to the university foundation dates. The Environmental score of the universities founded in 2007 and later was statistically lower than those founded in other years *(Table 1)*.

Additive scores of students in the attitudes of choosing ECs according to the class they are studying were found to be statistically lower in 4th and 5th-grade students compared to other classes. The personal score of the students studying in the 5th grade was observed to be lower than the other classes. The structural score is statistically similar according to classes. Instructor scores of 3rd and 4th-grade students are higher than others. The environmental score was lower in 1st and 2nd-grade students compared to other grades *(Table 1)*.

The degree of participation of the students to the factors (additive, personal, structural, instructor, and environmental) in the ECs preference attitude scale was determined statistically. The frequency and percentage values of the obtained data are presented in *Table 2*.

Discussion

Universities generally contribute to economic growth and national competitiveness by equipping students with higher-order thinking and academic skills [17]. In university education, students are offered many ECs apart from the compulsory courses and are given a choice that can meet the student's wishes [18]. In this freedom of choice, the friend

Table 1. *Factors affecting the choice of ECs according to socio-demographic data*

Factors		n	%	Factor 1 Additive	Factor 2 Personal	Factor 3 Structural	Factor 4 Instructor	Factor 5 Environmental
The range of points to be obtained from the factor (min.-max.)				6 - 30	5 - 25	10 - 50	4 - 20	3 - 15
Descriptive statistics				M (IQR)	M (IQR)	M (IQR)	M (IQR)	M (IQR)
Gender	Female	n=401	52.4%	27.0 (6.0)	21.0 (5.0)	38.0 (8.0)	15.0 (5.0)	11.0 (6.0)
	Male	n=364	47.6%	26.0 (6.0)	20.5 (5.0)	39.0 (9.0)	15.0 (5.0)	11.0 (5.0)
Test Statistics (*z* value)				0.579	2.214	1.535	0.911	1.804
P				0.562	**0.027**	0.125	0.362	0.071
Geographical Region	Mediterranean	n=124	16.2%	27.0 (6.0)^{ab}	21.0 (4.0)	39.0 (8.0)	15.0 (3.0)	10.0 (5.0)^{ac}
	Eastern Anatolia	n=98	12.8%	25.0 (5.2)^{b}	20.0 (5.2)	39.0 (9.2)	15.0 (5.0)	12.0 (6.0)^{b}
	Aegean	n=113	14.8%	27.0 (6.0)^{bc}	21.0 (4.0)	40.0 (8.0)	16.0 (5.0)	12.0 (5.0)^{b}
	Southeastern Anatolia	n=95	12.4%	28.0 (7.0)^{ab}	22.0 (5.0)	38.0 (10.0)	15.0 (4.0)	11.0 (5.0)^{bc}
	Central Anatolia	n=110	14.4%	25.0 (7.0)^{b}	20.0 (5.0)	38.0 (8.5)	14.0 (5.0)	11.0 (4.0)^{b}
	Black Sea	n=84	11.0%	25.0 (7.0)^{b}	21.0 (4.0)	39.5 (7.0)	14.0 (5.0)	12.0 (5.7)^{b}
	Marmara	n=141	18.4%	27.0 (6.0)^{ac}	21.0 (5.0)	38.0 (9.0)	15.0 (5.0)	9.0 (6.0)^{a}
Test Statistics (*H* value)				13.412	11.313	11.052	3.167	27.606
P				**0.037**	0.079	0.087	0.788	**<0.001**
Founding Date of the Faculty	1985 and before	n=118	15.4%	26.0 (6.0)	20.0 (4.5)	39.0 (9.0)	14.5 (5.0)	11.0 (6.0)^{a}
	1986-2006	n=445	58.2%	27.0 (7.0)	21.0 (5.0)	39.0 (8.0)	15.0 (5.0)	11.0 (6.0)^{a}
	2007 and after	n=202	26.4%	27.0 (6.0)	21.0 (4.2)	38.0 (8.2)	15.0 (4.0)	10.0 (6.0)^{b}
Test Statistics (*H* value)				3.113	4.409	0.371	2.149	20.951
P				0.211	0.110	0.831	0.342	**<0.001**
Grade	1st grade	n=146	19.1%	28.0 (6.0)^{a}	22.0 (4.0)^{a}	38.5 (7.2)	15.0 (5.0)^{a}	10.0 (5.0)^{a}
	2nd grade	n=207	27.1%	27.0 (6.0)^{a}	21.0 (5.0)^{ab}	37.0 (10.0)	14.0 (4.0)^{a}	10.0 (6.0)^{a}
	3rd grade	n=148	19.3%	26.0 (6.0)^{ab}	21.0 (5.0)^{ab}	39.0 (9.0)	16.0 (5.0)^{ab}	11.5 (6.0)^{b}
	4th grade	n=119	15.6%	25.0 (6.0)^{b}	21.0 (4.0)^{ab}	39.0 (7.0)	16.0 (4.0)^{ab}	12.0 (6.0)^{b}
	5th grade	n=145	19.0%	25.0 (8.0)^{b}	20.0 (5.0)^{b}	38.0 (9.5)	15.0 (5.0)^{a}	12.0 (6.0)^{b}
Test Statistics (*H* value)				21.888	9.600	8.041	10.357	30.429
P				**<0.001**	**0.048**	0.090	**0.035**	**<0.001**

M: Median, IQR: Interquartile range, H value: Kruskal Wallis H test, z: Mann Whitney U test

Table 2. Frequency and percentage values of the ECs preference attitude scale item pool

Questions	Frequency and Percentage Values									
	Absolutely I agree		I agree		I am undecided		I do not agree		Absolutely I do not agree	
	n	%	n	%	n	%	n	%	n	%
Factor 3: Structural										
1. “The content of the course” is effective in my choice of ECs.	359	46.9	284	37.1	52	6.8	38	5.0	32	4.2
2. The “course selection system” has an effect on my choice of ECs.	212	27.7	305	39.9	100	13.1	95	12.4	53	6.9
3. “The way the course is taught (traditional lecture, student research, student presentation, etc.)” is effective in my choice of ECs.	252	32.9	290	37.9	92	12.0	80	10.5	51	6.7
4. “Class hours (morning or afternoon)” is effective in my choice of ECs.	227	29.7	225	29.4	103	13.5	109	14.2	101	13.2
5. “The fact that it is close to the courses I have taken so far and that I have been successful in” is effective in my choice of EC.	233	30.5	296	38.7	111	14.5	76	9.9	49	6.4
6. “ECs I have taken before” are also effective in choosing EC.	200	26.1	290	37.9	119	15.6	94	12.3	62	8.1
7. “Whether the course is applied or not” is effective in my choice of EC.	273	35.7	259	33.9	123	16.1	54	7.1	56	7.3
8. “Whether the course I will take is up-to-date or not” is effective in my choice of ECs.	308	40.3	273	35.7	97	12.7	47	6.1	40	5.2
9. “Whether the course is encouraging for research or not” is effective in my choice of EC.	256	33.5	258	33.7	128	16.7	74	9.7	49	6.4
10. “No absenteeism problem” is effective in my choice of EC.	268	35.0	195	25.5	119	15.6	97	12.7	86	11.2
Factor 4: Instructor										
11. “My views about the lecturer giving the course” are effective in my choice of EC.	439	57.4	216	28.2	46	6.0	33	4.3	31	4.1
12. “The academic career of the lecturer (Prof.Dr.–Assoc. Prof.– Asst.Prof.)” is effective in my choice of ECs.	152	19.9	170	22.2	154	20.1	139	18.2	150	19.6
13. “The faculty members I consulted” are effective in my choice of ECs.	145	19.0	212	27.7	150	19.6	133	17.4	125	16.3
14. “Examination system (written, oral, test, etc.) of the lecturers giving the course” is effective in my choice of ECs.	298	39.0	246	32.2	87	11.4	78	10.2	56	7.3
Factor 1: Additive										
15. “The fact that it can contribute to my professional life” is effective in my choice of ECs.	474	62.0	214	28.0	43	5.6	17	2.2	17	2.2
16. “The fact that it can contribute to my academic development” is effective in my choice of ECs.	420	54.9	226	29.5	69	9.0	26	3.4	24	3.1
17. “The fact that the course can contribute to my personal development” is effective in my choice of ECs.	394	51.5	247	32.3	70	9.2	23	3.0	31	4.1
18. “The fact that the course can contribute to my general culture” is effective in my choice of ECs.	327	42.7	279	36.5	90	11.8	31	4.1	38	5.0
19. “The fact that it can increase my theoretical knowledge” is effective in my choice of ECs.	314	41.0	280	36.6	98	12.8	36	4.7	37	4.8
20. “The fact that it can increase my practical skills” is effective in my choice of ECs.	389	50.8	230	30.1	76	9.9	36	4.7	34	4.4
Factor 2: Personal										
21. My personal interests are effective in my choice of ECs.	440	57.5	233	30.5	37	4.8	25	3.3	30	3.9
22. My “personal abilities” are effective in choosing ECs.	339	44.3	252	32.9	97	12.7	42	5.5	35	4.6
23. My personal expectations are effective in my choice of ECs.	375	49.0	264	34.5	66	8.6	28	3.7	32	4.2
24. My “expectations about academic life” are effective in my choice of ECs.	325	42.5	235	30.7	105	13.7	47	6.1	53	6.9
25. “The fact that my grade point average can increase” is effective in my choice of ECs.	319	41.7	216	28.2	108	14.1	69	9.0	53	6.9
Factor 5: Environmental										
26. “Students who have taken that course before” are effective in my choice of ECs.	271	35.4	221	28.9	98	12.8	90	11.8	85	11.1
27. “Courses chosen by my circle of friends” are effective in my choice of ECs.	189	24.7	220	28.8	134	17.5	109	14.2	113	14.8
28. “The opinions of upper-class students even though they do not take that course” are effective in my choice of ECs.	172	22.5	219	28.6	104	13.6	122	15.9	148	19.3

factor is stated as the essential element of the theme of factors consisting of the person responsible for the course, the content of the course, credit filling, and transportation factors [19]. Students can be affected by their friends' preferences in choosing ECs, and their preferences can also change according to the number of people choosing the course [20]. In the study, it was determined that the ECs preference attitudes of the universities differ according to the foundation date, and the "environmental" score of the students studying at the universities founded before 2007 was statistically higher than the students studying at the universities founded in other years *(Table 1)*. The students prioritize the environmental factors *(Table 2*, Factor-5) that may guide them compared to the students in other institutions. The friend factor is influential in choosing ECs, possibly due to the students' need for experience and knowledge.

It is reported that universities with a long history in the big cities [21] and universities located in metropolitan cities are relatively more prestigious and preferred. It has also been revealed that the social demand for higher education in Türkiye is a conscious demand driven by the popularity and quality of universities rather than an effort to enter any higher education institution [22]. Especially Istanbul and Ankara are among the centers where universities are most located in Türkiye. This situation has severe opportunities regarding population, economic development levels, technical infrastructure, and faculty members and naturally affects the differences between regions [23]. In the study, it was determined that the student's attitudes towards ECs preferences changed according to the regions where they were educated, and the "environmental" score of the students studying in the Marmara region was statistically lower than those studying in other regions *(Table 1)*. When the possibilities of the Marmara region are evaluated, it can be stated that the expectations of the students who may have chosen the Marmara region as career-centered may also be high. It can be said that they chose the course with a more decisive demand such as the contribution they can make to their personal and professional life (additive factor) *(Table 1*, Factor-1) without being affected by the friend and student factor *(Table 1*, Factor-5).

The fact that students can choose ECs according to their needs and interests in university education affects their academic success positively. It contributes positively to their characteristics, such as motivation and self-efficacy. Motivation stems from the individual's personal, social, educational, and professional needs, and is closely related to academic success [24]. ECs recommendation systems, which are tried to be developed in this direction, not only benefit undergraduate students who need advice on course selection in various fields but also focus on improving course selection processes during pre-registration [25]. The study determined that the "additive" scores of the students studying in the Eastern Anatolia, Central Anatolia, and Black Sea regions were statistically low *(Table 2*, Factor-1). In contrast, the "environmental" scores were high *(Table 2*, Factor-5). The ECs to be opened within the scope of the interests, wishes, and needs of the students studying in the mentioned regions may contribute to their motivation and self-efficacy perceptions in parallel with the above study data. Thus, they can also come to the fore in "additive" factors such as professional, academic, and personal factors that are important in choosing ECs. In this context, offering ECs to students according to their academic profiles with ECs suggestion systems can also be beneficial.

It has been reported that students studying at veterinary schools have intensified their applied courses (clinic, laboratory, etc.) since the 3rd -grade. Therefore, they have difficulty coping with stress due to the intense curriculum content [26]. The essential relationship between instructor motivations and students' learning experiences must be addressed but explored in depth. A good instructor can motivate students even in the most boring subject and significantly increase their learning ability [27]. Instructors' motivation is considered an important factor for their cognition and experience and for students' learning experiences [28]. The students' opinions about the instructor are also very effective in selecting ECs [29]. In the study, when the students' attitudes towards ECs preference according to the class they study were examined, it was determined that the "instructor" score of the 3rd and 4th-grade students was higher than the other classes *(Table 1*, Factor-4). In the ECs preference of the students, it was found that the opinions of the lecturer who gave the course had the highest rate (57.4%, n=439) within the education factor *(Table 2*, Factor-4). In parallel with the above study data, the instructor factor greatly affected the students. Due to the pressure created by the intensive course curriculum and the anxiety of passing the course and grade, it can be stated that all the characteristics of the faculty members, such as their behaviors, scientific capacity, teaching, and grading, are effective factors in the choice of ECs. In addition, the high "environmental" scores of 3rd, 4th and 5th-grade students *(Table 1*, Factor-5) indicate that students take into account the opinions of others (environmental factor) about perceived instructor quality when choosing courses. This situation supports using the instructor and social impact framework in analyzing the student's course selection.

The study of McKenzie and Schweitzer [30] on 1st-grade students at a university in Australia revealed that academic achievement from the past is the most important

determinant of university adjustment. Some studies [31-33] indicate that university students' level of adjustment to higher education life, according to gender, shows no differentiation. 1st-grade veterinary students' concerns about the quality of teaching, the number of courses, the course grades, and the standards and general requirements in some courses are reported as important stress factors [26]. For students in the 2nd and higher grade, the change in interest is much less. A study on determining the shred ECs preferences of 2nd-grade undergraduate students states that the selected courses may differ according to the student's field of interest [8]. In the study, the 1st and 2nd-grade students were less affected by environmental factors (students taking the course, their friends, and the opinions of upper-class students) in choosing ECs compared to other classes (P<0.05) *(Table 1*, Factor-5). In light of this, it can be stated that this situation may have occurred due to the role of "person-environment harmony" and students' interests. The role of these factors in choosing ECs should be investigated in more detail.

It is important to determine students' ECs expectations. This information can strengthen and support intrinsic and/or extrinsic motivations and thus help improve student engagement and learning outcomes [34]. Many factors influence choosing ECs, including personal and professional development, interests, and attractiveness. It is reported that students firstly believe that these courses will contribute to their personal development, secondly, they think that these courses will be beneficial for their professional development, and finally, they prefer these courses because they think that they are suitable for their field [24]. In a study conducted with 3rd and 4th-grade students of the Biology, Physics, and Chemistry departments of the Faculty of Arts and Sciences, the factors affecting the course selection of female students were; In the first degree, it is reported that the opinions of the faculty members, the way the course is taught in the 2nd- grade, and personal interests in the 3rd- grade are effective [29]. In the study, when the effect of gender on the attitude toward ECs preference was examined, it was seen that the "personal" score of female students was higher than that of males *(Table 1)*. In addition, it was seen that the ability to contribute to the professional life was the first choice in the preference of ECs (62.0%, n=474), personal interests were the second (57.5%, n=440), and the views about the instructor who gave the course were the third (57.4%, n=439) (*Table 2*). Female students emphasize to personal development in their ECs preferences because they think that these courses will contribute to their personal development and increase their grade point averages *(Table 2*, Factor 2). Ulusoy et al.[24] are parallel with the study data, and Tezcan and Gümüş [29] prioritize the additive, personal, and instructor factors, respectively, unlike the study data, pointing out that the order of factors may change among students studying in different faculties. In addition, following the findings of Tezcan and Gümüş [29], it can be said that the teaching style of the instructor and the grading system may be an effective factor that should be taken into account in the choice of ECs in general.

As a result, the fact that the courses can contribute to the professional life and that their interests are at the forefront in the ECs preferences indicates that the students are open to self-development and make an effort to access the information they need. Therefore, a wide range of ECs based on improving student participation and learning outcomes is needed so that students can choose courses that can contribute positively to their characteristics, such as intrinsic motivation, interests, and self-efficacy, and thus strengthen their potential to increase learning and achievement levels.

In the future, it is thought that determining students' perspectives on ECs during their education, together with their justifications, will help enrich the content of in-field and out-of-field ECs and increase the variety of ECs according to needs, taking into account the advantages and disadvantages of faculties in different geographical regions. Orientation activities are critical, especially for 1st- grade students, to know about their faculties and the courses they will take in terms of person-environment harmony. Therefore, it can be said that restructuring such promotional activities in the light of the obtained data, determining new roadmaps for the needs that may change in the future, and putting more emphasis on study skills and academic success in terms of career development, taking into account the gender factor, can make a difference in choosing the right course. It is also thought that revealing the reasons why one EC is preferred over another will help in the planning of EC offers and available resources (facility, equipment, etc.), and thus allow sufficient resources to be allocated to ECs with high demand.

Availability of Data and Materials

The data supporting this study's findings are available on request from the corresponding author (A. Yiğit). The data are not publicly available due to privacy or ethical restrictions.

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Competing Interests

The authors declared that there is no conflict of interest.

Author Contributions

G. Aslım, A. Yaşar and A. Yiğit conceived and executed the idea, G. Aslım and A. Yaşar designed questionnaire, G. Aslım, M. A. Tekindal and E. Çelik collected data, G. Aslım and M. A. Tekindal analyzed results, G. Aslım, A. Yaşar, E. Çelik and A. Yiğit wrote the manuscript, G. Aslım and A. Yiğit detailed revision of the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the research and approved it for publication.

Ethical Approval

This study was carried out with the approval of Selcuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee (SÜVDAMEK) dated 27.02.2020 and numbered 2020/24.

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RESEARCH ARTICLE

Host Preferences of Vector *Culicoides* (Diptera, Ceratopogonidae, *Culicoides* Latreille) Species in Türkiye

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Abstract: Determination of host preferences of blood-feeding arthropods is an important criterion for how ectoparasite-host interactions, host selection, and feeding behaviour affects pathogen transmission. This study aimed to examine the host's blood from engorged *Culicoides* species collected from cattle farms in Turkey to shed light on their host preferences. For this purpose, we investigated the blood of 5 different hosts (cattle, sheep, dog, horse, and human) in 7 *Culicoides* species/species complexes by multiplex-PCR analysis, considering the domestic animals on the farms. Engorged *Culicoides* specimens were collected from places in Turkey with different geographical and ecological characteristics. A total of 1225 female *Culicoides* belonging to 7 different species/species complexes namely *C. obsoletus* complex (*C. obsoletus* and *C. scoticus*) (n:450), *C. schultzei* complex (n:234), *C. imicola* (n:208), *C. punctatus* (n:162), *C. newsteadi* (n:144), *C. lupicaris* (n:24), and *C. pulicaris* (n:3) were analysed for host blood identification. Abdomens of the engorged midges were separated from their body and pooled according to the date of collection and species. A total of 69 pools consisting of 1-28 specimens were analysed by multiplex-PCR and only cattle blood was detected in all pools. This study presents the first data on the identification of host preference of some *Culicoides* species in Turkey.

Keywords: *Blood meal preferences, Culicoides, multiplex-PCR, Türkiye.*

Türkiye'de Vektör *Culicoides* (Diptera, Ceratopogonidae, *Culicoides* Latreille) Türlerinin Konak Tercihlerinin Belirlenmesi

Öz: Kanla beslenen artropodların konak tercihlerinin tanımlanması, ektoparazit-konak etkileşimleri, eklembacaklıların konak seçimi ve beslenme davranışının patojen bulaşmasını nasıl etkilediği konularında önemli bir kriterdir. Bu çalışmanın amacı, Türkiye'deki sığır çiftliklerinden toplanan doymuş *Culicoides* türlerinden konakçı kanını inceleyerek konak tercihlerine ışık tutmaktır. Bu amaçla çiftliklerde bulunan evcil hayvanlar göz önünde bulundurularak 7 *Culicoides* tür/tür kompleksinde 5 faklı konağın (sığır, koyun, köpek, at ve insan) kanını multiplex-PCR testi ile araştırdık. Doymuş *Culicoides* örnekleri Türkiye'de farklı coğrafi ve ekolojik özelliklere sahip yerlerden toplanmıştır. *Culicoides obsoletus* complex (including *C. obsoletus* and *C. scoticus*) (n:450), *C. schultzei* complex (n:234), *C. imicola* (n:208), *C. punctatus* (n:162), *C. newsteadi* (n:144), *C. lupicaris* (n:24) ve *C. pulicaris* (n:3) olarak belirlenen 7 farklı tür/tür kompleksine ait toplam 1225 adet *Culicoides* konak kanı tespiti için analiz edildi. Doymuş dişilere ait abdomenler gövdelerinden ayrılarak toplanma tarihlerine ve türlerine göre havuzlar oluşturuldu. Toplam 1-28 sinekten oluşan 69 havuz multiplex-PCR ile analiz edildi ve tüm örneklerde sadece sığır kanı tespit edildi. Çalışmamız Türkiye'de *Culicoides* türlerinin konak tercihinin belirlenmesi konusunda ilk verileri sunmaktadır.

Anahtar sözcükler: *Kan emme tercihi, Culicoides, multiplex-PCR, Türkiye.*

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Introduction

Culicoides (Diptera: Ceratopogonidae) are among the smallest hematophagous flies in the world, measuring 1-3 mm in size. The genus is of great interest for its role as biological vector of pathogens of medical and veterinary importance. In addition to several nematode and protozoan species, more than 50 arboviruses have been isolated from *Culicoides* species [1-3]. Their role in transmitting diseases such as bluetongue virus (BTV), epizootic haemorrhagic disease virus (EHDV), African horse sickness virus (AHSV), Akabane virus, and Schmallenberg virus which causes economic losses in the livestock industry is the main reason for large scale investigation of this genus [4,5]. They are found on nearly all major landmasses from sea level to 4000 m [3]. A total number of 1347 species have been described so far [6]; 72 species have been reported from Turkey [7,8].

Culicoides species can cause severe itchy skin reactions in humans and animals, forcing the hosts to relocate or to flee indoors [1]. Their ability to transmit the Oropouche virus (OROV), the etiological agent of the febrile illness Oropouche fever, among humans has been the most important role of *Culicoides* species in public health to date [3]. A wide variety of insights can be obtained by examining the blood on which arthropods feed, such as the evolution of host specificity between vertebrates and their ectoparasites, ectoparasite-host interactions, the ecology of infectious diseases, how arthropod host selection and feeding behaviour affect pathogen transmission, and the economic and wellness effects of ectoparasite infestation on domestic animals and wildlife [9].

The vast majority of *Culicoides* species feed on mammals and birds; however, reptiles and amphibians are also in the host spectrum of these flies [1,10-12]. Although feeding frequency varies by species and environmental conditions, host availability plays an important role in the feeding behaviour of biting midges in general. Most of the *Culicoides* species are mammalophilic and ornithophilic, although some species are known to feed on reptiles and frogs [1,13-16]. As a result of the molecular identification of vertebrate hosts of *Culicoides* species in Europe, 45 different host species, including 33 bird species and 12 mammal species, were identified [17].

While serological methods were used to determine host preferences in the past [18,19], these have now been replaced by molecular methods. Advances in polymerase chain reaction (PCR)-based assays and molecular techniques for blood-meal analysis using direct sequencing of the cytochrome b (*cytb*) gene allow for species-level identification of hosts with a much higher degree of accuracy than has been achieved with previous serological assays [9-12,20-26]. This study aimed to identify the host's blood in engorged *Culicoides* species collected from cattle farms with other domesticated animals to understand their feeding preferences. This is the first study in Turkey to analyse the host's blood based on a segment of the mitochondrial gene, *cytb*, in *Culicoides* species.

Material and Methods

Study Area

The stations where *Culicoides* specimens were collected were selected from places in Turkey with different geographical and ecological characteristics (*Table 1*).

Collection of *Culicoides* Specimens

In this study, Onderstepoort Veterinary Institute (OVI) type light traps working with 220V, 8 Watt black fluorescent light and downward fan with a 4 mm net around to prevent the entry of large arthropods were used. Light traps were placed approximately 1.5-2 m above ground out of animals' reach and operated one hour before sunset until one hour after sunrise in cattle farms, where there were at least 20 cattle. There were few sheep, dogs, and a few horses in and around these farms. The samples were obtained from 24 cattle farms in 20 provinces by running traps once a month for 14 months between April-2019 and October-2020 (*Table 1*). Collected *Culicoides* specimens were morphologically identified under the stereomicroscope using the identification key for Palaearctic *Culicoides* species [27]. *Culicoides schultzei* complex consists of several species (*C. oxystoma*, *C. schultzei*, *C. subschultzei*, *C. kingi*, *C. rhizophorensis*, *C. enderleini*, *C. nevilli*, and *C. neoschultzei*) [28]. Since there is no detailed identification key on the complex, specimens were identified as *C. schultzei* complex. In this study, the abdomens of engorged fully females were separated and pooled according to their species and date of collection, DNA extraction was performed and the DNA samples were stored at -20°C until use.

DNA Extraction and Validation with Blood Samples

Blood of from domestic hosts (cattle, sheep, dog, and horse) was obtained from the animal hospital of Kafkas University Faculty of Veterinary Medicine and human blood obtained from the University Research Hospital. DNA extraction was conducted from each blood sample by using QIAmp DNA Mini Kit (Qiagen, GmbH, Hilden, Germany) in order to obtain positive controls in the PCR assay. These hosts were chosen for the assay because they were potential targets where traps were placed. Abdomens of engorged *Culicoides* females were separated from their bodies and pooled (1-28 per tube) in separate tubes according to date and species. Each pool was digested in 200 mL lysis buffer in tubes with steel balls and extracted according to the kit procedure (Analytik Jena AG, AJ Innuscreen GmbH, Berlin, Germany).

Table 1. *Summary of locations where blood-fed Culicoides were collected*

Province	District/Village	Species	Latitude	Longitude	Altitude (m)	Type of Animals Around the Trap	Blood-Fed Midges, n	Trap Localisations
Artvin	Ardanuç	*C. newsteadi*	41.114820	42.066060	489	Cattle, dog	40	Indoor
		C. obsoletus complex					217	
		C. punctatus					121	
		C. lupicaris					23	
Ankara	Çubuk	*C. newsteadi*	40.118740	32.944540	956	Cattle, sheep, dog	2	Outdoor
Edirne	Merkez/ Budakdoğanca	*C. newsteadi*	41.760760	26.340980	116	Cattle, dog, horse	71	Outdoor
		C. obsoletus complex					5	
		C. punctatus					13	
		C. pulicaris					3	
Edirne	Merkez/İskender	*C. newsteadi*	41.630751	26.669324	93	Cattle, dog, horse	21	Indoor
		C. punctatus					4	
Antalya	Kaş	*C. newsteadi*	36.339800	29.327030	11	Cattle, dog	4	Outdoor
Erzincan	Merkez/Çatalören	*C. newsteadi*	39.664770	39.518180	1191	Cattle, dog	6	Outdoor
Trabzon	Sürmene	*C. obsoletus* complex	40.891111	40.056944	75	Cattle, dog	32	Indoor
Rize	Fındıklı	*C. obsoletus* complex	41.253880	41.156770	30	Cattle, dog	137	Indoor
Mersin	Anamur	*C. obsoletus* complex	36.133480	32.859470	54	Cattle, sheep, dog	8	Indoor
		C. imicola					84	
Mersin	Silifke	*C. schultzei* complex	36.335278	34.000833	0,3	Cattle, dog	16	Outdoor
Samsun	Atakum	*C. obsoletus* complex	41.433580	36.084770	204	Cattle, dog	15	Indoor
		C. lupicaris					1	
Bursa	İznik	*C. obsoletus* complex	40.541110	29.834200	850	Cattle, sheep, dog	2	Indoor
Kastamonu	Araç	*C. obsoletus* complex	41.218780	33.381380	852	Cattle, sheep, dog	23	Indoor
Erzurum	Şenkaya	*C. obsoletus* complex	40.640000	42.338889	1247	Cattle, dog	2	Indoor
Erzurum	Şenkaya/Aydoğdu	*C. punctatus*	40.701246	42.471634	1631	Cattle, dog	20	Indoor
Kırklareli	Vize	*C. obsoletus* complex	41.706903	27.704541	430	Cattle, sheep, dog	2	Indoor
Balıkesir	Karasi	*C. obsoletus* complex	39.894986	27.843145	280	Cattle, sheep, dog	3	Indoor
Giresun	Merkez	*C. obsoletus* complex	40.910409	38.313453	35	Cattle, dog	4	Indoor
Burdur	Merkez	*C. punctatus*	37.633690	30.106700	875	Cattle, dog	4	Outdoor
Hatay	Arsuz	*C. imicola*	36.406389	35.891111	7	Cattle, dog	49	Outdoor
		C. schultzei complex					101	
Diyarbakır	Sur	*C. imicola*	37.961110	40.428700	641	Cattle, dog	5	Indoor
Diyarbakır	Bismil	*C. schultzei* complex	37.826090	40.615240	554	Cattle, dog	52	Outdoor
Gaziantep	Nurdağı	*C. schultzei* complex	37.190680	36.843299	506	Cattle, sheep, dog	50	Outdoor
		C. imicola					70	
Adana	Kozan	*C. schultzei* complex	37.363219	35.716955	90	Cattle, sheep, dog	15	Outdoor

Species-specific Multiplex PCR Assay

The primer sets used in this study are given in *Table 2*. Separate reactions were prepared for each primer set *(Fig. 1)*. Multiplex-PCR was performed with UNREV1025 in a total volume of 25 µL using 2.5 µL of 10X PCR buffer, 2.5 µL of 2 mM $MgCl_2$, 1 µL of 10 mM dNTP mix, 1 µL of each primer (10 pmol), 0.125 µL Taq polymerase and 4 µl template DNA. PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 sEC, 57°C for 1min, 72°C for 1min, and final elongation at 72°C for 5min. The PCR products were loaded onto 1.5% agarose gel stained with SYBR® Safe DNA (Thermo Fisher Scientific, Invitrogen) and visualized under UV light.

Table 2. *Primer set used for the identification of blood-meal in Culicoides abdomens*			
Primer Name	**Primer Sequence (5'→3')**	**Length (bp)**	**Origin**
UNREV1025	GGTTGTCCTCCAATTCATGTTA		Kent and Norris [29]
Human741	GGCTTACTTCTCTTCATTCTCTCCT	334	Kent and Norris [29]
Cow121	CATCGGCACAAATTTAGTCG	561	Kent and Norris [29]
Horse	CCCTACATCGGTACTACCC	500	Pitzer at al.[30]
Forward primer UNIV1	GACCAATGATATGAAAAACCATCGTTGT		Garros at al.[21]
Dog, *Canis lupus* familiaris	CAAGCATACTCCTAGTAAGGATCCG	170	Garros at al.[21]
Forward primer UNIV2	TGAGGACAAATATCATTYTGAGGRGC		Garros at al.[21]
Sheep *Ovis aries*	GGCGTGAATAGTACTAGTAGCATGAGGATGA	336	Garros at al.[21]
Cow, *Bos taurus*	TAAGATGTCCTTAATGGTATAGTAG	287	Garros at al.[21]
Forward primer UNIV3	TTTTTTTTTTTTCGVTCHATYCCHAAYAAACTAGG		Garros at al.[21]
Horse, *Equus caballus*	TACGTATGGGTGTTCCACTGGC	208	Garros at al.[21]

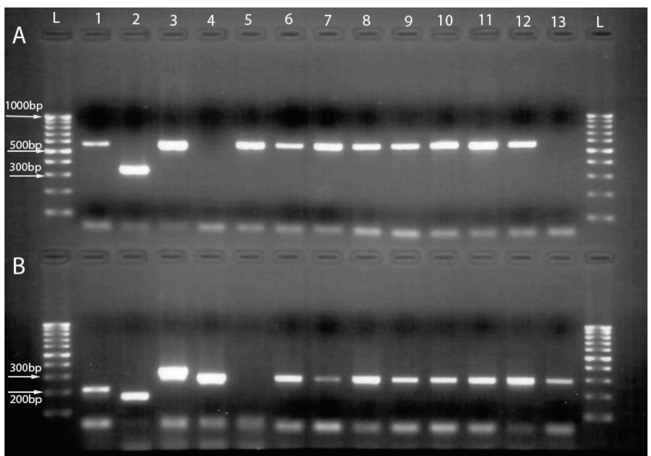

Fig 1. Species-specific PCR amplifications. **A-** Unrev1025 primer set used; L: 100 bp molecular DNA ladder, 1. blood DNA template from a horse, 2. blood DNA template from human, 3. blood DNA template from cattle, 4. Negative control, 5-12. Samples. 13. Negative control **B**-UNIV1, UNIV2 and UNIV3 primer sets used; L: 100 bp molecular DNA ladder, 1. blood DNA template from a horse, 2. blood DNA template from a dog, 3. blood DNA template from sheep, 4. blood DNA template from cattle, 5. Negative control, 6-13. Samples

Results

In this study, 1225 engorged *Culicoides* females were identified from 24 traps in 20 provinces, which belong to 7 different *Culicoides* species/species complexes: *C. obsoletus* complex *(C. obsoletus* and *C. scoticus)* (n:450), *C. schultzei* complex (n:234), *C. imicola* (n:208), *C. punctatus* (n:162), *C. newsteadi* (n:144), *C. lupicaris* (n:24) and *C. pulicaris* (n:3). Engorged females of *C. obsoletus* complex were collected from 12 different traps, *C. newsteadi* from 5 different traps, *C. punctatus* from 4 different traps, *C. lupicaris* from 2 different traps, *C. pulicaris* from only 1 trap, *C. imicola* from 4 different traps and *C. schultzei* complex from 5 different traps *(Table 1)*. Engorged abdomens were separated and pooled according to the date of collection and species. A total of 69 pools consisting of 1-28 specimens were analysed. DNA from human, sheep, cattle, dog, and horse blood were amplified with species-specific *cytb* primers *(Fig. 1)*. As a result of the molecular analysis of 69 pools, all 7 *Culicoides* species/ species complexes were found to be fed only from cattle *(Table 3)*.

Table 3. *Blood-meal identifications by multiplex-PCR of engorged Culicoides.*					
***Culicoides* Species**	**Cow *(Bos taurus)***	**Sheep *(Ovis aries)***	**Horse *(Equus caballus)***	**Human *(Homo sapiens)***	**Dog (Canis lupus)**
C. newsteadi	+	-	-	-	-
C. obsoletus complex (*C. obsoletus/C. scoticus*)	+	-	-	-	-
C. punctatus	+	-	-	-	-
C. lupicaris	+	-	-	-	-
C. pulicaris	+	-	-	-	-
C. imicola	+	-	-	-	-
C. schultzei complex	+	-	-	-	-

Discussion

Molecular techniques developed to identify vertebral hosts that blood-feeding arthropods feed on, allow the host to be determined at the species level [9,17]. In the identification of host preferences of engorged *Culicoides* species, species-specific primers based on the *cytb* that amplify the known host DNA [20,21] and general primers that amplify the conserved genetic regions of all vertebrate species are used [10,22,23,25,26]. In this study, we performed a multiplex-PCR test using specific primers based on *cytb* polymorphism and determined the host blood (bovine, sheep, dog, equine and human) in 7 *Culicoides* species/species complexes. Multiplex-PCR test is a fast, cost-effective, and efficient method that can be routinely applied in the laboratory, where large numbers of samples are tested simultaneously. It is widely used to identify the host preference of various blood-feeding arthropods [17,21,30]. However, it requires preliminary selection to identify primer sets for targeted hosts, which may cause a significant limitation in samples from undisturbed habitats with a wider host range [17,21]. We did not encounter the stated adverse effects as there were a limited number of target hosts (cattle, sheep, dog, horse, and human) in and around the farms where the light traps were placed. In this study, as a result of multiplex-PCR, 7 *Culicoides* species/species complexes (*C. obsoletus* complex, *C. schultzei* complex, *C. imicola*, *C. punctatus*, *C. newsteadi*, *C. lupicaris*, and *C. pulicaris*) were found to feed on cattle but not on other hosts. This may be due to the small number of other hosts in the farms, the higher CO2 emission, the larger mass of cattle and the greater number of cattle in the farms [20]. It has been stated that the host selection of *Culicoides* species may be limited by the presence of hosts in their environment [15,17]. Lassen et al.[12] found 74% cattle blood from the collected samples of *Culicoides* species in their study, and they stated that the result was due to placing the traps close to cattle. In our study, all the traps were placed in cattle farms. As a result, cattle appear to be more attractive hosts for *Culicoides* due to their greater number, less mobility than other animals, and limited defensive movements. In a study on the host preference of *Culicoides* between cattle, horse, and sheep, it was found that *Culicoides* preferred cattle more in an area where cattle and sheep coexist, while more species and individuals were collected from horses [31]. It has been reported that cattle not only attract *Culicoides* but are also important hosts of for these species [13]. Studies on host preferences are also performed by direct aspiration of species from hosts, sweep nets or drop nets. Comparison of host preference between cattle and sheep with these methods showed that cattle are far more attractive hosts for *Culicoides* species in general [13,32]. The structure of the farms (indoor or outdoor), seasonal behavioural changes, and the number of hosts are important for host selection of *Culicoides* species [12].

Culicoides imicola is one of the most important vector species among the species analysed for host identification in this study [3]. *C. imicola* is an important vector for bluetongue virus (BTV) and is distributed in the south and southeast regions of Türkiye [33]. There are few number of researches on the host preference of this species. Slama et al.[34] identified host preferences of *C. imicola* in 96 engorged females and found that they feed on cattle, humans, sheep, goats, and dogs. Martínez-de la Puente et al.[23] found in their study that *C. imicola* feed on 6 mammal species. In a study carried out in South Africa, it has been detected that *C. imicola* and *C. subschultzei* fed on human, zebra, and kudu blood [35]. Both species (*C. imicola* and *C. schultzei* complex) were found to feed on blood from cattle in this study.

Newly engorged female specimens are important in identifying the host source with high accuracy. However, it is known that it is quite difficult to collect these newly engorged females. It represents only a small portion of the total individuals captured [17]. For this reason, it is stated that host preference identification can also be made with *Culicoides* at different stages of digestion [36]. In our study, 1225 new engorged *Culicoides* specimens were analysed. In future studies, choosing samples from both newly engorged specimens and specimens at different stages of digestion (partially or completely) will expand the number of samples for host identification studies.

Studies carried out in Turkey so far have mainly focused on the *Culicoides* species fauna [7,8,37,38]. Our study presents the first data on the identification of host preference of *Culicoides* species in Turkey. In this study, we found that 7 *Culicoides* species/species complexes (*C. obsoletus* complex, *C. schultzei* complex, *C. imicola*, *C. punctatus*, *C. newsteadi*, *C. lupicaris* and *C. pulicaris*) did not feed on other hosts but on cattle by multiplex-PCR assays. Although there were other animals on the farms, the fact that they prefer only cattle can be attributed to the low number of other hosts on the farms. It may also be due to cattle being more attractive hosts for *Culicoides* species found in the farms in this study.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (A. Deniz) on reasonable request.

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Ethical Approval

Not necessary

Competing Interests

The authors declared that there is no conflict of interest

Author Contributions

The study was designed by AD and CK. Fly samples were collected by AD, CK, HB, AİE and OS. Culicoides species identification was made by CK, UE and ST. Multiplex-PCR diagnosis was made by AD, HB and ZV. The original draft was prepared by AD. All authors contributed to the review and proofreading of the article.

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RESEARCH ARTICLE

Diagnostic Value of Serum H-FABP and NT-proBNP Levels in Determining Cardiac Damage in Cattle with Bovine Respiratory Disease Complex

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Abstract: Cardiac complications associated with respiratory diseases are well-documented in humans, but there are not many studies with cattle. Therefore, this study aimed to investigate cardiac damage in cattle with bovine respiratory disease complex (BRDC) with serum heart-type fatty acid-binding protein (H-FABP), N-terminal pro-peptide natriuretic type B (NT-proBNP) and other known cardiac damage biomarkers [cardiac troponin I (cTnI), creatine kinase-myocardial band (CK-MB), creatine kinase (CK), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH)]. The material of this study consisted of 20 cattle, aged 4-6 months with BRDC (infected group) and 10 healthy cattle aged 4-6 months (control group). The study findings revealed that leukocyte parameters and serum H-FABP, NT-proBNP, cTnI, CK-MB, CK, LDH, and AST levels were higher in cattle with BRDC (P=0.000). Heart rate and respiratory rate showed a strong positive correlation with cardiac damage markers. ROC analysis revealed that serum H-FABP levels with a cut-off value of 0.45 ng/ml were more sensitive (100%) and specific (100%) than the rest in determining cardiac damage. It was concluded that cardiac damage occurred in cattle with BRDC, and H-FABP was more sensitive and specific in detecting cardiac damage. It is anticipated that the use of biomarkers to detect cardiac injury in BRDC will be important for determining prognosis and guiding treatment.

Keywords: *BRDC, Cardiac damage, Cattle, cTnI, H-FABP, NT-proBNP*

Sığırların Solunum Sistemi Hastalığı Kompleksinde Kardiyak Hasarın Belirlenmesinde Serum H-FABP ve NT-proBNP Düzeylerinin Tanısal Değeri

Öz: Solunum yolu hastalıkları ile ilişkili kardiyak komplikasyonlar insanlarda iyi tanımlanmasına rağmen sığırlarda bu alanda çok az çalışma yapılmıştır. Bu nedenle, bu çalışmada sığır solunum hastalığı kompleksi (BRDC) olan sığırlarda serum kalp tipi yağ asidi bağlayıcı protein (H-FABP), N-terminal pro-peptid natriüretik tip B (NT-proBNP) ve bilinen diğer kardiyak hasar biyobelirteçleri [kardiyak troponin I (cTnI), kreatin kinaz-miyokardiyal band (CK-MB), kreatin kinaz (CK), aspartat aminotransferaz (AST) ve laktat dehidrogenaz (LDH)] ile kardiyak hasarın araştırılması amaçlandı. Çalışmanın materyalini BRDC'li (enfekte grup) 4-6 aylık 20 sığır ve 4-6 aylık 10 sağlıklı sığır (kontrol grubu) oluşturdu. Verilerimiz, BRDC'li sığırlarda lökosit parametreleri ve serum H-FABP, NT-proBNP, cTnI, CK-MB, CK, LDH ve AST düzeylerinin daha yüksek olduğunu ortaya koydu (P=0.000). Kalp ve solunum hızı, kardiyak hasar belirteçleri ile güçlü pozitif korelasyon gösterdi. ROC analizi, 0,45 ng/ml eşik değerine sahip serum H-FABP düzeylerinin kardiyak hasarı belirlemede diğer tanısal belirteçlere göre daha duyarlı (%100) ve özgül (%100) olduğunu ortaya çıkardı. Sonuç olarak, BRDC'li sığırlarda kardiyak hasarın meydana geldiği ve H-FABP'nin kardiyak hasarı tespit etmede daha duyarlı ve özgül olduğu sonucuna varıldı. BRDC'de kardiyak hasarı saptamak için biyobelirteçlerin kullanılmasının, prognozu belirlemek ve tedaviyi yönlendirmek için önemli olacağı tahmin edilmektedir.

Anahtar sözcükler: *BRDC, Kardiyak hasar, Sığır, cTnI, H-FABP, NT-proBNP*

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Introduction

Bovine respiratory disease complex (BRDC) is a well-defined multifactorial disease as a complex or syndrome involving the interaction of viruses, bacteria, and stress factors [1]. It is the most ubiquitous and severe disease in calf-rearing herds, with significant rates of morbidity (65-80%) and mortality (47-75%) [2,3]. *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* are common bacteria associated with BRDC [4]. Among the most significant viral pathogens linked to BRDC are infectious bovine rhinotracheitis virus (IBRV), bovine viral diarrhea virus (BVDV), parainfluenza-3 virus (PIV3), and bovine respiratory syncytial virus (BRSV) [5-7]. Due to the production losses and high treatment costs associated with BRDC, it is a financially significant disease in calves [8].

For the identification of BRDC agents, whole blood, nasal swab samples, nasopharyngeal swab samples, and bronchoalveolar lavage fluid can be used [9,10]. However, regular surveillance of infectious organisms is impractical and expensive for many farms. Because of this, clinical scoring systems-one of the more practical techniques for BRDC diagnosis-have been created [11-13]. Characteristic clinical signs are traditionally used to make the clinical diagnosis of BRDC [11]. Fever, cough, ocular or nasal discharge, abnormal breathing, and auscultation of abnormal lung sounds are the symptoms that are used to diagnose respiratory disease in calves [12]. Clinical scoring methods assign various values to some of these symptoms, and animals with a total score of 4 or higher are referred to as "BRDC positive." Usually, 90% of positive cases and controls are correctly classified by these techniques [12].

Our key hypothesis for this investigation was that respiratory system infections would cause cardiac damage from tachycardia and hypoxia. The information that follows is also relevant to our hypothesis. Increased blood cardiac troponin I (cTnI) concentrations have been associated with severe BRDC in weaned calves [14] and with increased disease severity in community-acquired pneumonia in humans [15]. Lung abscesses, consolidation, vasculitis, fever, hypoxia, septicaemia, complement activation, initiation of coagulation, increased acute phase proteins, exo- and endotoxin generation, and other conditions can all develop in BRDC that may cause cardiac damage [16-18].

The most often utilized biochemical indicators for identifying cardiac damage are cTnI, creatine kinase-myocardial band (CK-MB), creatine kinase (CK), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) [19]. A novel cardiac marker called heart-type fatty acid-binding protein (H-FABP) is utilized to diagnose acute myocardial infarction in its early stages (within the first two hours) and to detect myocyte damage. In comparison to CK-MB and troponin, studies on humans have shown that H-FABP is a better diagnostic marker for early diagnosis (6 hours), with high sensitivity (79%) and specificity (93%) [20]. Natriuretic peptides have become crucial diagnostic and therapeutic monitoring tools for cardiac problems in recent years [21,22]. In the event of pressure and volume overload, the ventricular myocardium responds by releasing the hormone N-terminal pro-peptide natriuretic type B (NT-proBNP) and remains elevated in the blood for up to 60-120 minutes [23]. The most often utilized natriuretic peptide at the moment, more specifically in veterinary medicine, is NT-proBNP [21,22].

Studies in human medicine have demonstrated that cardiac problems are frequent in community-acquired pneumonia, are related to more severe illness, and may predict prognosis [18,24-26]. There are also studies in veterinary medicine evaluating heart damage in respiratory system diseases [14,27]. This study's main goal was to detect cardiac damage in calves with BRDC using serum levels of H-FABP, NT-proBNP, and other known cardiac biomarkers (cTnI, CK-MB, CK, AST and LDH). This study also aims to assess, using ROC analysis, the performance of these cardiac biomarkers in detecting cardiac damage.

Material and Methods

Ethical Approval

This study was performed in accordance with the approved ethical rules of Atatürk University (protocol no. 2022/6, decision number: 109) and for each cattle written informed consent was obtained from the owner.

Animals and Protocol Design

The study material included 30 cattle of 4-6 months old, Simmental breed and both genders. The cattle were divided into two groups as BRDC (infected, n=20) and healthy (control, n=10) based on clinical examination and complete blood count findings. During the clinical examination, the rectal temperature (RT), heart rate (HR) and respiratory rates (RR) of all calves were measured and noted. Animals that received a total score of 5 or higher under the scoring system created by Love et al.[12] were recognized as BRDC. Calves with abnormal ear or head carriage (normal, ear flick or head shake: 0, ear droop or head tilt: 5), calves with nasal discharge (none: 0, any: 4) and one other clinical finding [cough (none or induced cough: 0, spontaneous cough: 2), ocular discharge (none: 0, any: 4), rectal temperature (<39.2°C: 0, ≥39.2°C: 2), and abnormal respiration (absent: 0, present: 2)], or calves that have any three clinical signs are defined as BRDC cases according to this scoring system.

Blood Sampling

Blood samples from all the calves were taken from *v. jugularis externa* and collected into tubes with EDTA (Vacutainer, K2E 3.6 mg, BD, UK) and gel (Vacutainer, BD, UK) for haematological and biochemical analyses. Blood samples in gel tubes were kept at room temperature and centrifuged at 3000 rpm for 10 min. The obtained serum samples were stored at -80°C until the day of the biochemical analysis. Haematological analyses were finished right away.

Haematological Analyses

White blood cell (WBC), lymphocyte (LYM), monocyte (MON), neutrophil (NEU), eosinophil (EOS), basophil (BAS), and platelet (PLT) levels of the cattle were determined by a haematology analyser (Abacus Junior Vet5, Hungary).

Biochemical Analyses

Using approved commercial bovine-specific enzyme-linked immunosorbent assay (ELISA) kits, the manufacturer's recommendations were followed to assess the serum concentrations of H-FABP and NT-proBNP (Sunred Biological Technology, Shanghai, China). The intra-assay and inter-assay coefficients of variation (CV) for H-FABP were found to be 10% and 12%, respectively, with a minimum detectable concentration (MDC) of 0.08 ng/mL. The MDC for NT-proBNP was 10 ng/mL, and the intra-assay and inter-assay CVs were 10% and 12%, respectively. A commercial immunoassay system was used to measure the levels of cTnI in the serum in accordance with the one-step sandwich method (Unicel Beckman Coulter Access II, USA). The similarity of the troponin I sequence between cattle and humans is > 96%, allowing for the reliable application of this assay in cattle [28]. Troponin can be measured by the immunoassay system between 0.01 and 100 ng/mL. Serum CK, CK-MB, LDH, and AST activities were determined using a biochemistry autoanalyzer (Beckman Coulter, AU5800, USA) employing commercial enzyme kits.

Statistical Analyses

For statistical analysis, SPSS software (Version 25.0, SPSS Inc., Chicago, IL, USA) was utilized. A Shapiro-Wilk test was used to assess the data distribution between the groups (BRDC and Healthy groups). The Independent-Samples t-Test was used to compare parametric variables (LYM, PLT, H-FABP, NT-proBNP, CK-MB, AST, RT, HR, and RR). The Mann-Whitney U test was used to compare nonparametric variables (WBC, MON, NEU, EOS, BAS, cTnI, CK, and LDH). The correlation among parameters was measured by the Pearson Correlation test. The diagnostic efficacy of serum H-FABP and NT-proBNP in identifying cardiac damage was assessed using Receiver Operating Characteristic (ROC) analysis. For parametric variables, all results were shown as mean ± standard deviation (SD), and for nonparametric variables, median and range (min, max). All statistical comparisons were performed at the significance level of P<0.05.

Results

Clinical Findings

High temperature (T: 39.44±0.74), tachycardia (149.6±22.72 beats per minute), tachypnea (50.4±9.39 breaths per minute), anaemic and moderately cyanotic mucosa, cough, dyspnoea, nasal and ocular discharge, and anorexia were common clinical signs in cattle with BRDC. When compared to the control group, the infected cattle's RT (P< 0.019), HR, and RR (P=0.000) were significantly higher *(Table 1)*.

Haematological Findings

According to the haematologic findings, the WBC, LYM, BAS, and PLT values of the BRDC group were higher than those of the healthy group (P<0.05) *(Table 1)*.

Biochemical Findings

The mean H-FABP, NT-proBNP, CK-MB, and AST concentrations and the median cTnI, CK and LDH concentrations in the BRDC group were significantly higher than the healthy group (P=0.000) *(Table 1)*.

Serum H-FABP levels were very strongly positively correlated with LDH (r=0.831, p=0.000), strongly correlated with NT-proBNP (r=0.733, P=0.000) and AST (r=0.659, P=0.000), and moderately positively correlated with cTnI (r=0.559, P=0.001), (CK-MB (r=0.531, P=0.003) and CK (r=0.489, P=0.006). Serum NT-proBNP levels were strongly positively correlated with LDH (r=0.724, P=0.000), moderately positively correlated with CK-MB (r=0.561, P=0.001), AST (r=0.538, P=0.002), and CK (r=0.462, P=0.010), and weakly positively correlated with cTnI (r=0.370, P=0.044) *(Table 2)*.

There was a very strong positive correlation between heart rate and H-FABP (r=0.855, P=0.000) and LDH (r=0.828, P=0.000). Heart rate was strongly positively correlated with NT-proBNP (r=0.633, P=0.000), CK (r=0.600, P=0.000), and AST (r=0.755, P=0.000) whereas moderately positively correlated with cTnI (r=0.537, P=0.002) and (CK-MB (r=0.476, P=0.008). Respiration rate was moderately positively correlated with H-FABP (r=0.570, P=0.001) and NT-proBNP (r=0.531, P=0.003) whereas strongly positively correlated with CK (r=0.640, P=0.000), LDH (r=0.705, P=0.000), and AST (r=0.766, P=0.000) *(Table 2)*.

Serum H-FABP levels were moderately positively correlated with WBC (r=0.409, P=0.025) and LYM

***Table 1.** Comparing haematological, biochemical and some clinical results between infected and control groups of cattle*

Parameters	Healthy	BRDC	P Value
WBC (x10³/μL)	6.95 (4.45-8.33)	9.97 (5.45-25-89)	0.014
LYM (x10³/μL)	3.32±1.41	4.82±0.80	0.009
MON (x10³/μL)	0.21 (0.08-0.71)	0.12 (0.06-0.38)	0.170
NEU (x10³/μL)	2.54 (0.52-5.34)	4.22 (1.63-21)	0.053
EOS (x10³/μL)	0.1 (0.05-0.20)	0.06 (0.02-0.10)	0.006
BAS (x10³/μL)	0.0 (0.00-0.01)	0.03 (0.01-0.07)	0.000
PLT (x10³/μL)	284±112	494±174	0.002
H-FABP (ng/mL)	0.37±0.79	0.65±0.81	0.000
NT-proBNP (ng/mL)	0.19±0.43	0.41±0.12	0.000
cTnI (ng/mL)	0.0398 (0.0196-0.0536)	0.0685 (0.0437-0.1512)	0.000
CK-MB (U/L)	136±45.2	382±113	0.000
CK (U/L)	122.5 (66-351)	318 (174-384)	0.000
LDH (U/L)	499 (270-1364)	2683 (2093-2971)	0.000
AST (U/L)	33±21.85	121±35.25	0.000
RT (°C)	38.97±0.28	39.44±0.24	0.019
HR (beats/min)	79±9.58	149.6±22.72	0.000
RR (breaths/min)	32.40±9.32	50.4±9.39	0.000

WBC: white blood cell; LYM: lymphocyte; MON: monocytes; NEU: neutrophil; EOS: eosinophil; BAS: basophil; PLT: platelet; H-FABP: heart type fatty acid binding protein; NT-proBNP: N-terminal pro-peptide natriuretic type B; cTnI: cardiac troponin I; CK-MB; creatine kinase myocardial band; CK; creatine kinase; LDH: lactate dehydrogenase; AST: aspartate aminotransferase; RT: Rectal temperature; HR: Heart rate (per min); RR: Respiratory rate (per min). Data are presented as mean ± standard deviation and or median (range).

***Table 2.** Correlation results of some haematological, clinical and cardiac damage parameters of cattle in the infected and control groups (Pearson Correlation)*

Parameters	WBC	LYM	RR	HR	H-FABP	NT-pro BNP	cTnI	CK-MB	CK	LDH	AST
WBC	1.000	0.449*	0.713**	0.626**	0.409*	0.295	0.063	0.088	0.344	0.483**	0.647**
LYM		1.000	0.327	0.550**	0.552**	0.551**	0.304	0.354	0.144	0.562**	0.355
RR			1.000	0.703**	0.570**	0.531**	0.157	0.321	0.640**	0.705**	0.766**
HR				1.000	0.855**	0.633**	0.537**	0.476**	0.600**	0.828**	0.755**
H-FABP					1.000	0.733**	0.559**	0.531**	0.489**	0.831**	0.659**
NT-proBNP						1.000	0.370*	0.561**	0.462*	0.724**	0.538**
cTnI							1.000	0.490*	0.248	0.413*	0.259
CK-MB								1.000	0.645**	0.743**	0.563**
CK									1.000	0.766**	0.860**
LDH										1.000	0.897**
AST											1.000

WBC: White blood cell; LYM: lymphocyte; RR: Respiratory rate (per min); HR: Heart rate (per min); H-FABP: heart type fatty acid binding protein; NT-proBNP: N-terminal pro-B-type natriuretic peptide; cTnI: cardiac troponin I; CK-MB; creatine kinase myocardial band; CK; creatine kinase; LDH: lactate dehydrogenase; AST: aspartate aminotransferase

(r=0.552, P=0.002). Serum NT-proBNP levels were moderately positively correlated with LYM (r=0.551, P=0.002) *(Table 2)*.

ROC analysis results of cardiac biomarkers were shown in *Table 3* and *Fig. 1*. The areas under the ROC curves (AUC) were found to be 1.000 for the H-FAB, 0.975 for the NT-proBNP, 0.970 for the cTnI, and 0.950 for the CK-MB parameter. The cut-off values of H-FABP, NT-proBNP, cTnI, CK-MB, CK, LDH, and AST parameters in showing cardiac damage were 0.45 ng/mL, 0.27 ng/mL, 0.0435 ng/mL, 185.9 U/L, 201 U/L, 1364 U/L and 63 U/L, respectively. The sensitivity and specificity values of the proposed diagnostic cut-off point for demonstrating cardiac injury were found to be 100% and 100% for H-FABP, 90% and 100% for NT-proBNP and CK-MB, and 100% and 90% for cTnI.

Table 3. ROC analysis results of H-FABP, NT-proBNP, cTnI, CK-MB, CK, LDH, and AST

Parameters	H-FABP (ng/mL)	NT-proBNP (ng/mL)	cTnI (ng/mL)	CK-MB (U/L)	CK (U/L)	LDH (U/L)	AST (U/L)
Area	1.000	0.975	0.970	0.950	0.900	1.000	0.990
Cut-off	>0.45	>0.27	>0.0435	>185.9	>201	>1364	>63
Sensitivity (%)	100	90	100	90	90	100	100
Specificity (%)	100	100	90	100	90	100	90
SEM	0.000	0.023	0.032	0.039	0.071	0.000	0.013
P value	0.000	0.000	0.000	0.000	0.000	0.000	0.000

H-FABP: heart type fatty acid binding protein; NT-proBNP: N-terminal pro-B-type natriuretic peptide; cTnI: cardiac troponin I; CK-MB; creatine kinase myocardial band; CK; creatine kinase; LDH: lactate dehydrogenase; AST: aspartate aminotransferase

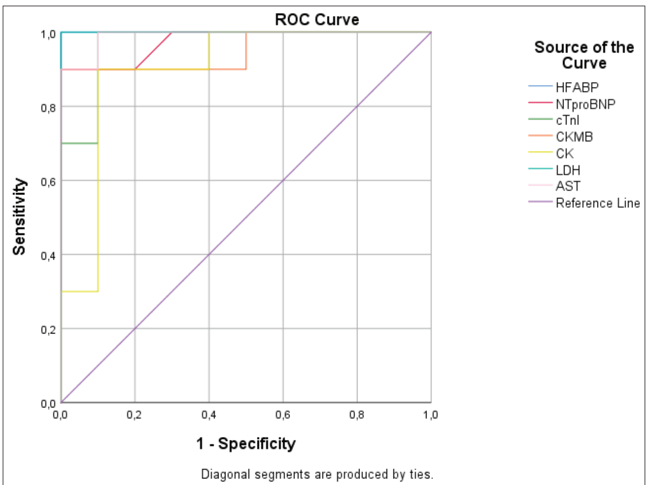

Fig 1. ROC curve analysis of H-FABP, NT-proBNP, cTnI, CK-MB, CK, LDH, and AST parameters

Discussion

In this study, we investigated changes in the levels of cardiac damage markers in cattle with BRDC. Consistent with our hypothesis, we found that cardiac damage occurred in cattle with BRDC with elevated H-FABP, NT-proBNP, cTnI, CK, AST and LDH levels. High respiratory and heart rates were also noticed and found to be strongly positively correlated with heart damage markers. H-FABP was found to be more sensitive (100%) and specific (100%) than other diagnostic markers in identifying the cardiac injury.

Cardiovascular problems such as cardiac arrhythmias [25] might arise in cases of pneumonia [18]. Serum cardiac troponin levels may rise due to bacterial endotoxins, cytokines, increased cardiac oxygen demands due to inflammation, ventilation-perfusion mismatches in acute pneumonia, myocardial contractility depression, catecholamine release, and tachycardia [18,29]. Hypoxia can affect heart function by leading to impaired oxygen delivery to cells and decreased tissue perfusion [30]. Myocardial oxygen demand increases when tachycardia develops as a result of hypoxemia in pneumonia cases [31], which may contribute to acute myocardial damage [15]. In this study, the BRDC group showed higher cardiac and respiratory frequency than the control group (P=0.000). Respiratory frequency was moderately positively correlated with H-FABP and NT-proBNP, on the other hand, heart rate was very strongly correlated with H-FABP and strongly positively correlated with NT-proBNP. These findings suggest that BRDC-induced hypoxia and tachycardia caused cardiac damage in cattle. Similar to this, Hanedan et al.[14] reported elevated heart and respiratory frequency in cattle with BRDC and verified heart damage with high cTnI.

In the early diagnosis of the acute coronary syndrome, it was found that H-FABP was more sensitive and specific than troponin I and CK-MB. The rapid release of H-FABP into the bloodstream following myocardial injury has made it a valuable early and accurate diagnostic marker for myocardial infarction in humans [32]. Similarly, in this study, we found that H-FABP was superior to NT-proBNP, cTnI and CK-MB in detecting cardiac damage by ROC analysis, and a similar feature may exist in cattle. Pharmaceutical treatments, such as anti-tachycardic drugs, were observed to lower H-FABP plasma levels [33]. In our study, the marker with the highest positive correlation coefficient with heart rate was H-FABP (r=0.855, P=0.000). Therefore, tachycardia may play a role in finding H-FABP as the most sensitive marker for detecting heart damage in cattle with BRDC. According to a study that evaluated the levels of H-FABP, NT-proBNP, and cTnI in dogs with dilated cardiomyopathy and degenerative valve disease, H-FABP may be a helpful marker because it was shown to be high, linked with the severity of the disease, and tended to predict a shorter survival time [34]. The importance of such sensitive markers in the veterinary field was emphasized after it was shown that H-FABP levels were high in a different study's assessment of heart damage in cattle with traumatic pericarditis [35]. The second most sensitive marker in BRDC for identifying cardiac damage, according to ROC analysis, was NT-proBNP. As a result of both acute and chronic pneumonia in cattle, pulmonary artery pressures have been shown to rise [36]. Considering that NT-proBNP is released into the blood circulation in

case of pressure and volume overload [23], we speculate that pneumonia, tachycardia and possible pulmonary hypertension are effective in finding NT-proBNP as a high and second sensitive marker in cattle with BRDC.

As is generally known, BRDC is a condition characterised by severe inflammation [37]. In calves with BRDC, leucocytosis and neutrophilia connected with acute respiratory inflammation have been documented [38-40]. Similarly, in this study, the BRDC group had higher WBC (P=0.014), LYM (P=0.009), and BAS (P=0.000) values than that of the healthy group. Serum NT-proBNP levels were shown to be moderately positively correlated with WBC and LYM, but serum H-FABP levels only with WBC. As a result, infection-related cardiac damage occurs in BRDC. To support this inference, it has been reported that pulmonary interstitial disease or severe pneumonia may cause cardiac damage [41]. Additionally, *Histophilus somnus* has been linked to endocarditis [42,43]. This study's shortcoming is the lack of detection of etiological agents in animals with BRDC. However, the study primarily aimed to reveal whether cardiac damage occurred in BRDC-affected animals. I recommend that future studies should investigate how cardiac damage varies depending on the etiological agents in animals with BRDC.

In conclusion, this study demonstrated that cardiac damage occurred in cattle with BRDC with elevated levels of H-FABP, NT-proBNP, cTnI, CK-MB, CK, LDH and AST. In addition, H-FABP and NT-proBNP were superior to other markers in showing this damage, respectively. Cardiac damage markers in BRDC may be elevated as a result of increased cardiac oxygen demands caused by inflammation, ventilation-perfusion mismatches, hypoxia, tachycardia, and pulmonary hypertension due to acute respiratory distress syndrome. It is important to understand the role of cardiac dysfunction in BRDC as this would help clinicians both assess the risk of death in BRDC cases and determine their treatment accordingly.

Availability of Data and Materials

On reasonable request, the corresponding author will provide the data provided in this study.

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Competing Interests

The author declares no conflict of interest.

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RESEARCH ARTICLE

Molecular Characterization and Toxins Optimization of Indigenous *Clostridium perfringens* Toxinotype B Isolated from Lamb Dysentery Clinical Cases

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Abstract: *Clostridium perfringens* toxinotype B is causative agent of lamb dysentery in Pakistan. For assessment of gene expression, it is preferred to optimize toxin production potential of indigenous isolates. Current study aimed to confirm the local isolates of *C. perfringens* toxinotype B on molecular basis followed by optimization of major toxins under varied physical and chemical conditions. The isolates were identified using microbiological and biochemical assays and confirmed by PCR followed by nucleotide sequencing of 16S rRNA amplified gene. These nucleotide sequences were submitted to NCBI GenBank® and accession numbers retrieved were MW867097, MW867098, MW867099, MW867100 and MW867101. Confirmation of toxinotype B was done by PCR amplification of alpha beta and epsilon toxin genes. Major toxins were optimized at varied physical (temperature and pH) and chemical (reinforced clostridial media, Robertson's cooked meat media, egg meat media, fluid thioglycolate media and iron milk media) conditions. Higher hemolytic units of alpha toxin (21.45±0.53 HU/mL), epsilon toxin (16.57±0.19 HU/mL) and higher cytotoxic units of beta toxin (18.65±0.34 HU/mL) were produced at 37°C with pre-adjusted pH 6.0 in Robertson cooked meat media. The ELISA percentages of alpha (14.27%, 13.92%, 13.67%, 13.56%, 13.45%), beta (12.43%, 12.81%, 12.39%, 12.61% and 13.07%) and epsilon (13.93%, 14.78%, 14.28%, 14.03% and 13.25%) toxins were also higher at same conditions. These optimized conditions can be used for major toxin gene expression studies of *C. perfringens* toxinotype B.

Keywords: *Alpha, Beta, Clostridium perfringens toxinotype B, Epsilon, Gene expression, Lamb dysentery, Robertson's cooked meat medium*

Kuzu Dizanteri Klinik Olgularından İzole Edilen Lokal *Clostridium perfringens* Toksinotip B'nin Moleküler Karakterizasyonu ve Toksin Optimizasyonu

Öz: *Clostridium perfringens* toksinotip B, Pakistan'da kuzu dizanterisinin yapıcı etkenidir. Gen ekspresyonunun değerlendirilmesi için lokal izolatların toksin üretim potansiyelinin iyileştirilmesi gerekmektedir. Bu çalışma, *C. perfringens* toksinotip B lokal izolatlarını moleküler düzeyde doğrulamayı ve takiben çeşitli fiziksel ve kimyasal koşullar altında majör toksinlerin üretiminin iyileştirilmesini amaçlamıştır. İzolatlar mikrobiyolojik ve biyokimyasal testler ile tanımlanmış ve PCR ile doğrulandıktan sonra ,16S rRNA amplifiye geninin nükleotid dizilimi yapılmıştır. Bu nükleotid dizileri NCBI GenBank®'a gönderilmiş ve MW867097, MW867098, MW867099, MW867100 ve MW867101 erişim numaraları alınmıştır. Toksinotip B'nin doğrulanması, alfa, beta ve epsilon toksin genlerinin PCR ile amplifikasyonu ile gerçekleştirilmiştir. Başlıca toksinler çeşitli fiziksel (sıcaklık ve pH) ve kimyasal (zenginleştirilmiş klostridiyal besiyeri, Robertson pişmiş et besiyeri, yumurtalı et besiyeri, sıvı tiyoglikolat besiyeri ve demir süt besiyeri) koşullarda optimize edilmiştir. Robertson pişmiş et besiyerinde, pH 6.0'da ve 37°C'de daha konsantre hemolitik alfa toksin (21.45±0.53 HU/mL) ve epsilon toksin (16.57±0.19 HU/mL) ve daha konsantre sitotoksik beta toksin (18.65±0.34 HU/mL) üretilmiştir. Aynı koşullarda alfa (%14,27, %13,92, %13,67, %13,56, %13,45), beta (%12,43, %12,81, %12,39, %12,61 ve %13,07) ve epsilon (%13,93, %14,78, %14,28, %14,03 ve %13,25) toksinlerinin ELISA yüzdeleri daha yüksek saptanmıştır. Optimize edilmiş bu koşullar, *C. perfringens* toksinotip B'nin majör toksin gen ekspresyon çalışmalarında kullanılabilir niteliktedir.

Anahtar sözcükler: *Alfa, Beta, Clostridium perfringens toxinotype B, Epsilon, Gen ekspresyonu, Kuzu dizanterisi, Robertson's pişmiş et besiyeri*

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Introduction

Clostridium perfringens type B is causative agent of lamb dysentery. It usually progresses in early days of life. Neonatal lambs catch infection from dam or its environment, then its number increases in the gut especially with heavy lactation by the dam [1]. The result is enterotoxemia complemented by ulceration of small intestine, extensive hemorrhages and enteritis. In per-acute cases, sudden death is primary sign, without premonition. In acute cases, feeding termination and increased abdominal ache are complemented by dysentery followed by recumbence, unconsciousness and fatality in less than 24 hours. Pine is the chronic form of this disease which occurs in older lambs which demonstrates chronic abdominal cramps without diarrhea [2].

C. perfringens virulence is based upon presence of approximately twenty diverse extracellular enzymes and toxins [3]. The organism has been recently classified into seven toxinotypes or strains viz; A to G due to the presence of iota (ι), epsilon (ε), beta (β) and alpha (α) toxins along with recently included necrotic enteritis B-like (NetB) and enterotoxin (CPE) toxins [4]. Additionally, each strain of *C. perfringens* has the ability of production of minor toxins viz; BEC, NetF, PFO, CPB2 etc. There is no single strain or toxinotype known which can produce all the minor toxins [5].

Alpha (α or CPA) toxin encoding gene *(cpa* or *plc)* is located in the house keeping region of the chromosome which makes it very conserved, stable and produced by *C. perfringens* all toxinotypes [6]. In contrast, the genes which code major toxins resides on large plasmids, hence, in other words, the scheme of *C. perfringens* toxinotyping is based on the presence or absence of plasmids. Actually, among the major toxins, toxinotype B produces ETX, CPB and CPA toxins [7]. Beta toxin is an etiological agent in necrotizing enterocolitis. The reported 50% lethal dose (LD50) of beta toxin is 310 ng/kg [8]. Epsilon (ε-toxin) form pores in infected cells, which results in loss of K^+ and increase in cytoplasmic level of Cl^- and Na^+. Epsilon toxin also causes slightly increase in Ca^+ ions in cytoplasm of infected cells. ETX induced release of K^+, loss of ions activate cell death by necrosis or ATP exhaustion [9].

For gene expression studies, molecularly confirmed *C. perfringens* isolates may be optimized for maximum toxins production under varied physical and chemical parameters. It has been reported that *C. perfringens* has proteolytic as well as saccharolytic activity in thioglycolate medium, reinforced clostridial medium, iron milk medium, egg meat medium and cooked meat medium. In prior studies, differences in the hemolytic activity of toxinotype B and D were reported. There is dire need to quantify the toxin production potential of *C. perfringens* toxinotype B in these culture media. Therefore, current exploration was executed for optimization of chemical and physical conditions to maximize the production of alpha beta and epsilon toxins of *C. perfringens* toxinotype B.

Material and Methods

Ethical Approval

All animal experiments were approved by Institutional Animals Ethical Review Committee of University of Veterinary and Animal Sciences, Lahore, Pakistan and carried out according to the International Ethics law and regulations. All efforts were made to minimize the animal sufferance.

Sample Collection

A total of 35 samples from Lahore and Kasur districts were collected to isolate *C. perfringens* toxinotype B from lambs. Rectal swabs from lambs showing signs of enterotoxaemia were transferred into anaerobic medium by deep insertion and transported to Anaerobiology Section, Quality Operational Laboratory, Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore using cold chain (4°C) for further processing [10].

Isolation of *C. perfringens*

Bacterial enrichment from collected sample was performed as described previously [11]. Peptone water 0.1% (pH 6.8±0.2) supplemented with 0.05% L-cysteine HCl was inoculated with cotton swab fecal samples by cutting the swab stick with a sterile scissor in sterile environment. Bacterial strain isolation and identification were made as described previously but with minor modifications [12]. Briefly, 1:100 dilution of the sample suspension in peptone water (0.1%) was prepared and incubated at 70°C for 30 min. Suspensions were inoculated into reinforced clostridial media (RCM) broth and incubated for 48 h at 37°C in anaerobic atmosphere. Then broth culture (100µL) was spread on perfringens agar supplemented with D-cycloserine and incubated in anaerobic jar at 37°C for 48 h. Colony characteristics were observed carefully and pinpoint black colonies were further subjected to biochemical tests using oxidase, catalase, hemolytic activity on blood agar containing sheep blood, gelatin hydrolysis and lecithinase activity along with fermentation of glucose, maltose, mannitol, salicin, dulcitol, inositol and lactose for identification of *C. perfringens* [13].

Molecular Confirmation of *C. perfringens*

DNA of biochemically confirmed isolates was extracted by using a commercial kit (Exgene™ GeneAll). Extracted DNA was subjected to amplification of 16S rRNA gene for molecular confirmation of *C. perfringens* [14]. Briefly,

Polymerase chain reaction (PCR) reaction mixture of 25 µL was prepared by mixing 12.5 µL (2X) PCR master mix, 1µL of each forward and reverse primers (10 pmol) (*Table 1*), 2 µL of extracted DNA and 8.5 µL nuclease free water. Amplification of 16S rRNA gene was carried out at 94°C for 10 min (initial denaturation) followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min. Gel electrophoresis was performed using 1.8% agarose with ethidium bromide 0.5 µg/mL. Amplified products of 16S rRNA gene were subjected to nucleotide sequencing and FASTA files were retrieved [15]. The nucleotide sequences were submitted to national center for biotechnology information (NCBI) through GenBank® after screening for chimeras by using JUSTbio and accession numbers were retrieved. Phylogenetic analysis of these sequences were performed using molecular evolutionary genetics analysis (MEGA) [16].

Confirmation of *C. perfringens* Toxinotype B

The extracted genome of biochemically confirmed isolates was also processed for amplification of alpha, beta, epsilon and iota toxin genes of *C. perfringens* [17]. Reaction mixtures were prepared as described above. The amplification was carried out at 94°C for 10 min (initial denaturation) followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 45 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 min. Gel electrophoresis was performed using 1.8% agarose with ethidium bromide 0.5µg/mL.

Optimization of Toxin Production ability under Physico-Chemical Conditions

For optimization of toxin production, bacterial inoculum was prepared in normal saline supplemented with L-cysteine HCl (0.05%) as reducing agent following the methods described previously [18]. Fresh growth from perfringens agar was used for this purpose.

To analyze the impact of physical and chemical changes on *C. perfringens* type B major toxins, confirmed isolates (n=5) were cultured on reinforced clostridial (RC) media and incubated at 32°C, 35°C, 37°C, 39°C and 42°C to know the optimum temperature for maximum toxin production. Likewise, the same isolates (n=5) were cultured on reinforced clostridial (RC) media with pre-adjusted pH at 6.0, 7.0, 8.0 respectively to know the optimum pH for maximum toxin production. Later on, same isolates (n=5) were cultured on Robertson's cooked meat, egg meat, fluid thioglycolate and iron milk media and incubated at pre-optimized temperature and pH [19].

Quantification of Alpha Beta and Epsilon Toxins

Cell-free supernatant from broth culture was obtained by centrifugation at 8000 rpm for 5 min. Supernatant were poured into small aliquots of 1.5 mL and stored at 4°C for future use. For detection of alpha, beta and epsilon toxins, Bio-X Diagnostics MULTISCREEN Ag-ELISA kit was used and sandwich ELISA assay was performed for *C. perfringens* type B isolates as per manufacturer's recommendations [20]. Optical density of Ag-ELISA plate was recorded at 450nm wavelength. Percentage absorbance of the corresponding toxin was calculated as:

$$Prcentage\ Absorbannce = \frac{Optical\ density\ of\ the\ test\ sample}{Positive\ control\ value\ of\ the\ respective\ toxin} \times 100$$

For quantification of alpha toxin cell-free supernatant was used untreated but for epsilon toxin, cell-free supernatant was activated with 1% trypsin solution and mixed in a 9:1 ratio and incubated at 37°C for 30 min. Hemolytic activity of alpha and epsilon toxins were assessed by micro titer plate assay by the methods described previously [21,22]. Toxins in supernatant was two folds diluted (v/v) serially in phosphate buffer saline using 96 well flat bottom microtiteration plates. Washed sheep red blood cells (1%) suspension in sterile phosphate buffer saline was prepared. In each well, 100 µL of 1% suspension of sheep red blood cells were added. These micro titer plates were incubated

Table 1. Primers used for molecular confirmation of C. perfringens toxinotype B

Genes	Primers	Primer Sequences	Product Size
16S rRNA	8FLP(F)	5`AGTTTGATCCTGGCTCAG-3`	1500 bp
	XB4(R)	5`-GTGTGTACAAGGCCCGGGAAC-3`	
Alpha (*cpa*)	CPAlphaF	5'-GCTAATGTTACTGCCGTTGA-3'	324 bp
	CPAlphaR	5'-CCTCTGATACATCGTGTAAG-3'	
Beta (*cpb*)	CPBetaF3	5'-GCGAATATGCTGAATCATCTA-3'	195 bp
	CPBetaR3	5'-GCAGGAACATTAGTATATCTTC-3'	
Epsilon (*etx*)	CPEpsilonF	5'-TGGGAACTTCGATACAAGCA-3'	376 bp
	CPEpsilonR2	5'-AACTGCACTATAATTTCCTTTTCC-3'	
Iota (*iap*)	CPIotaF2	5'-AATGGTCCTTTAAATAATCC-3'	272 bp
	CPIotaR	5'-TTAGCAAATGCACTCATATT-3'	

with shaking for 60 min at 37°C. Optical densities were measured at 595 nm by spectrophotometer and 50% hemolytic units (HU/mL) were documented.

The cytotoxicity of baby Hamster kidney 21 (BHK 21) cell line was used for quantification of beta toxin. Sterile Glasgow minimal essential medium (GMEM) containing 8-10% fetal calf serum in 96 well flat bottom microtiteration plates were used to culture BHK 21 cells. Each well of microtiteration plate was inoculated with $1x10^5$ cells. After 24 h of incubation, two fold serially diluted beta toxin was added in the wells containing cell growth and incubated at 37°C with supply of 5% CO_2. Subsequently, the cells were washed with sterile PBS and stained as described previously but with minor modifications [23]. Optical densities were recorded by ELISA plate reader at 570 nm. Cell survival percentage was valued by the formula given below.

$$\text{Cell Survival percentage} = \frac{\text{Optical Density of Test} - \text{Optical density of Negative Control}}{\text{Optical density of Possitive Control} - \text{Optical density of Negative Control}} \times 100$$

Statistical Analysis

All the experimental data was analyzed by using SPSS software and represented as mean±SE. Statistical significance was determined using the analysis of repeated measurements variance followed by post hoc, Duncan's multiple range (DMR) test.

Results

Biochemical Characterization of *C. perfringens*

Out of 35 samples collected from clinical cases of lamb dysentery, 11 were positive for anaerobic growth as cultured on reinforced clostridial media and perfringens agar. The isolates (n=10) were characterized as *C. perfringens* based on colonial morphology, microscopic appearance and biochemical profile. All isolates (n=10) were negative for catalase and oxidase test whereas six (6) isolates showed double hemolysis, two (2) showed beta hemolysis and two (2) depicted partial hemolysis. Moreover, all isolates (n=10) were positive for lecithinase and gelatin hydrolysis test. Furthermore, isolates (n=10) fermented sugars viz., fructose, mannitol, maltose, lactose, sucrose and glucose with gas production.

Molecular Confirmation

Biochemically characterized isolates were further confirmed through 16S rRNA gene amplification by polymerase chain reaction (PCR) and amplicons of 1500 bp were subjected to nucleotide sequencing. The FASTA files received were analyzed through BLAST (mega blast) for similarity. BLAST represented that these isolates were 100% identical to aligned sequences (data base) with 95 to 97% query coverage and E value was <0.0. Out of 10 sequenced isolates, 09 isolates were considered as *C. perfringens* on the basis of 16S rRNA gene. The accession numbers of nucleotide sequences of *C. perfringens* isolates (n=5) received were MW867097, MW867098, MW867099, MW867100 and MW867101. Confirmed isolates were targeted for toxinotype identification by amplification of alpha, beta and epsilon toxin genes using toxin specific primers by conventional PCR and amplified DNA bands were visualized on agarose gel (1.8%) under UV. All isolates revealed alpha toxin gene nucleic acid band of 324 bp whereas, seven (7) isolates revealed beta toxin gene amplicon of 197 bp and nine (9) isolates were positive for epsilon gene (376 bp) (*Fig. 1*). On the basis of molecular characterization, seven (7) isolates were confirmed as toxinotype B and two (2) isolates were confirmed as toxinotype D.

Phylogenetic Analysis of *C. perfringens* Based on 16S rRNA Gene Sequences

Phylogenetic analysis of submitted 16S rRNA gene sequences was carried out using MEGA. Dendrogram for *C. perfringens* was completed using 16S rRNA sequences, bootstrap as phylogeny method and 2000 bootstrap replication in neighbor joining algorithm. In dendrogram, current *C. perfringens* sequences were represented with accession numbers in stained boxes. MW867101.1, sequence 46% evolutionary related to the MW867097.1 which is 35% evolutionary related to MW867098.1 and FJ978611.1 kept as out group in evolutionary tree. MW867099.1 displayed 41% evolutionary relation to MW8697100.1. Phylogenetic tree represented that MW332247.1 distantly related to MT463463.1 and MT464449.1 (*Fig. 2*).

Optimization of Toxin Production by *C. perfringens* Toxinotype B

A higher hemolytic units (11.03±0.16 HU/mL) of alpha toxin of *C. perfringens* toxinotype B were produced at 37°C incubation temperature. The alpha toxin of *C. perfringens* type B isolates produced higher hemolytic units (11.03±0.16 HU/mL) at 37°C temperature of incubation. Similarly, ELISA percentages for alpha toxin were higher (8.92, 9.65, 9.42, 9.34 and 9.19%) at 37°C temperature. Whereas, least hemolytic units of alpha toxin (02.72±0.53 HU/mL) and least ELISA percentages for alpha toxin (1.50,1.25, 1.21, 1.71 and 0.98%) were produced at 42°C incubation temperature.

Higher hemolytic units (16.72±0.15 HU/mL) of alpha toxin of *C. perfringens* toxinotype B were produced at 37°C on pre-adjusted pH 6.0 and ELISA percentages for alpha toxin were also higher (10.32, 10.29, 10.08, 10.65 and 10.23%) on the same temperature and pre-adjusted pH. Meanwhile, least hemolytic units of alpha toxin (09.39±0.31 HU/mL)

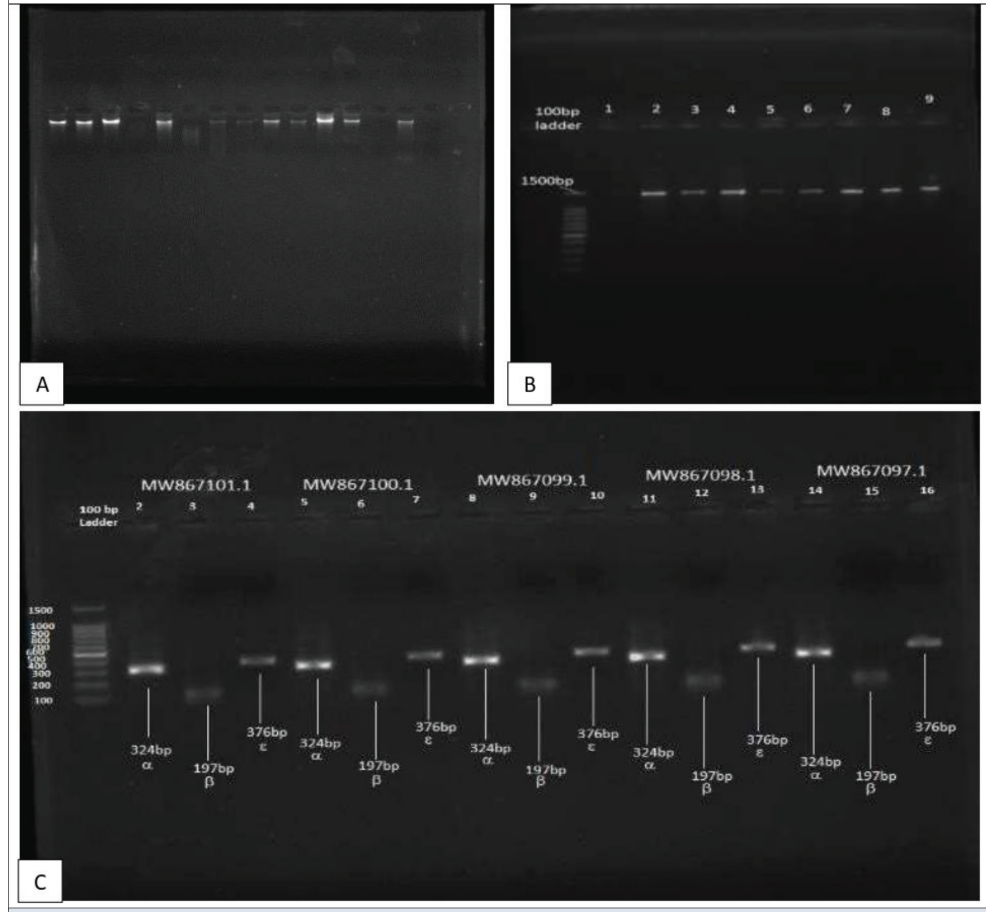

Fig 1. Agarose electrophoresis gels for molecular confirmation of *C. perfringens* Toxinotype Type B. A- Extracted DNA, B- 16S rRNA gene amplification, C- Alpha, beta and epsilon toxin amplification for confirmation of Toxinotype B

and ELISA percentages (5.63, 5.21, 5.72, 5.93 and 5.72%) were produced at pre- adjusted pH 8.0. Higher hemolytic units (21.45±0.53 HU/mL) of alpha toxin of *C. perfringens* toxinotype B were produced in Robertson cooked meat medium (RCMM) at 37°C temperature of incubation with pre-adjusted pH 6.0. Likewise, ELISA percentages for alpha toxin were also higher (14.27, 13.92, 13.67, 13.56 and 13.45%) in Robertson cooked meat medium at 37°C temperature of incubation and pre-adjusted pH 6.0. On the other side, least hemolytic units (06.21±0.36HU/mL) and ELISA percentages (3.45, 3.29, 3.75, 3.98 and 3.69%) of alpha toxin were recorded in egg meat medium. Significant differences were observed among the alpha toxin hemolytic units produced by *C. perfringens* toxinotype B under the influence of varied temperature, pH and culture media *(Table 2)*.

The higher cytotoxic units (9.32±0.19CU/mL) of beta toxin of *C. perfringens* toxinotype B were produced at 37°C temperature of incubation and the ELISA percentages for beta toxin were also higher (6.38, 6.21, 6.87, 5.98 and 6.47%) at 37°C incubation temperature. Whereas, least cytotoxic units (01.73±0.19 CU/mL) and ELISA percentages (0.79, 0.86, 0.94, 0.91 and 0.65%) of beta toxin were observed at 32°C temperature for incubation. The beta toxin of *C. perfringens* toxinotype B isolates produced higher cytotoxic units (14.63±0.28 CU/mL) at 37°C on pre-adjusted pH at 6.0. Similarly, ELISA percentages for beta toxin were also higher (8.84, 8.98, 9.13, 9.29 and 8.79%) on the same temperature and pre-adjusted pH. While, least cytotoxic units (05.84±0.28 CU/mL) and ELISA percentages (4.23, 4.15, 3.98, 3.95 and 4.07%) of beta toxin were observed at pre-adjusted pH 8.0. The cytotoxic units of beta toxin produced by *C. perfringens* type B were higher (18.65±0.34 CU/mL) in Roberson cooked meat medium upon 37°C incubation temperature with pre- adjusted pH 6.0. It was also observed that ELISA percentages for beta toxin were higher (12.43, 12.81, 12.39, 12.61 and 13.07%) at 37°C incubation temperature with pre-adjusted pH 6.0 in Roberson cooked meat medium. It was also reported that least cytotoxic unit (04.76±0.29 CU/mL) and ELISA percentages (4.72, 4.34, 4.23, 4.07 and 4.83%) were verified in iron milk medium. Significant differences were observed among the cytotoxic units of beta toxin produced by *C. perfringens* type B under the

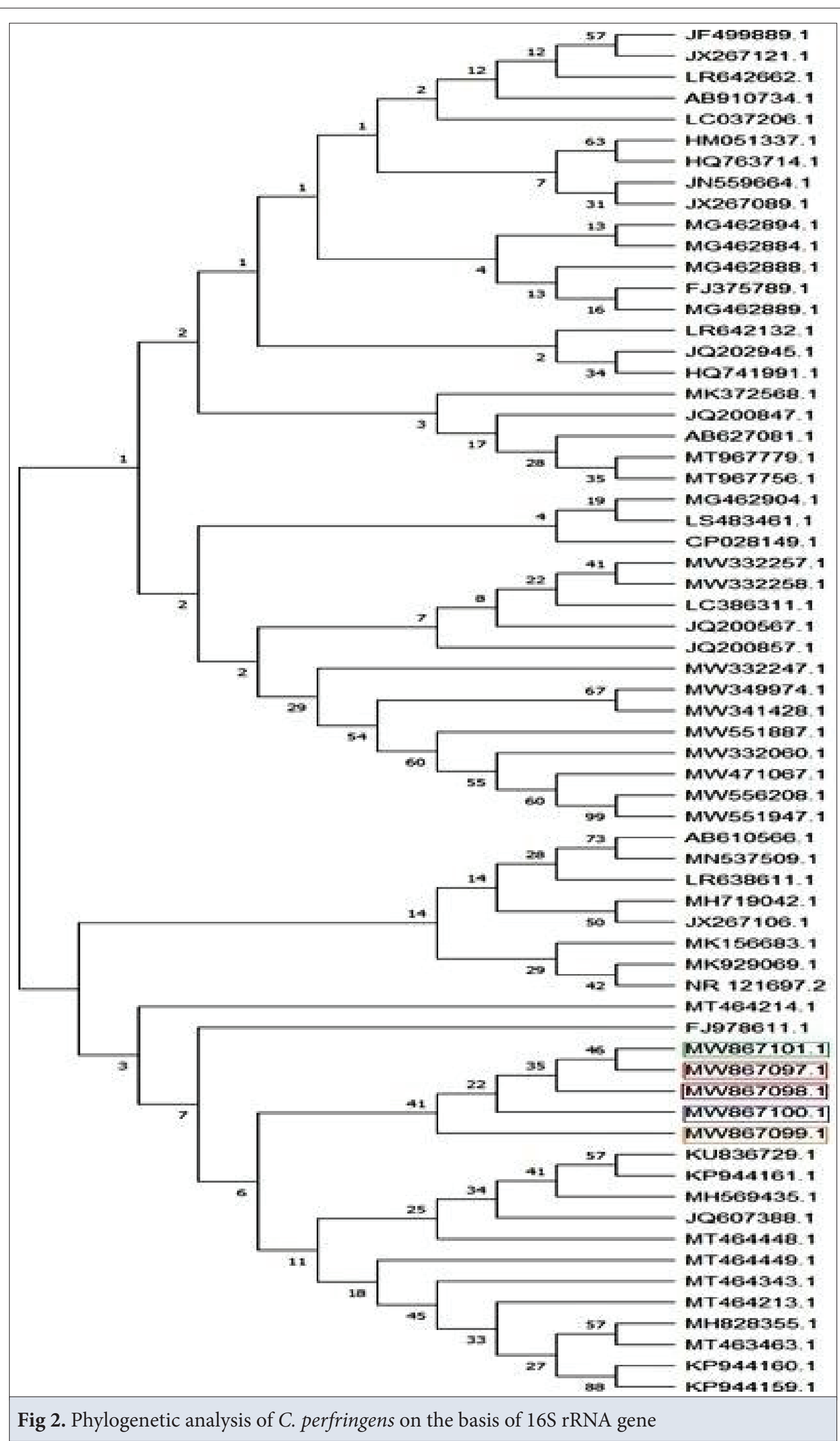

Fig 2. Phylogenetic analysis of *C. perfringens* on the basis of 16S rRNA gene

influence of varied temperature, pH and culture media *(Table 3)*.

The epsilon toxin of *C. perfringens* type B isolates produced higher hemolytic units (08.52±0.29 HU/mL) when incubated at 37°C temperature. Similarly, the ELISA percentages of epsilon toxin were also higher (7.14, 7.02, 6.94, 6.49 and 6.87%) at 37°C of incubation temperature. The least hemolytic units (01.36±0.08 HU/mL) and ELISA percentages (0.89, 0.97, 1.20, 1.18 and 1.31%) of epsilon toxin were recorded at 32°C temperature. The epsilon toxin of *C. perfringens* type B isolates produced higher hemolytic units (11.96±0.45 HU/mL) at 37°C on pre-adjusted pH at 6.0. Likewise, ELISA percentages for epsilon toxin were also higher (9.49, 9.32, 9.73, 9.56 and 9.18%) on the same temperature and pre adjusted pH. The least reported hemolytic units (06.97±0.31 HU/ mL) and ELISA percentages (3.94, 3.82, 3.91, 3.71 and 3.62%) of epsilon toxin were observed at pre- adjusted pH 8.0. The hemolytic units of epsilon toxin produced

Table 2. *Optimization of alpha toxin of C. perfringens type B under different physico-chemical conditions (hemolytic units and ELISA)*

Parameters	Hemolytic Units (HU/mL)	ELISA (%)				
	(Mean±SE)	MW867097.1	MW867098.1	MW867099.1	MW867100.1	MW867101.1
Temperature						
32°C	02.96±0.27[c]	2.12	2.65	2.42	2.84	2.34
35°C	05.97±0.37[b]	3.24	3.13	3.71	3.19	3.29
37°C	11.03±0.16[a]	8.92	9.65	9.42	9.34	9.19
39°C	06.75±0.48[b]	4.34	4.18	4.63	4.21	4.71
42°C	02.72±0.53[c]	1.50	1.25	1.21	1.71	0.98
pH						
6.0	16.72±0.15[a]	10.32	10.29	10.08	10.65	10.23
7.0	11.03±0.16[b]	8.92	9.65	9.42	9.34	9.19
8.0	09.39±0.31[b]	5.63	5.21	5.72	5.93	5.72
Culture Media						
Reinforced Clostridial Medium	16.72±0.15[b]	10.32	10.29	10.08	10.65	10.23
Egg Meat Medium	06.21±0.36[c]	3.45	3.29	3.75	3.98	3.69
Iron Milk Medium	07.89±0.32[c]	4.41	4.67	4.83	4.29	4.81
Robertson Cooked Meat Medium	21.45±0.53[a]	14.27	13.92	13.67	13.56	13.45
Fluid Thioglycolate Medium	14.34±0.27[b]	9.23	9.24	9.52	9.17	9.06

[a,b,c] *Values with in column with different superscript differ significantly at P<0.05*

Table 3. *Optimization of beta toxin of C. perfringens type B under different physico-chemical conditions (cytotoxic units and ELISA)*

Parameters	Cytotoxic Units (CU/mL)	ELISA (%)				
	(Mean±SE)	MW867097.1	MW867098.1	MW867099.1	MW867100.1	MW867101.1
Temperature						
32°C	01.73±0.19[c]	0.79	0.86	0.94	0.91	0.65
35°C	04.74±0.57[b]	1.71	1.65	1.81	1.39	1.56
37°C	09.32±0.19[a]	6.38	6.21	6.87	5.98	6.47
39°C	04.03±0.37[b]	2.10	2.03	1.91	1.87	1.90
42°C	01.92±0.32[c]	1.30	0.98	1.18	1.03	0.87
pH						
6.0	14.63±0.28[a]	8.84	8.98	9.13	9.29	8.79
7.0	09.32±0.19[b]	6.38	6.21	6.87	5.98	6.47
8.0	05.84±0.28[c]	4.23	4.15	3.98	3.95	4.07
Culture Media						
Reinforced Clostridial Medium	14.63±0.28[b]	8.84	8.98	9.13	9.29	8.79
Egg Meat Medium	05.48±0.76[c]	5.68	5.74	5.86	5.43	5.21
Iron Milk Medium	04.76±0.29[c]	4.72	4.34	4.23	4.07	4.83
Robertson Cooked Meat Medium	18.65±0.34[a]	12.43	12.81	12.39	12.61	13.07
Fluid Thioglycolate Medium	12.49±0.71[b]	10.30	09.89	10.28	10.78	10.24

[a,b,c] *Values with in column with different superscript differ significantly at P<0.05*

Table 4. *Optimization of epsilon toxin of C. perfringens type B under different physico-chemical conditions (hemolytic units and ELISA)*

Parameters	**Hemolytic Units (HU/mL)**	**ELISA (%)**				
	(Mean±SE)	**MW867097.1**	**MW867098.1**	**MW867099.1**	**MW867100.1**	**MW867101.1**
Temperature						
32°C	01.36±0.08[c]	0.89	0.97	1.20	1.18	1.31
35°C	03.54±0.28[b]	2.87	3.03	3.24	3.23	2.98
37°C	08.52±0.29[a]	7.14	7.02	6.94	6.49	6.87
39°C	03.82±0.17[b]	3.82	3.67	3.73	3.85	3.29
42°C	01.68±0.09[c]	1.48	1.67	1.73	1.82	1.38
pH						
6.0	11.96±0.45[a]	9.49	9.32	9.73	9.56	9.18
7.0	08.05±0.29[b]	7.14	7.02	6.94	6.49	6.87
8.0	06.97±0.31[b]	3.94	3.82	3.91	3.71	3.62
Culture Media						
Reinforced Clostridial Medium	11.96±0.45[b]	9.49	9.32	9.73	9.56	9.18
Egg Meat Medium	03.17±0.29[c]	3.94	4.63	4.25	4.16	4.72
Iron Milk Medium	04.01±0.18[c]	6.54	5.96	6.07	5.84	6.23
Robertson Cooked Meat Medium	16.57±0.19[a]	13.93	14.78	14.28	14.03	13.25
Fluid Thioglycolate Medium	09.62±0.29[b]	9.78	9.76	9.45	9.30	9.89

[a,b,c] *Values with in column with different superscript differ significantly at P<0.05*

by *C. perfringens* type B were higher (16.57±0.19HU/mL) in Robertson's cooked meat medium upon 37°C incubation temperature with pre- adjusted pH 6.0. Additionally, ELISA percentages for epsilon toxin were higher (13.93, 14.78, 14.28, 14.03 and 13.25%) at 37°C incubation temperature with pre- adjusted pH 6.0 in the Robertson cooked meat medium. Furthermore, least hemolytic units of epsilon toxin (03.17±0.29HU/mL) and ELISA percentages (3.94, 4.63, 4.25, 4.16 and 4.72%) were recorded in egg meat medium. Significant differences were observed among the hemolytic units of epsilon toxin produced by *C. perfringens* type B under the influence of varied temperature, pH and culture media *(Table 4)*.

Discussion

C. perfringens is a rod shaped, gram positive, spore forming, non-motile, anaerobic pathogenic bacteria of domestic animals and human [24]. *C. perfringens* divided in to five types A, B, C, D and E on the basis of four types of major toxins produced by these types [24,25]. In many earlier studies, a range of specified media (Motility nitrate medium, reinforced clostridial medium, modified ducan strong medium, lactose gelatin medium and tryptose sulphite cycloserine medium) provided higher isolation rates. In this study we used two types of media, reinforced clostridial medium and tryptose sulphite cycloserine medium to get wide range of results. All of the positive samples gave straw color colonies on TSC agar plates as per previous report [26]. Typical black color colonies were sign of specificity for *C. perfringens* positive isolates on the surface of TSC agar plates [27].

To isolate *C. perfringens* form lambs, fecal samples / rectal swabs (n=35) were collected from Lahore and Kasur districts of Punjab province and processed based on microscopic studies, biochemical profile and molecular characteristics. On the basis of biochemical profile, 10 (28.57%), samples were characterized as having *C. perfringens*. In a study of the prevalence of *C. perfringens*, out of 177 samples (25 from goats and 152 from sheep) collected from diarrheic animals were screened for *C. perfringens* toxinotypes. Out of these 177 samples, 125 (70.62%) were found positive for *C. perfringens*, of which 15 (60%) were from goats and 110 (72.36%) were from sheep [28].

In current study, on the basis of 16S rRNA gene amplification it was revealed that 9/35 were confirmed as *C. perfringens*. Similarly it was reported that PCR for 16S rRNA gene is reliable method for detection of *C. perfringens* from a group of individual showing similar type of biochemical profile [29]. In present study, the toxinotypes confirmed as type B were 7/35 (20%) while type D were 2/35 (5.71%). Whereas, a team of researchers conducted a study in Nigeria on 245 samples, which were tested by toxin-antitoxin neutralizing tests, of which 127

were type A, 17 were type B, 14 were type C, 44 were type D, 19 were type E. According to that study 90.20% isolates were *C. perfringens* [30].

Out of 41 isolates which were positive for *C. perfringens* on biochemical basis, it was observed that 38 isolates were positive after PCR amplification of 16S rRNA gene and sequencing as *C. perfringens*. It was revealed that 16S rRNA followed by gene sequencing can be opted as alternative tool for definitive confirmation of *C. perfringens* [31]. Earlier it was elaborated that diverse clostridial species were identified with the help 16S rRNA gene amplification and sequencing [32]. In 1994, Wang and coworkers identified *C. perfringens* on the basis of 16S rRNA gene amplification by using species specific primers [33]. DNA-based techniques (PCR and hybridization) have been developed for *C. perfringens* typing and are a reliable alternative method to testing in laboratory animals [6]. However, there is variability to in-vitro production of toxins of *C. perfringens*, so it is problem in using immunological tests. Molecular methods (genotyping), which are mainly based on polymerase chain reaction (PCR) have become the standard for toxin typing of *C. perfringens* [34]. In current study, out of 38 isolates 16 were positive for *cpa* toxin gene while 14 isolates contained both *cpa* and *etx* toxin genes and 8 isolates were positive for *cpa*, *cpb* and *etx* toxin genes.

C. perfringens type B bacteria produced higher hemolytic units (21.45±0.53) and ELISA % (14.27, 13.92, 13.67, 13.56 and 13.45) of alpha toxin when cultured in Robertson Cooked Meat Medium (RCMM) under anaerobic conditions at 37°C with pre adjusted pH 6.0. It was reported that after inoculation active growth started within ~3-4 hours and gas production in broth cultures was also observed as bubbling. In fermenter and stationary culture, *C. perfringens* active growth started within 2 and 3 h [35]. In broth culture, there is decreased production of alpha toxin units in present study. Previously, *C. perfringens* type B hemolytic titer were observed in culture supernatant of reinforced clostridial (RC), thioglycolate (TG),egg meat (EM) and iron milk (IM) medium [19]. After 4 hours of growth in RC medium alpha toxin hemolytic units of type B were 66 HU/mL. In Present study results were in contrast to this study, because type B produced low hemolytic units of alpha toxin.

It was reported that *C. perfringens* produces various acids viz; propionic acid, butyric acid and acetic acid in routine culture media due to multiple metabolic activities resulting in decreased pH [36]. It was established that highest hemolytic units by *C. perfringens* were reported at pH 4.2 to 5.8 [19]. In present research hemolytic activity of alpha and epsilon toxin was observed at pH 6. Brandi and colleagues recorded that the *C. perfringens* type B growth dropped the pH from 7 to 5.2 due to production of organic acid production. It was claimed that from pH 5.5 to 8.00 *C. perfringens* grow well. It is difficult to maintain the constant pH during cultivation, so it severely affected the microbial and enzyme activity of *C. perfringens* [37].

C. perfringens toxinotype B bacteria produce higher cytotoxic units (18.65±0.34 CU/mL) and ELISA % (12.43, 12.81, 12.39, 12.61 and 13.07) of beta toxin when cultured in Robertson Cooked Meat Medium (RCMM) under anaerobic conditions at 37°C with pre-adjusted pH 6.0. It was reported that type B cell free supernatant lethality without trypsin treatment was found to be of beta toxin. But after trypsin pre-treatment both beta and epsilon toxins are important and a part of beta toxin still remains active. *C. perfringens* Type B was grown in TGY medium at 37°C and beta toxin level was 16.5±2.3 μg/mL [18]. It was found that beta toxin variant was having increase trypsin sensitivity and more cytotoxicity. This increase cytotoxicity was attributable and compensates for increase trypsin sensitivity of beta toxin variant CN 3685. Beta toxin in monomeric form liable to trypsin activity but after oligomer formation on the host cell the complex becomes trypsin resistant. Beta toxin produced pore in cell membrane. In present research beta toxin was cytotoxic for BHK21 cell line. After treatment with toxin for 24 hours swelling, round and clumping of cells was observed.

C. perfringens type B bacteria produced higher hemolytic units (16.57±0.19 HU/mL) and ELISA % (13.93, 14.78, 14.28, 14.03 and 13.25) of epsilon toxin when cultured in Robertson Cooked Meat Medium (RCMM) under anaerobic conditions at 37°C with pre adjusted pH 6.0. In a previous study, *C. perfringens* type B epsilon toxin produced 1040 HU/mL hemolytic units. After treatment with 1% trypsin, epsilon toxin activated and there were increased hemolytic units observed. In thioglycolate and RC medium, after trypsin treatment hemolytic units of epsilon toxin were 2056 HU/mL [19]. Hemolytic units of epsilon toxin of type B did not increase after 1% trypsin treatment in present study. During epsilon toxin production in synthetic medium fresh caprine heart and liver tissue used and minimum lethal dose (MLD) was 4000/mL. For toxin production temperature, pH, stirring rotation per min were 37°C, 7.0 and 100-200rpm. Glucose (0.2%) was supplemented in intervals of every 2 hours for toxin maximum production [35]. But there low hemolytic and $TCID_{50}$ units of alpha, beta and epsilon toxins were observed. But in RCMM maximum production of alpha, beta and epsilon toxins was observed.

In conclusion, as per results of current study, toxin production potential of *C. perfringens* toxinotype B isolated from lamb dysentery cases can be maximized in robertson's cooked meat medium with pre-adjusted pH 6.0 at 37°C of incubation temperature. Gene expression

studies under these conditions may result in higher expression of alpha, beta and epsilon toxins genes.

Availability of data and materials

Given data in this paper is to be submitted accurately and come from the University of Veterinary and Animal Sciences, Lahore, Pakistan. Data that support the findings of this study are available on request from the corresponding author (A.A. Anjum). The data are not publically available due to privacy and ethical restrictions.

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Conflict of Interest

It is certified that there is no conflict of interest in any part of the manuscript or among the authors.

Author Contributions

MMKS and AAA performed experiments regarding isolation of *C. perfringens* from clinical cases along with molecular toxinotyping for confirmation of *C. perfringens* and written the initial draft while YFC performed phylogenetic analysis and proofread the final draft of the manuscript whereas, TY and AA performed the experiments regarding optimization of toxin production and edited the final version of the manuscript.

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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.

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. The length of the text should be no longer than 15 pages in total.

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.

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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.

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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