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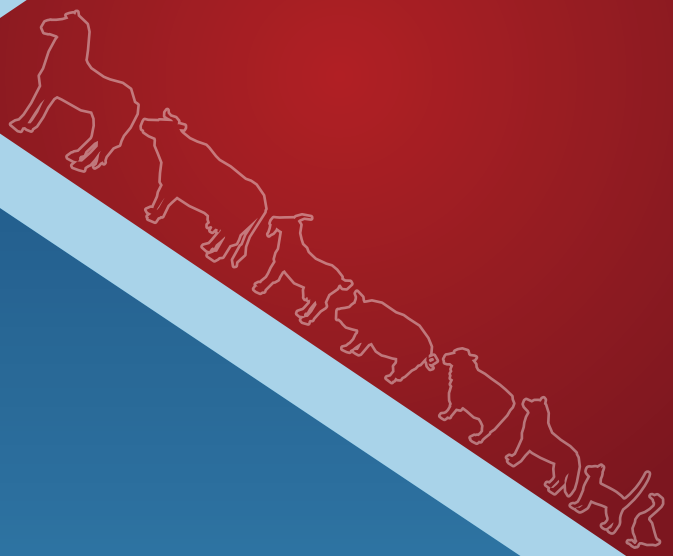

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Research Article

The Expression Profile of Some Homeobox Proteins in the Bovine Liver During Prenatal Development

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Abstract: Homeobox proteins play critical roles in controlling processes such as morphogenesis and organogenesis in many organisms. Some of these proteins are known to affect the formation, development and regeneration of the liver. In this context, the present study was aimed at demonstrating the localization and expression intensity of some homeobox proteins in the bovine fetal liver during the different stages of gestation, determining whether or not these proteins are found in the structural components of the liver, and identifying their potential physiological roles. The study material comprised of 27 clinically healthy bovine fetuses, which were obtained from slaughterhouses and belonged to different stages of gestation. The fetuses were grouped according to their crown-rump length (CRL) measurements. Liver samples were taken from each study group and subjected to routine histological processing, followed by immunohistochemical staining. The staining results showed that, throughout gestation, the expression intensities of the homeobox proteins HOXA10, HOXA11, HOXB6, TLX1, Dlx-5 and HLX were stronger in the hepatocytes, compared to the hepatic artery, vena interlobularis and bile ducts. However, the expression intensity of HLX was determined to have significantly decreased during the second and third trimesters of gestation, compared to the first trimester. In conclusion, the expression of the investigated homeobox proteins at differing and similar levels in the hepatocytes, hepatic artery, vena interlobularis and bile ducts of the bovine fetal liver during gestation could be interpreted as an important indicator of these proteins being involved in the development and physiological activity of the fetal liver.

Keywords: *Bovine, Fetus, Hepatocytes, Homeobox proteins, Liver*

Prenetal Gelişim Süresince Sığır Karaciğerindeki Bazı Homeobox Proteinlerinin Ekspresyonu

Öz: Homeobox proteinleri, birçok organizmada morfogenezis ve organogenezis gibi süreçlerin kontrol edilmesinde kritik roller üstlenmektedir. Bu proteinlerin bir kısmının karaciğerin oluşumu, gelişimi ve rejenerasyonuna da etki ettiği bilinmektedir. Bu nedenle çalışmamız; Homeobox proteinlerinin sığır fötal karaciğerinde gebeliğin farklı dönemlerinde bazı homeobox proteinlerinin lokalizasyonu ve ekspresyon yoğunluğunu göstermek, karaciğerin yapısal bileşenlerine katılıp katılmadığını ve olası fizyolojik rollerini belirlemek amacı ile yapılmıştır. Çalışmada kesimhanelerden temin edilen gebeliğin farklı dönemlerine ait ve klinik olarak sağlıklı 27 adet fetüs kullanıldı. Kullanılan fetüslerin gruplandırılması da alın-sağrı uzunluğu (Crown-Rump Lenght; CRL) ölçümüne göre yapıldı. Belirlenen her gruptan alınan karaciğer örnekleri rutin histolojik prosedürlerinden geçirilerek immunohistokimya boyamasına tabi tutuldu. Boyama sonucunda gebelik dönemlerine göre karaciğer hepatositleri, arteria hepatika, vena interlobularis ve ductus biliferilerdeki HOXA10, HOXA11, HOXB6, TLX1, Dlx-5 ve HLX ekspresyon yoğunlukları karşılaştırıldığında gebelik süresince hepatositlerdeki reaksiyonun diğerlerine oranla daha güçlü olduğu görüldü. Ancak, HLX ekspresyon yoğunluğunun gebeliğin 2. ve 3. trimesterlarında gebeliğin 1. trimesterına göre anlamlı bir şekilde azaldığı belirlendi. Sonuç olarak bazı homeobox proteinlerinin fötal sığır karaciğerindeki hepatositlerde, arteria hepatika, vena interlobularis ve ductus beliferuslarda gebeliğin her döneminde benzer ve farklı düzeylerde ekspresse olması bu proteinlerin fötal karaciğerin gelişiminde ve fizyolojik aktivitesinde rol oynadıklarının önemli bir kanıtı olabilir.

Anahtar sözcükler: *Sığır, Fetüs, Hepatositler, Homeobox proteinler, Karaciğer*

Introduction

The liver, which is the largest and most functional visceral organ of the body, develops from the intestinal endoderm in the mid-third week of embryonic development [1,2], and owing to its hematopoietic role, displays a rapid development in the prenatal period, such that it constitutes nearly 10% of the fetal weight by the 10th week of gestation.

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The hematopoietic activity of the liver continues until the last two months of the prenatal period, and progressively decreases until parturition, such that only very small hematopoietic islets are observed at the time of birth [2,3].

The liver plays a critical role in both viability and some digestive processes. This organ is involved in metabolic processes such as hematopoiesis and blood volume regulation in the embryonic period, as well as in protein synthesis, immunity, the endocrine control of growth signal pathways, and physiological processes such as metabolite deposition, bile secretion and detoxification [4,5].

The liver is composed of different types of embryonic cells (hepatocytes, biliary epithelial cells-cholangiocytes, stellate cells, Kupffer cells and hepatic sinusoidal endothelial cells). Each of these different cell types have unique tasks, which complement each other in the functioning of the liver. Hepatocytes, which comprise the primary epithelial cell population of the liver, make up the majority (60%) of the hepatic volume and undertake multiple tasks. Hepatocytes have been demonstrated to be regulatory cells that are critical to nutrient transport as well as fetal growth and development. On the other hand, Kupffer cells are described as resident hepatic macrophages. These cells are capable of responding to pathogenic stimuli carried by the hepatic portal circulation, and depending on a series of contributing factors, may play pro- or anti-inflammatory roles in hepatic wound healing [5,6].

Homeobox genes encode the homeodomain proteins, which regulate development, differentiation and morpho-genesis in various organisms, including animals and plants [7]. By means of hematopoietic differentiation, tissue-specific homeobox proteins are reported to show effect on cell division, cell development and hepatic regeneration [8]. HOX proteins, which are a subunit of the homeobox proteins, are classified under subtypes, which are referred to as HOX/Hox A/a, B/b, C/c and D/d and are localized to different chromosomes, in humans and mice [9].

HOXA10, which is a sub-member of the HOX proteins and belongs to cluster A on chromosome 7, plays critical roles in gene expression, morphogenesis, differentiation, fertility, embryonic viability and hematopoietic lineage [10]. This particular protein has also been indicated to regulate the proliferation, migration and invasion of cells in various organ and tissue tumors [11].

HOXA11 is a transcription factor, which provides certain positional identities to cells and takes part in the regulation of the developmental system. Moreover, HOXA11 has also been reported to regulate uterine development in females, and to be expressed in the thymus, placenta, lungs, prostate and liver [12]. HOXB6, similar to other mammalian HOX proteins, serves as a DNA-binding transcription factor [13]. Thereby, HOXB6 has been reported to have influence on neurogenesis, renal development and hematopoiesis, as well as on the proliferation and differentiation of multiple cells and tissues [13,14]. The physiological functions determined for other members of the homeobox protein family include splenogenesis and the development of certain sensory neurons for TLX1, the development of the forebrain and craniofacial structures, osteogenesis, chondrogenesis, neurogenesis and hematopoiesis for Dlx-5, and the development of visceral organs such as the gallbladder, liver and intestines as well as the differentiation of hematopoietic cells for HLX [15-19]. The primary regulators of hepatic development were identified by the use of rodent, fish and frog models in preliminary research. These regulators include extracellular signal molecules, intracellular signal transduction pathways and transcription factors. While members of the family of transcription factors have been described as being proteins required for hepatic specification, homeobox proteins have been listed among the major regulatory factors of hepatic development [20]. In this context, the present study was aimed at i) determining the localization and expression intensity of some homeobox proteins during the development of the liver in the bovine fetus ii) identifying the gestational stage during which the intensity of expression, demonstrated by immunohistochemistry, differs iii) and determining the potential physiological roles of the selected homeobox proteins.

Material and Methods

The study material comprised of 27 clinically healthy Holstein bovine fetuses without sex differentiation, which belonged to different gestational stages and were obtained from private slaughterhouses. Fetal age was estimated by measuring the crown-rump length (CRL) and using the formula described by Harris et al.[21]. Following age estimation *(Table 1)*, the fetuses were assigned to one of the three groups established for the different gestational trimesters as follows: the first trimester (days 69-89 of gestation/9 fetuses), the second trimester (days 99-178 of gestation/9 fetuses), and the third trimester (days 190-269 of gestation/9 fetuses). Hepatic tissues samples were taken from the fetuses included in each group. These tissue samples were first fixed in 10% formalin-alcohol solution for 18 h, then dehydrated through a graded series of alcohol, cleared in methyl benzoate and benzene, and embedded in paraffin. Five-micrometer-thick cross-sections were cut from the paraffin blocks. For immunohistochemical staining, these sections were mounted on glass slides coated with 3-aminopropyltriethoxysilane (APS) (Sigma-Aldrich Chemicals, St. Louis, MO, USA).

Immunohistochemistry

The serial sections, after being mounted onto adhesive glass slides, underwent immunohistochemical (IHC)

Table 1. *Estimation of fetal age*

Parameter	Number of Samples																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Fetal crown-rump length (CRL, in cm)	6	7.5	8	10	11	12.5	13	13.5	14.5	18	19.5	21	22.5	24	28	34.5	47.5	50	55	58	60	65	66.5	70	79	82	87
Fetal age (Day)	69	73	75	79	82	86	87	88	90	99	102	106	110	114	123	140	172	178	190	198	202	214	219	227	249	257	269

Table 2. *Primary antibodies used for immunohistochemistry (IHC)*

Antibodies	Clonality/Isotype	Host	Reactivity	Dilution	Catalog Number
HOXA10	Polyclonal/IgG	Rabbit	Human, Mouse	1/100	St John's Laboratory, model no: STJ193159
HOXA11	Polyclonal/IgG	Rabbit	Human	1/100	Invitrogen, PA5-57341
HOXB6	Polyclonal/IgG	Goat	Human, Mouse, Rat, Dog, Cattle, Pig	1/100	St John's Laboratory, model no: STJ73348
TLX1	Polyclonal/IgG	Rabbit	Human, Mouse, Rat	1/100	Invitrogen, cat no: PA5-34553
Dlx-5	Polyclonal/IgG	Rabbit	Human, Mouse, Rat	1/100	St John's Laboratory, model no: STJ92725
HLX	Polyclonal/IgG	Rabbit	Human	1/100	Invitrogen, PA5-44857

staining using the streptavidin-peroxidase procedure. Once dried, the sections were first deparaffinized (2x5 min in xylol), then rehydrated through a graded series of ethanol, and transferred into distilled water. Subsequently, tissue endogenous peroxidase activity was blocked by maintaining the sections in 3% H_2O_2 solution in methanol for 20 min followed by 3x5 min washes in phosphate-buffered saline (PBS) (pH: 7.4, 0.01 M). Next, the preparations were incubated in citrate buffer solution (pH: 6) at 95ºC for 30 min to expose the antigenic regions for antibody binding, and at the end of the incubation period, were left in the same solution until being cooled to room temperature. Subsequently, the sections were incubated in a blocking solution (Ultra V Blok, catalogue number: TA-125-UB, Thermo Scientific) for 15 min to block the non-specific binding of the primary antibody, and after the discard of the solution, were incubated with the primary antibodies listed in *(Table 2)* overnight at 4ºC. The next day, after being washed 3x5 min in PBS, the sections were incubated with biotinylated secondary antibody (Biotinylated Goat Anti-Polyvalent, catalogue number: TP-125-BN, Thermo Scientific) at room temperature for 20 min. Following another round of 3x5 min washes in PBS, the sections were treated with streptavidin peroxidase (Thermo Fisher Scientific, catalogue number: TA-125-HR) at room temperature for 20 min. Subsequently, 3.3 diaminobenzidine (DAB Substrate, Thermo Scientific, catalogue number: TA-125-HD) was dropped onto the slides and treatment was allowed for 5-15 min. After being washed in distilled water, nuclear staining was performed with Mayer's hematoxylin for 2 min. Next, the sections were washed under running tap water for 5 min, dehydrated through a graded series of alcohol, cleared in xylol, and finally embedded in Entellan and covered with a coverslip. The accuracy of the immunohistochemical method applied was demonstrated with the use of positive controls, which comprised of bovine uterine and feline testicular tissue samples. On the other hand, the negative controls comprised of hepatic tissue samples, which were incubated with PBS instead of primary antibody.

Semi-quantitative Assessment

The immunoreactions demonstrated for some homeobox proteins in the hepatic tissue samples were observed at different magnifications (10X, 20X and 40X) under a Nikon Eclipse E400 (Nikon, Tokyo, Japan) research microscope equipped with a digital camera (Nikon Coolpix 4500), and were assessed semi-quantitatively for the intensity score. The intensity scores were determined on the basis of the intensity of the positive staining of the cells. All regions of the liver were screened by two independent senior researchers (UT and HS) for the scoring of the immunohistochemical staining. Scoring was performed on a 3-point scale as follows: 0 - negative (no staining observed in the cells at high microscopic magnification), 1 - weak (stained cells observed only at high microscopic magnification), 2 - moderate (stained cells easily observed at low microscopic magnification), 3 - strong (stained cells observed at very low microscopic magnification) [22]. Semi-quantitative assessment was made for each hepatic portal area components and adjacent hepatocytes (hepatocytes, branch of the hepatic artery, branch of the vena interlobularis and bile ducts).

Statistical Analysis

Statistical analyses were made with the SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) software package. All values are given in mean ± standard deviation. Data normality was assessed with the Shapiro-Wilk test. The non-parametric Kruskal-Wallis test was used to analyze any statistically

significant difference in the immunohistochemical staining intensity score for HOXA10, HOXA11, HOXB6, TLX1, Dlx-5 and HLX of the hepatocytes, hepatic artery, vena interlobularis and bile ducts of the bovine fetal liver during the different trimesters of gestation or between these cell and tissue types. Differences between the cell types for the staining intensity score of each antibody were determined with the Mann-Whitney U test. The results are given in mean ± standard deviation (SD) and statistical significance was set at P<0.05 *(Fig. 1, Fig. 2-k)*.

Results

Immunohistochemical staining demonstrated varying intensities of positive reactions for the proteins HOXA10, HOXA11, HOXB6, Dlx-5, TLX1 and HLX in the bovine fetal liver during the different stages of gestation.

Strong immunoreactions were observed for HOXA10, HOXA11 and HOXB6 in the hepatocytes during all three trimesters of gestation *(Fig. 1-a,b,c)*. Immunoreactions for HOXA10 in some hepatic arteries were weak any during

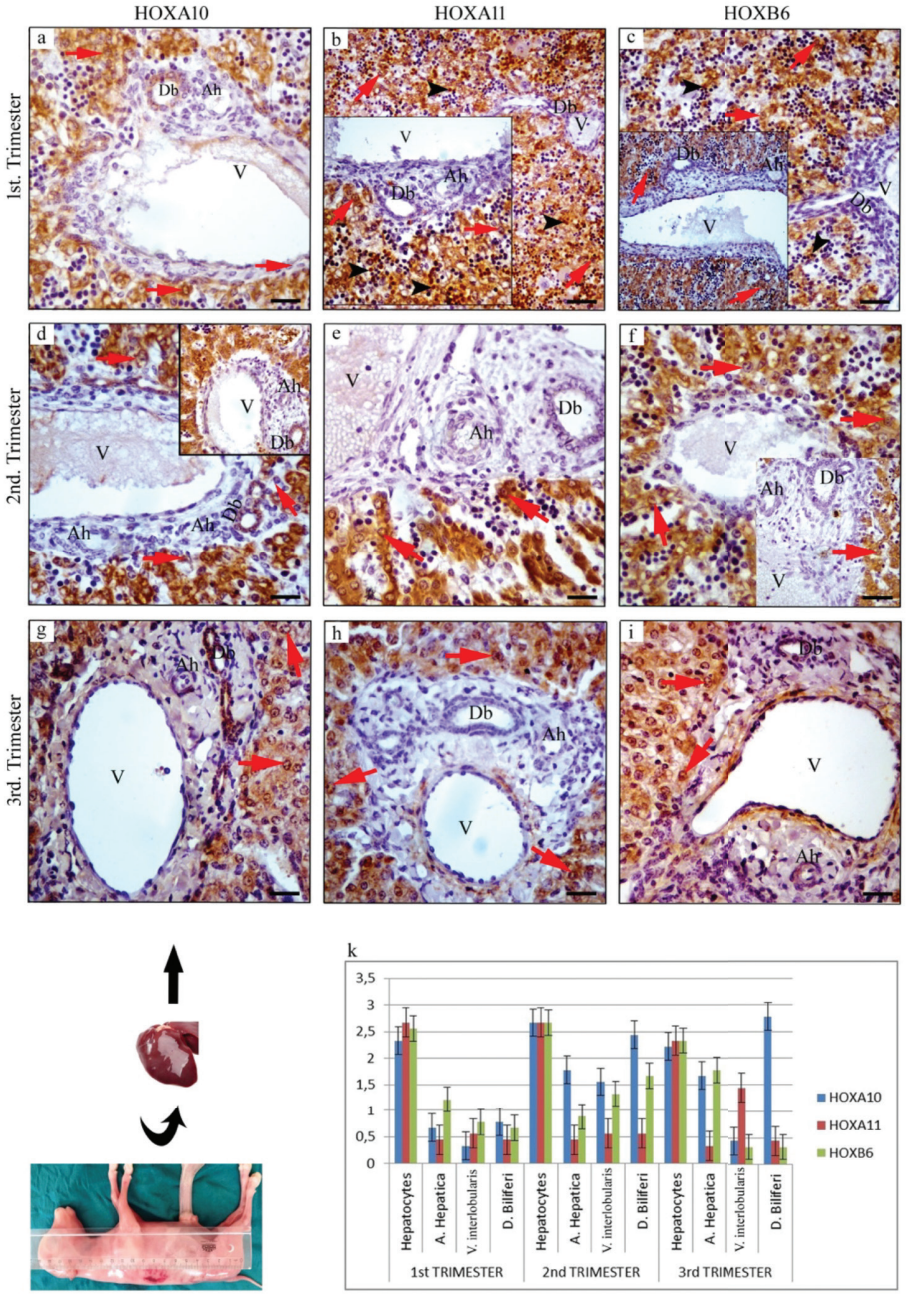

Fig 1. Expression of HOXA10, HOXA11 and HOXB6 in fetal bovine liver, in the 1st trimester (73 days) (a, b, c), 2nd trimester (102 days) (d, e, f), 3rd trimester (214 days) (g, h, i), Statistical graph of HOXA10, HOXA11 and HOXB6 staining intensity (k). *Red arrow:* Hepatocytes, Black arrowhead: Lymphocyte, Ah: Arteria hepatica, V: Vena interlobularis, Db: Ductus biliferi. Scale Bar: 25 µm (a, d, e, f, g, h, i), 50 µm (b, c)

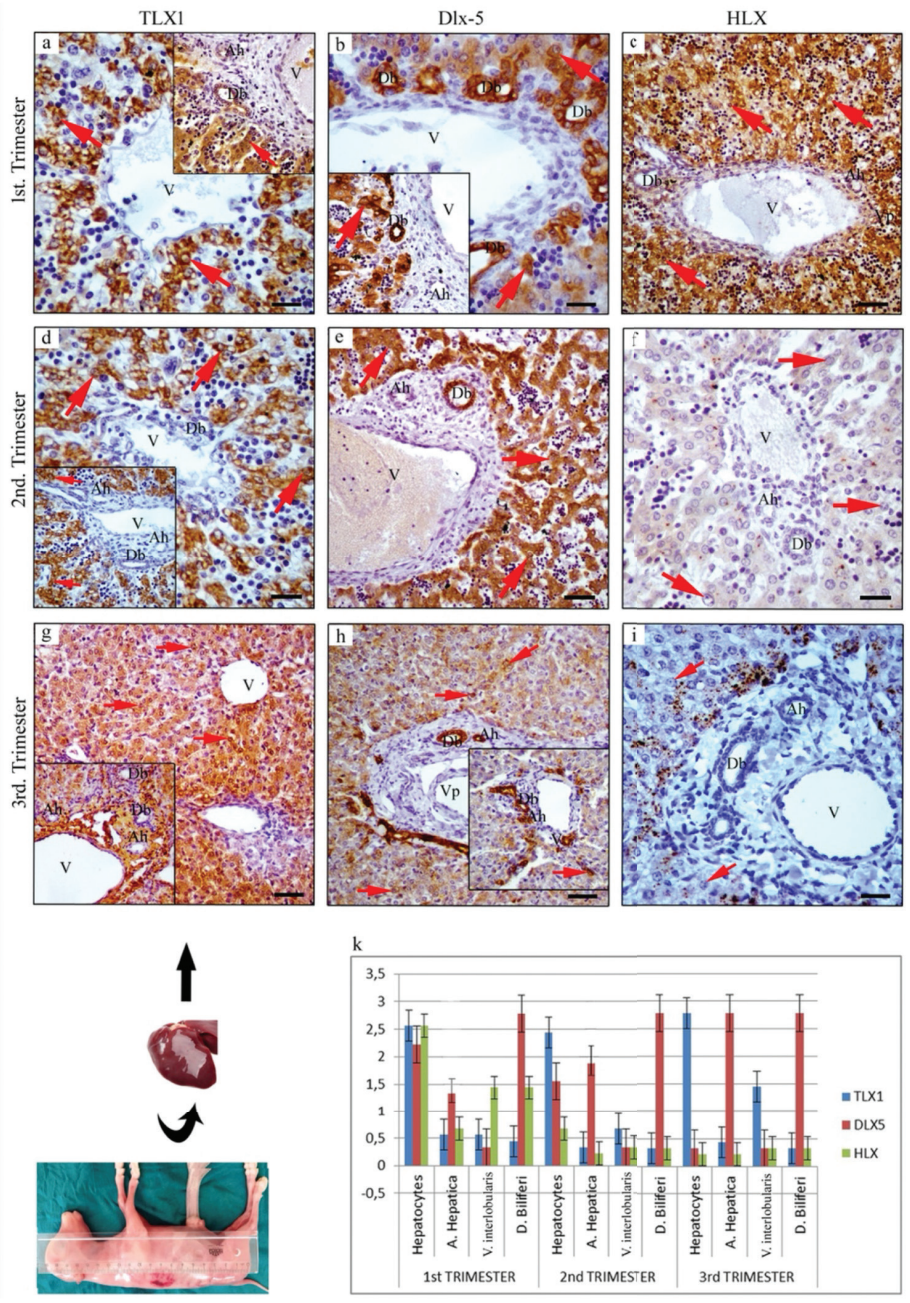

Fig 2. Expression of TLX1, Dlx-5 and HLX in fetal bovine liver, in the 1st trimester (69 days) (a, b, c), 2nd trimester (114 days) (d, e, f), 3rd trimester (227days) (g, h, i), Statistical graph of TLX1, Dlx-5 and HLX staining intensity (k). *Red arrow:* Hepatocytes, Ah: Arteria hepatica, V: Vena interlobularis, Db: Ductus biliferi. Scale Bar: 25 µm (a, b, d, f, h, i), 50 µm (c, e, g, h)

the first trimester and moderate during the second and third trimesters. Immunoreactions in some vena interlobularis were weak any during the first and third trimesters and moderate during the second trimester. On the other hand, immunoreactivity in the intrahepatic bile ducts was weak during the first trimester and strong during the second and third trimesters *(Fig. 1-a,d,g)*. Immunoreactions were weak observed for HOXA11 in the hepatic artery and intrahepatic bile ducts throughout the three trimesters of gestation. Furthermore, immunoreactions in the vena interlobularis were weak during the first and second trimesters and moderate during the third trimester *(Fig. 1-b,e,h)*. HOXB6 immunoreactivity in the hepatic artery was weak throughout gestation, but relatively stronger during the last trimester compared to the first and second trimesters. On the other hand, in some vena interlobularis and intrahepatic bile ducts, immunoreactions were weak any during the first and third trimesters, and stronger during the second trimester *(Fig. 1-c,f,i)*. Furthermore, immunoreactions for HOXA11 and HOXB6 in the lymphocytes were strong during the first trimester of gestation *(Fig. 1-b,c)*.

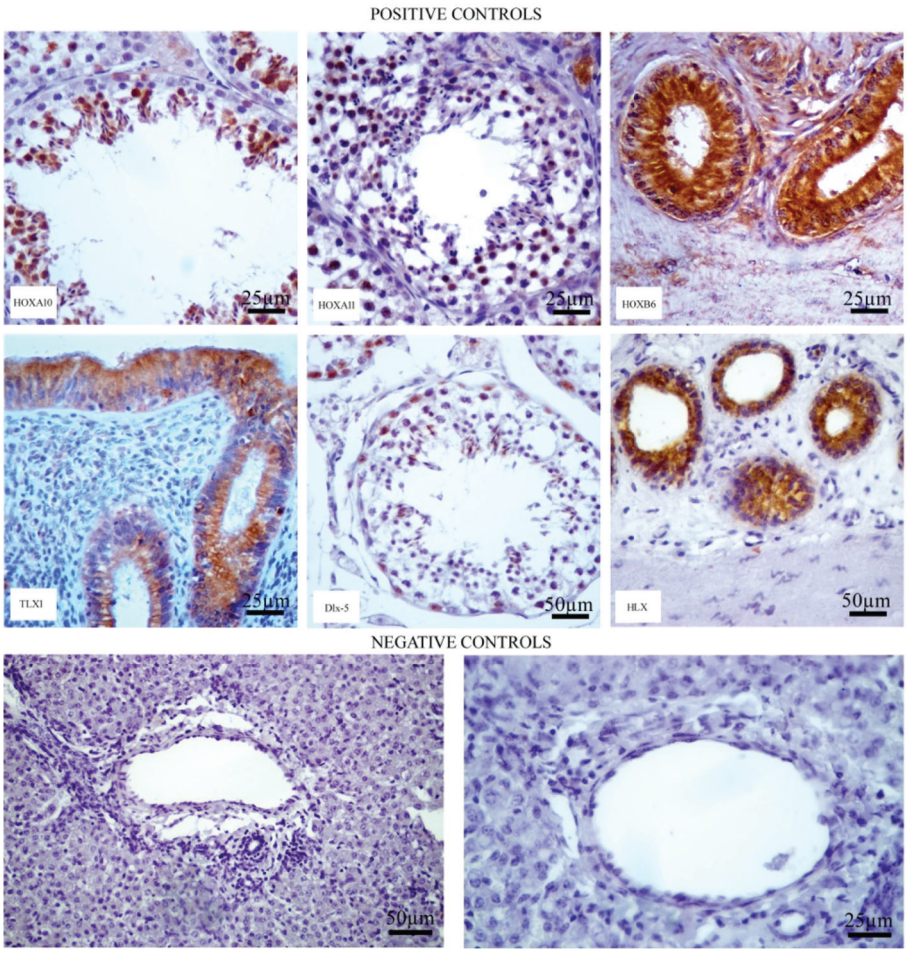

Fig 3. Positive expression of HOXA10, HOXA11, Dlx-5 proteins in testis, HOXB6, TLX1 and HLX proteins in uterine tissue. Negative expression of HOXA10, HOXA11, Dlx-5, HOXB6, TLX1 and HLX proteins in fetal bovine liver

During all three trimesters, the intensity of immunoreactions for TLX1 in the hepatocytes ranged from moderate to strong. On the other hand, immunoreactivity in some hepatic artery and intrahepatic bile ducts was weak any throughout gestation. In the vena interlobularis, immunoreactions ranged from weak to moderate during the third trimester, and were relatively stronger in intensity compared to the first and second trimesters *(Fig. 2-a,d,g)*. In the hepatocytes, immunoreactions for Dlx-5 were of moderate intensity during the second and third trimesters, and were stronger during the first trimester. On the other hand, Dlx-5 immunoreactions were moderate to strong in some hepatic artery and intrahepatic bile ducts during all three trimesters ($P<0.05$), but were weak any in the vena interlobularis *(Fig. 2-b,e,h)*. While the HLX protein induced strong immunoreactions in the hepatocytes during the first trimester ($P<0.05$), immunoreactivity was weak any and even negative in some hepatocytes during the second and third trimesters. Immunoreactions for HLX were weak any in the hepatic artery throughout gestation. Furthermore, immunoreactions in the vena interlobularis and intrahepatic bile ducts were weak during the second and third trimesters, but were relatively stronger during the first trimester *(Fig. 2-c,f,i)*.

The accuracy of the staining was confirmed by the use of positive controls (bovine uterine and feline testicular tissues) and negative controls *(Fig. 3)*.

Discussion

Homeobox proteins are critical to the identity of the various structures/tissues localized to the anterior-posterior axis of the developing embryo, as well as to organogenesis and cell differentiation [23,24]. Known to be expressed during the very early period of mammalian development, Hox proteins have also been observed in all three embryonic germ layers (and are of ectodermal origin in the nervous system, mesodermal origin in the genitourinary system and endodermal origin in the digestive system), and have been reported to undertake critical roles in these layers [25]. Research has shown that some homeobox proteins (Hex) undertake basic roles in endodermal organs, such as the thyroid gland and liver [26]. The present study demonstrated both the expression and the localization of the investigated

homeobox proteins in the bovine fetal liver during gestation and revealed that expression showed relative differences with gestational stage. Thus, in agreement with previous research on homeobox proteins [20], the present study demonstrated that HOXA10, HOXA11, HOXB6, TLX1, Dlx-5 and HLX could play major roles in the morphogenesis and cell differentiation of the bovine fetal liver during gestation.

Depending on the chromosomal position of their encoding genes, the proteins HOXA10 and HOXA11 have been indicated to be expressed along the paramesonephric canal in the human fetus. Based on this information, these proteins have been reported to be involved in embryonic development and to affect uterine development and differentiation [27]. Several studies have shown that these particular proteins may have normal functional roles in the female genital system of mice as well as in skeletal and renal tissue development [28], the uterus of rats [29], humans [30], monkeys [31] and pigs [32], and the bovine placenta and feline testis [33,34]. On the other hand, these proteins have also been reported to be involved in the formation and progression of tumors in humans, such that in particular HOXA10 has been indicated to be present at levels higher than that of hepatocytes in hepatic cell cancer [11,35,36]. Furthermore, it has been determined that, apart from being expressed in normal hepatic tissue [12], HOXA11 also affects hepatocyte carcinoma and aids in the proliferation and invasion of these cancer cells [35]. In line with these studies, it has been determined that HOXA10 and HOXA11 are found in adult liver. However, Cauwelier and Speleman [12] and Yu et al.[35] have revealed that these proteins are expressed in the adult liver. In parallel with this report, the present study demonstrated that the homeobox proteins HOXA10 and HOXA11 are expressed in some cells and structures of the bovine fetal liver throughout gestation. While expression was determined to be strong and at similar levels in the hepatocytes during all three trimesters of gestation, it was ascertained that the expression of HOXA10 was stronger in the bile ducts during the second and third trimesters of gestation ($P<0.05$). In the hepatic artery, the expression of HOXA10 was relatively stronger than that of HOXA11, and occurred at stronger intensities during the second and third trimesters ($P>0.005$). The findings of the present study suggest that these proteins may have a modulating effect in the bovine fetal liver and contribute to the development of the liver and the physiological functions of the hepatocytes (nutrient transport). Our findings also suggest the particularly major involvement of HOXA10 in the division and proliferation of vascular endothelial cells and bile duct epithelial cells. Furthermore, HOXA11 having been determined to be strongly expressed in some lymphocyte-like cells during the first trimester of gestation suggests that this protein may contribute to the erythropoietic activity of the liver, the blood-forming organ of the fetal development period, as well as to the defense system and hematopoiesis.

In previous research, HOXB6 has been generally reported to be expressed in human hematopoietic progenitor/stem cells [37,38]. To our knowledge, there is no previous study on the role of HOXB6 in healthy fetal liver tissue. However, in available literature [39], this protein has been described as a SOX9 biomarker involved in the proliferation, differentiation and regeneration of liver progenitor cells, hepatocytes and bile duct epithelial cells in mice. Moreover, it has been reported that HOXB6 transcriptionally regulates the expression of the SOX9 biomarker, and thereby, affects the proliferation and differentiation of liver cells. In another study, it was determined that HOXB6 was expressed during the oncogenic processes of some tissues and organs (esophagus, hepatocytes) and affected the regulation of the proliferation, migration and invasion of cancer cells [40]. Similar to the case in the human and bovine placentae and feline testes, the present study demonstrated that HOXB6 was expressed in the bovine fetal liver, such that the expression intensity was strong in the hepatocytes, but ranged from weak to moderate in the hepatic artery, vena interlobularis and bile ducts during all three trimesters of gestation. Thus, in agreement with the findings of previous cancer research on HOXB6 [40], the present study revealed that this protein could also affect cell division, proliferation and migration in the bovine fetal liver. Similar to HOXA11, the determination of HOXB6 immunoreactivity in lymphocyte-like cells during the first trimester of gestation suggests that this protein could have a synergistic effect with HOXA11.

Although TLX1 is normally not expressed in hematopoietic cells, previous studies on TLX1 and Dlx-5 have shown that this protein is expressed in the fetal spleen and plays an important role in the development of this organ [41]. TLX1 has been described as an oncogene, the disrupted expression levels of which are associated with T-cell acute lymphoblastic leukemia (T-ALL) in humans [42]. In a previous study aimed at demonstrating the effects of TLX1 on cell differentiation and proliferation in mice, this protein was determined to be structurally expressed in fetal liver cells [43]. Similarly, the Dlx-5 gene encodes the transcription factors essential to embryonic and postnatal development. This protein is involved in the morphogenesis of the craniofacial structures, branchial arches, forebrain and sensory organs, postpartum homeostasis and particularly hematopoiesis, and if expressed irregularly, also in oncogenesis (cancer of the ovaries and lungs) [44]. According to the current information about the expression and presence of TLX1 and Dlx5 in the liver, it is known that these proteins have critical roles in the development of some tissues during

the embryonic period. In the present study, TLX1 induced strong immunoreactions in the hepatocytes during all three trimesters of gestation, whilst Dlx-5 induced weaker immunoreactions during the second and third trimesters. This suggests that these proteins could potentially affect the division, proliferation and physiological functions of hepatocytes. On the other hand, the expression of Dlx-5 having been observed to progressively decrease with the advance of gestation was considered to be related to the decrease in metabolic activity, division and proliferation rate of cells with gestational advance. Moreover, immunoreactions for TLX1 being scarcely any in the hepatic artery, vena interlobularis and bile ducts throughout gestation suggests that this protein has no effect on the mitotic activity of vascular endothelial cells and bile duct epithelial cells in the liver. Contrarily, immunoreactions having been observed for Dlx-5 in the bile ducts and moderate to strong in some hepatic artery throughout gestation could be interpreted as this protein contributing to angiogenesis, the division and differentiation of vascular endothelial cells and bile duct epithelial cells, as well as the production and secretion of bile.

HLX/Hlx has been reported to be expressed in mesodermal tissues, particularly the visceral mesenchyme, skeletal myoblasts, sclerotome and mesenchyme of the extremities during embryogenesis [45,46]. It has been reported that, in mice, Hlx is significantly expressed in mesodermal tissues, in particular the mesenchyme of the developing liver, gallbladder and intestines [46]. In previous research aimed at determining the functions of the Hlx gene by means of its targeted mutation in mice, it was observed that not only did the liver and intestines display anemia and hypoplasia, but also hepatocyte differentiation and liver growth were restricted [18]. It has also been demonstrated that while Hlx has critical roles in the development of the liver and intestines in mice, the protein sequence of mouse Hlx shows 86.5% homology to that of human HLX, which suggests that these proteins could have similar roles in both species [47]. Human research has shown that the mutation of HLX may cause various anomalies during embryonic development, including diaphragmatic hernia, short bowel and asplenia. In the present study, it was determined that HLX expression had significantly decreased during the second and third trimesters of gestation, when compared to the first trimester ($P<0.05$). Based on this finding, it was considered that, in parallel with the rapid division, growth and differentiation of cells during early gestation, the expression of HLX in bovine fetal liver cells was strong during this period, and this particular protein had a basic role in physiological processes. Furthermore, the decrease observed in the expression of HLX with the advance of gestation was considered to be associated with the reduced metabolic activity and decreased growth rate of the liver with gestational advance. On the other hand, HLX immunoreactions being scarcely any in the hepatic artery, vena interlobularis and bile ducts suggested that the homeobox protein HLX had no effect on the physiological functions of these structures. Based on these results, it is suggested that, similar to the case in mice and humans, HLX is also involved in the formation and development of the bovine liver, and its deficiency may cause liver anomalies.

In conclusion, the presence of the homeobox proteins HOXA10, HOXA11, HOXB6, TLX1, Dlx-5 and HLX in the hepatocytes, some hepatic artery, vena interlobularis and bile ducts of the bovine fetal liver during all three trimesters of gestation, and the expression of these proteins at varying levels, both suggest that these proteins may have significant roles in the development and physiological activity of the bovine fetal liver. Different from previous research on homeobox proteins, which have generally focused on cancer and developmental anomalies, the present study has shown that these proteins may positively contribute to the development of organs, such as the liver, as well as to the development of cells, such as hepatocytes, vascular endothelial cells and bile duct epithelial cells, and may be found in structural components. Thereby, this study provides valuable data for future research.

Availability of Data and Materials

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

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Competing Interests

The authors declare that there is no conflict of interest.

Authors' Contributions

UT, HS and MEA planned the study, designed the experiments and helped manuscript writing; MAK and NA helped with data analyses and bioinformatics and wrote the manuscript; UT, BGS and NA collected samples and conducted laboratory process; HS and MEA analysed the statistics data. All authors read and approved the final manuscript.

Ethical Approval

The materials used in our study were collected from the slaughterhouses of the province of Diyarbakir, and in accordance with the regulation on the working procedures and principles of animal experimentation ethics committees in the official gazette published on

February 15, 'Procedures with dead animals or tissues, slaughterhouse materials, waste fetuses' are not subject to HADYEK permission.

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Research Article

Cerebroprotective Effects of Yizhitongmai Granule and Decomposed Recipes on Vascular Dementia Rats Via the Nod-like Receptor Protein 3 Inflammasome Pathway

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Abstract: We aimed to evaluate the cerebroprotective effects of Yizhitongmai Granule and its decomposed recipes on vascular dementia (VD) rats via the Nod-like receptor protein 3 (NLRP3) inflammasome pathway. Sixty rats were randomly divided into Sham, VD Model, Yizhitongmai Recipe, Bushen Recipe, Tongluo Recipe and positive control groups (n=10). From 12 d after operation, Yizhitongmai Recipe, Bushen Recipe and Tongluo Recipe groups were gavaged with corresponding drug liquid. The drugs were administered at 2 mL once a day for 28 consecutive days. The reactive oxygen species (ROS), superoxide dismutase (SOD), total antioxidant capacity (T-AOC) and lactate dehydrogenase (LDH) in hippocampal tissues were detected using biochemical methods. Tumor necrosis factor-α (TNF-α), interleukin-18 (IL-18) and IL-1β were detected by enzyme-linked immunosorbent assay. Western blotting was performed to detect the expression levels of neuronal growth-associated protein-43 (GAP43), synaptophysin (SYN), aquaporin 4 (AQP4), NLRP3 and Caspase-1. Compared with the Model group, the number of apoptotic cells, levels of ROS, LDH, TNF-α, IL-18, IL-1β, NLRP3 and Caspase-1 decreased, and the levels of SOD, T-AOC, GAP43, SYN and AQP4 increased in the Yizhitongmai Recipe, Bushen Recipe and Tongluo Recipe groups ($P<0.05$). However, the Bushen Recipe and Tongluo Recipe groups had similar indices ($P>0.05$). Compared with the Bushen Recipe and Tongluo Recipe groups, the Yizhitongmai Recipe group had fewer apoptotic cells, decreased levels of ROS, LDH, TNF-α, IL-18, IL-1β, NLRP3 and Caspase-1, and increased levels of SOD, T-AOC, GAP43, SYN and AQP4 ($P<0.05$). Yizhitongmai Granule and its decomposed recipes can protect hippocampal neurons, relieve oxidative stress and inflammatory response caused by hypoperfusion brain injury.

Keywords: *Brain protection, Inflammasome, Nod-like receptor protein 3, Oxidative stress, Vascular dementia*

Yizhitongmai Granülü ve Dekompoze Tanımlarının Nod-benzeri Reseptör Protein 3 İnflamasom Yoluyla Vasküler Demans Sıçanlarındaki Serebroprotektif Etkileri

Öz: Yizhitongmai granülü ve dekompoze tariflerinin, vasküler demans (VD) sıçanları üzerindeki serebroprotektif etkilerinin Nod-like reseptör protein 3 (NLRP3) inflamatuar yolağı üzerinden değerlendirilmesini amaçladık. Altmış sıçan rastgele Sham, VD Model, Yizhitongmai grubu, Bushen grubu, Tongluo grubu ve pozitif kontrol gruplarına ayrıldı (n=10). Deneyden 12 gün sonra, Yizhitongmai grubu, Bushen grubu ve Tongluo grubuna karşılık gelen formülasyonlar verildi. İlaçlar, 28 gün boyunca hergün ve günde bir kez olmak üzere 2 mL şeklinde uygulandı. Hipokampal dokulardaki reaktif oksijen türleri (ROS), süperoksit dismutaz (SOD), toplam antioksidan kapasite (T-AOC) ve laktat dehidrojenaz (LDH) biyokimyasal yöntemlerle tespit edildi. Tümör nekrozis faktör-α (TNF-α), interlökin-18 (IL-18) ve IL-1β, ELISA ile tespit edildi. Nöronal growth-associated protein-43 (GAP43), sinaptofizin (SYN), aquaporin 4 (AQP4), NLRP3 ve Kaspaz-1'in ekspresyon seviyelerini saptamak için Western blot uygulandı. Model grubu ile karşılaştırıldığında, Yizhitongmai, Bushen ve Tongluo gruplarında apoptotik hücre sayısı, ROS, LDH, TNF-α, IL-18, IL-1β, NLRP3 ve Kaspaz-1 seviyeleri azalmış, SOD, T-AOC, GAP43, SYN ve AQP4 seviyeleri artmıştı ($P<0.05$). Ancak, Bushen grubu ile Tongluo grubu benzer indekslere sahipti ($P>0.05$). Bushen ve Tongluo gruplarıyla karşılaştırıldığında, Yizhitongmai grubunda daha az apoptotik hücre, ROS, LDH, TNF-α, IL-18, IL-1β, NLRP3 ve Kaspaz-1 seviyelerinde azalma ve SOD, T-AOC, GAP43, SYN ve AQP4 seviyelerinde artış saptandı ($P<0.05$). Yizhitongmai granülü ve dekompoze tarifleri, hipokampal nöronları koruyabilir, oksidatif stresi ve hipoperfüzyon beyin hasarının neden olduğu inflamatuar yanıtı hafifletebilir.

Anahtar sözcükler: *Beynin korunması, Inflamasom, Nod-like reseptör protein 3, Oksidatif stres, Vasküler demans*

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Introduction

Vascular dementia (VD) defined as an acquired cognitive impairment syndrome caused by hypoperfusion brain injury is the most common dementia disease following Alzheimer's disease [1,2]. According to a meta-analysis, the morbidity rate of VD is about 0.96% in China, and it frequently occurs in the elderly [3]. VD is primarily caused by ischemic stroke, hemorrhagic stroke, and acute/chronic hypoxic cerebrovascular disease, making it one of the important diseases seriously affecting the quality of life of the elderly [4,5]. The specific pathogenesis of VD remains unclear, and relevant research suggests that it is related to the cholinergic system, oxidative stress, inflammatory response and neuronal apoptosis, among which inflammatory response plays a key role [6]. Hypoperfusion brain injury can lead to inflammatory response and neurovascular unit injury, the latter of which is considered the major cause of cognitive impairment in VD in many studies [7,8]. The Nod-like receptor protein 3 (NLRP3) inflammasome pathway is the key for neurovascular unit cell pyroptosis, which is a crucial player in various brain diseases [9]. VD occurs secondary to cerebrovascular events. Traditional Chinese medicine suggests that blood stasis is an important pathological factor leading to the onset and progression of VD [10]. Meanwhile, the kidney can promote blood circulation, indicating that kidney deficiency and blood stasis are mutually causal [11]. Various pathological factors, such as phlegm and blood stasis, invade the brain and eventually damage to the collaterals. Therefore, brain collateral stasis is an inevitable result of VD which is treated by Chinese herbal medicine through nourishing the kidney and dredging brain collaterals [12].

Yizhitongmai Granule is an empirical traditional Chinese medicine prescription for the clinical treatment of VD, with effects of tonifying kidney and dredging collaterals, promoting blood circulation and removing blood stasis, and eliminating phlegm and inducing resuscitation, which can effectively raise the patients' cognitive ability and improve the activity of daily living [13]. In this study, the effects of Yizhitongmai Granule and its decomposed recipes on the cognitive ability, hippocampal tissue morphology, and levels of NLRP3 pathway-related molecules in VD model rats were compared, the cerebroprotective effect of Yizhitongmai Granule on VD rats was explored, and its target and possible molecular mechanism were investigated, thereby providing some references for the selection of prescriptions and drugs in the clinical treatment of VD.

Material and Methods

Ethical Approval

This study has been approved by the animal ethic committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University (Approval No. 2021120082), and all experiments were carried out as per related guidelines.

Laboratory Animals

Sixty SPF male SD rats (15 months old, 280-320 g) were purchased from Shandong Laboratory Animal Center [animal certificate No. SCXK (Shandong) 2017-007]. They were fed adaptively in the SPF room for 1 week before experiments. In the feeding period, the rats had free access to food and water.

Reagents

Positive drug Ginkgo biloba hevert tablets (Ginaton®) were purchased from Dr. Willmar Schwabe GmbH & Co. KG (Germany), and prepared with 1% sodium carboxymethyl cellulose into a suspension. Yizhitongmai recipe: 3 g Dragon's blood, 10 g earthworm, 3 g centipede, 10 g ginseng, 10 g Rhizome of rehmannia, 10 g Sharpleaf galangal fruit, 10 g Gastrodia elata, and 6 g Hirudo. Bushen recipe: 10 g Ginseng, 10 g Rhizome of rehmannia, 10 g Sharpleaf galangal fruit, and 10 g Gastrodia elata. Tongluo recipe: 3 g Dragon's blood, 10 g earthworm, 3 g centipede, and 6 g Hirudo. Crude drugs were bought from Anhui Tienho Herbal Source Co., Ltd. (China). Each milliliter of extract was equivalent to 4 g crude drug, and prepared by the Preparation Room of Shandong Provincial Hospital.

Hematoxylin-eosin (HE) staining kit (Cat. No. C0105S) and one-step terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay kit (Cat. No. C1086) were purchased from Shanghai Beyotime Biotechnology Co., Ltd. Reactive oxygen species (ROS) assay kit (Cat. No. E004-1-1), superoxide dismutase (SOD) assay kit (Cat. No. A001-3-2), total antioxidant capacity (T-AOC) assay kit (Cat. No. C0212-4-2) and lactate dehydrogenase (LDH) assay kit (Cat. No. A020-2-2) were purchased from Nanjing Jiancheng Bioengineering Institute. Enzyme-linked immunosorbent assay (ELISA) kits of tumor necrosis factor-α (TNF-α) (Cat. No. ml002859), interleukin-18 (IL-18) (Cat. No. ml002816) and IL-1β (Cat. No. ml037361) were purchased from Shanghai MLBio Co., Ltd. RIPA reagent (Cat. No. R0278) was bought from Sigma, USA. Pierce BCA protein quantification kit (Cat. No. 23225) and SuperSignal West Pico PLUS chemiluminescent substrate (Cat. No. 34580) were bought from Thermo Fisher, USA. Antibodies of neuronal growth-associated protein-43 (GAP43) (Cat. No. ab75810), synaptophysin (SYN) (Cat. No. ab32127), aquaporin 4 (AQP4) (Cat. No. ab9512), NLRP3 (Cat. No. ab270449) and Caspase-1 (Cat. No. ab207802) were bought from Abcam, UK. Other reagents were of commercially available and analytical grade.

Apparatus

A ZS-Morris water maze (Beijing Zhongshi Dichuang

Technology Development Co., Ltd.), an optical microscope and a fluorescence microscope (Leica, Germany), an automatic biochemical analyzer (Beijing Pulang New Technology Co., Ltd.), an HBS-ScanX full-wavelength microplate reader (Nanjing DeTie Laboratory Equipment Co., Ltd.), a Mini Gel Tank (Thermo Fisher, USA), an eBlot™ L1 rapid wet transfer system (Nanjing GenScript Biotechnology Co., Ltd.), and a contact nondestructive quantitative imager (Shanghai e-BLOT Optoelectronics Technology Co., Ltd.) were used.

Grouping and Modeling

The 60 rats were randomly divided into Sham group, VD Model group, Yizhitongmai Recipe group, Bushen Recipe group, Tongluo Recipe group and positive control group (n=10). The VD model was established in each group except for Sham group as follows [14]: The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate, and fixed in a supine position on the laboratory table. After skin preparation and disinfection, a median incision was made on the neck, the tissue was bluntly separated to expose the bilateral common carotid arteries, the bilateral common carotid arteries were ligated with surgical suture, and the incision was sutured. In Sham group, the bilateral common carotid arteries were only separated without ligation. All rats were injected with penicillin (2000 U) locally at the incision to prevent infection. No rats died after modeling.

Drug Intervention

From 12 d after operation, Yizhitongmai Recipe, Bushen Recipe, Tongluo Recipe and positive control groups were gavaged with corresponding drug liquid, while Sham and Model groups were gavaged with normal saline of the same volume. The drugs were administered at 2 mL once a day for 28 consecutive days.

Detection of Degree of Dementia by Morris Water Maze Test

Morris water maze test was performed in each group at 7 d after operation and after the end of drug administration. Four points were set as entry points in the east, west, south and north directions on the wall of a round pool (diameter: 1.2 m, height: 0.5 m, depth: 0.35 m, water temperature: 25°C). A black platform (diameter: 10 cm, height: 33 cm) was placed in the center of the pool. The rats were put into the pool randomly from the entry point, and the time for rats to swim to the platform was recorded. If the rat failed to find the platform within 120 s, it was guided to the platform by the experimenter, and the latency was recorded as 120 s. After the rats stayed on the platform for 30 s, the test was repeated from a new entry point. The training test lasted for 4 d, during which the external environment of the water maze remained the same. At 5 d, the platform was withdrawn, the rats were put into the water from the pool wall, and the number of times of crossing the original position of platform within 120 s was recorded.

Observation of Hippocampal Morphology by HE Staining

After the second Morris water maze test, all rats were sacrificed by decapitation, and the hippocampus tissues were harvested. Part of hippocampus tissues were fixed with 4% paraformaldehyde for 24 h and prepared into paraffin sections. The remaining part was frozen at -80°C. The paraffin sections were stained using HE staining kit, and the hippocampal morphology in each group was observed and photographed under an optical microscope.

Detection of Hippocampal Neuronal Apoptosis by TUNEL Assay

After deparaffinization and hydration, the paraffin sections were incubated with DNase-free proteinase K (20 μg/mL) at 37°C for 20 min, and washed with 1×PBS for 3 times. After drying, the sections were added dropwise with 50 μL of TUNEL reagent on the surface, covered with a cover glass, and incubated at 37°C away from light for 1 h. After washing with 1xPBS for 3 times, the sections were added dropwise with antifade mounting medium, sealed, observed and photographed under a fluorescence microscope away from light. Positive cells were counted in 5 randomly-selected fields in each group, and the average was taken as the apoptosis status of hippocampal tissues.

Detection of Cerebrovascular Endothelial Cell Function

Part of the hippocampal tissues were prepared into homogenate with an appropriate amount of pre-cooled lysis buffer, fully lysed and centrifuged at 10.000 rpm and 4°C for 10 min. The supernatant was harvested for BCA quantification. After sample preprocessing according to the instructions of the biochemical assay kit, the levels of ROS, SOD, T-AOC and LDH in hippocampal tissues were measured in strict accordance with the operation steps.

Detection of Inflammatory Factors in Hippocampal Tissues

The total protein of hippocampal tissues was harvested. After sample preprocessing according to the instructions of the ELISA kit, the levels of TNF-α, IL-18 and IL-1β in hippocampal tissues were measured in strict accordance with the operation steps.

Detection of GAP43, SYN, AQP4, NLRP3 and Caspase-1 Expressions in Hippocampal Tissues by Western Blotting

The total protein of hippocampal tissues was harvested and prepared into samples. Then the sample was separated by gel electrophoresis and transferred onto a membrane,

and the target band was cut and blocked with blocking buffer made of 5% skim milk powder on a shaker at room temperature for 1 h. Later, the sample was incubated with primary antibodies diluted with blocking buffer (1:500) at 4°C overnight. The next day, the membrane was taken out, equilibrated to room temperature, washed and incubated with corresponding secondary antibodies (1:5000) at room temperature for 2 h, followed by washing and reaction with electrochemiluminescence solution away from light for 5 min. The results were collected using a quantitative imager.

Statistical Analysis

According to a previous literature, 8-12 animals were commonly selected for each group [15], so 10 rats were set for each group in this study. SPSS 21.0 software was used for statistical analysis. Measurement data were subjected to the tests of normal distribution and homogeneity of variance. The normal distributed data were expressed as mean ± standard deviation (`X±s), and compared by the independent-samples *t* test between two groups and by one-way analysis of variance among groups. P<0.05 was considered statistically significant.

Results

Dementia Degree

At 7 d after operation (11 d), the escape latency was significantly prolonged and the number of platform-crossing times were reduced in Model group, Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group compared with those in Sham group (P<0.05). After the end of drug administration (44 d), the escape latency was prolonged and the number of platform-crossing times were reduced in Model group, Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group compared with those in Sham group (P<0.05). Compared with Model group, Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group had shortened escape latency and an increased number of platform-crossing times (P<0.05). Escape latency and number of platform-crossing times had no statistically significant differences between Bushen Recipe group and Tongluo Recipe group (P>0.05). Compared with Bushen Recipe group and Tongluo Recipe group, Yizhitongmai Recipe group had shortened escape latency and an increased number of platform-crossing times (P<0.05). There were shorter escape latency and more platform-crossing times at 44 d than those at 11 d in Sham group, Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group (P<0.05) *(Fig. 1)*.

HE Staining Results

It was observed by HE staining that the hippocampal neurons were neatly arranged, and the cells had a regular shape and normal morphology, with clearly visible nucleoli in Sham group. In Model group, the neurons were disorderly arranged, the cells had large intercellular space and abnormal morphology, and the number of cells declined. In Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group, the cells were arranged neatly, the intercellular space was reduced, the cells had good morphology, and the number of cells rose compared with those in Model group, and Yizhitongmai Recipe group exhibited more significant improvement than Bushen Recipe group and Tongluo Recipe group *(Fig. 2)*.

TUNEL Staining Results

The results of TUNEL staining showed that compared with that in Sham group, the number of apoptotic cells rose in Model group, Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group (P<0.05). Compared with that in Model group, the number of apoptotic cells declined in Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group (P<0.05). There was no statistically significant difference in the number of apoptotic cells between Bushen Recipe group and Tongluo Recipe group (P>0.05). Yizhitongmai Recipe group had fewer apoptotic cells than Bushen Recipe group and Tongluo Recipe group (P<0.05) *(Fig. 3)*.

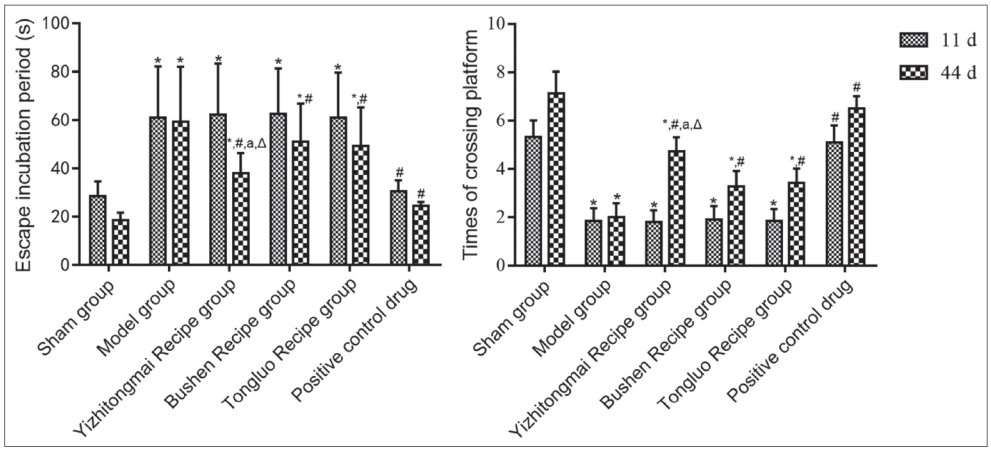


Fig 1. Morris water maze test results. Compared with Sham group, *P<0.05; compared with Model group, #P<0.05; compared with Bushen Recipe group, ΔP<0.05; compared with Tongluo Recipe group, aP<0.05

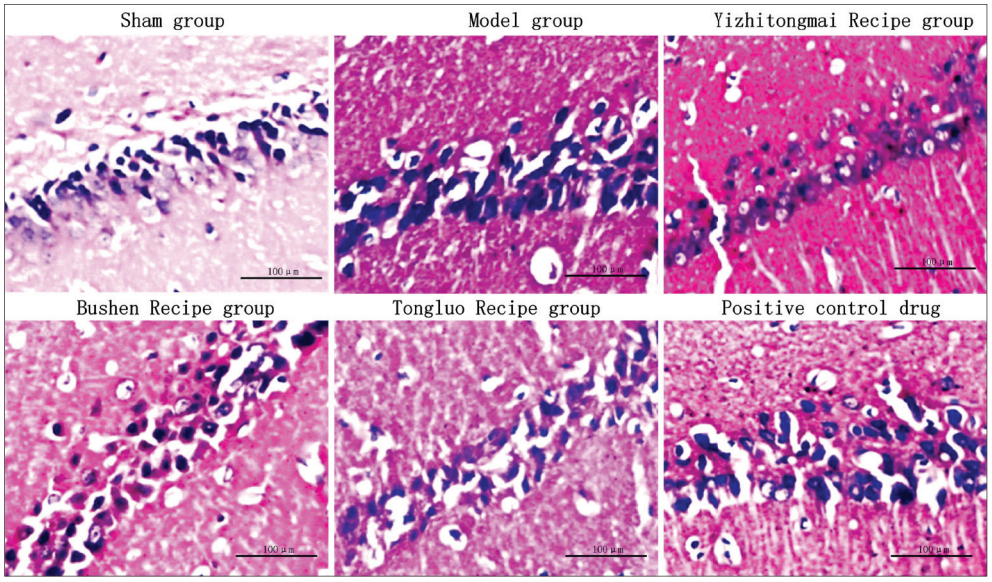


Fig 2. HE staining results (x400). Compared with Sham group, *P<0.05; compared with Model group, #P<0.05; compared with Bushen Recipe group, ΔP<0.05; compared with Tongluo Recipe group, aP<0.05

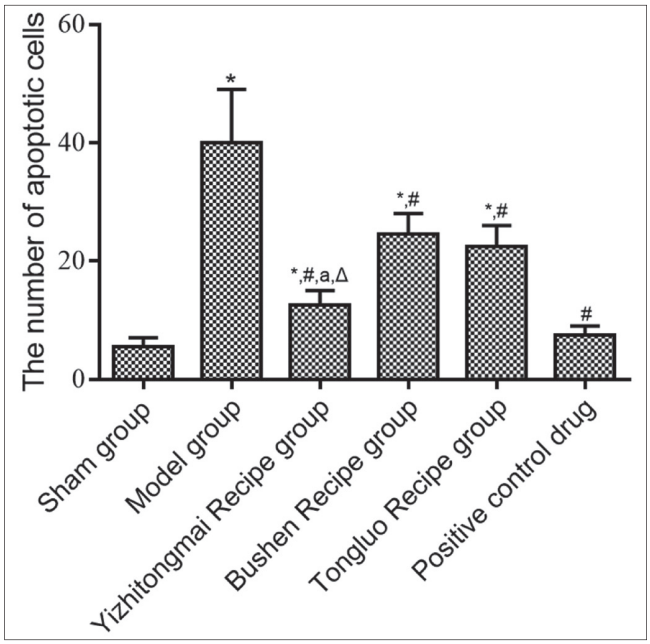


Fig 3. TUNEL staining results. Compared with Sham group, *P<0.05; compared with Model group, #P<0.05; compared with Bushen Recipe group, ΔP<0.05; compared with Tongluo Recipe group, aP<0.05

Cerebrovascular Endothelial Cell Function

Compared with Sham group, the levels of ROS and LDH increased (P<0.05), but the levels of SOD and T-AOC decreased in Model group, Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group (P<0.05). Compared with Model group, the levels of ROS and LDH decreased (P<0.05), whereas the levels of SOD and T-AOC increased in Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group (P<0.05). There were no statistically significant differences in ROS, SOD, T-AOC and LDH levels between Bushen Recipe group and Tongluo Recipe group (P>0.05). Compared with Bushen Recipe group and Tongluo Recipe group, Yizhitongmai Recipe group had decreased levels of ROS and LDH (P<0.05), and increased levels of SOD and T-AOC (P<0.05) *(Fig. 4)*.

Levels of Inflammatory Factors

The levels of TNF-α, IL-18 and IL-1β were higher in Model group, Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group than those in Sham group (P<0.05), while they were lower in Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group than those in Model group (P<0.05). There were no statistically significant differences in the levels of TNF-α, IL-18 and IL-1β between Bushen Recipe group and Tongluo Recipe group (P>0.05). Compared with Bushen Recipe group and Tongluo Recipe group, Yizhitongmai Recipe group had decreased levels of TNF-α, IL-18 and IL-1β (P<0.05) *(Fig. 5)*.

Expressions of GAP43, SYN, AQP4, NLRP3 and Caspase-1

Compared with those in Sham group, the levels of NLRP3 and Caspase-1 were increased (P<0.05), and the levels of GAP43, SYN and AQP4 were decreased in Model group, Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group (P<0.05). Compared with those in Model group, the levels of NLRP3 and Caspase-1 were decreased (P<0.05), and the levels of GAP43, SYN and AQP4 were increased in Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group (P<0.05). There were no statistically significant differences in the above-mentioned indexes between Bushen Recipe group and Tongluo Recipe group (P>0.05). Compared with Bushen Recipe group and Tongluo Recipe group, Yizhitongmai Recipe group had decreased levels of NLRP3 and Caspase-1 (P<0.05), and increased levels of GAP43, SYN and AQP4 (P<0.05) *(Fig. 6)*.

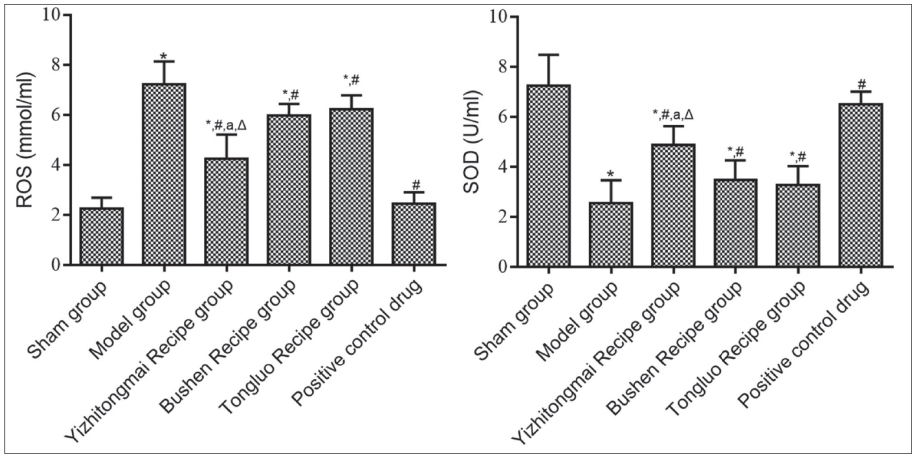
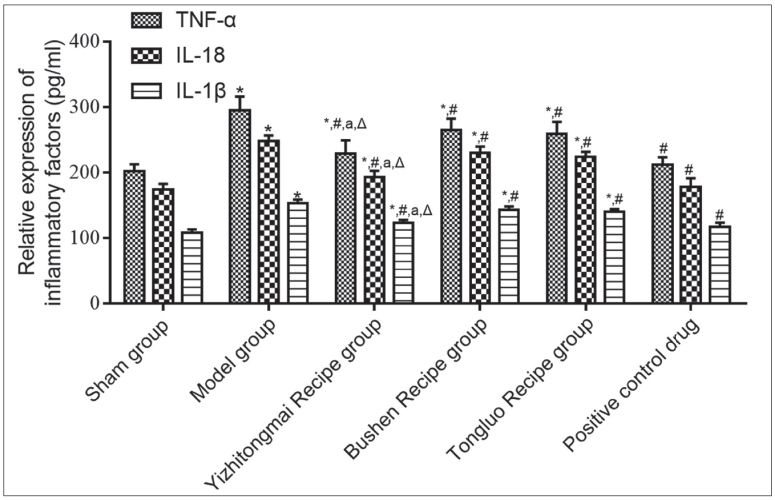


Fig 4. Biochemical detection results. Compared with Sham group, *P<0.05; compared with Model group, #P<0.05; compared with Bushen Recipe group, ΔP<0.05; compared with Tongluo Recipe group, aP<0.05

Fig 5. ELISA results. Compared with Sham group, *P<0.05; compared with Model group, #P<0.05; compared with Bushen Recipe group, ΔP<0.05; compared with Tongluo Recipe group, aP<0.05



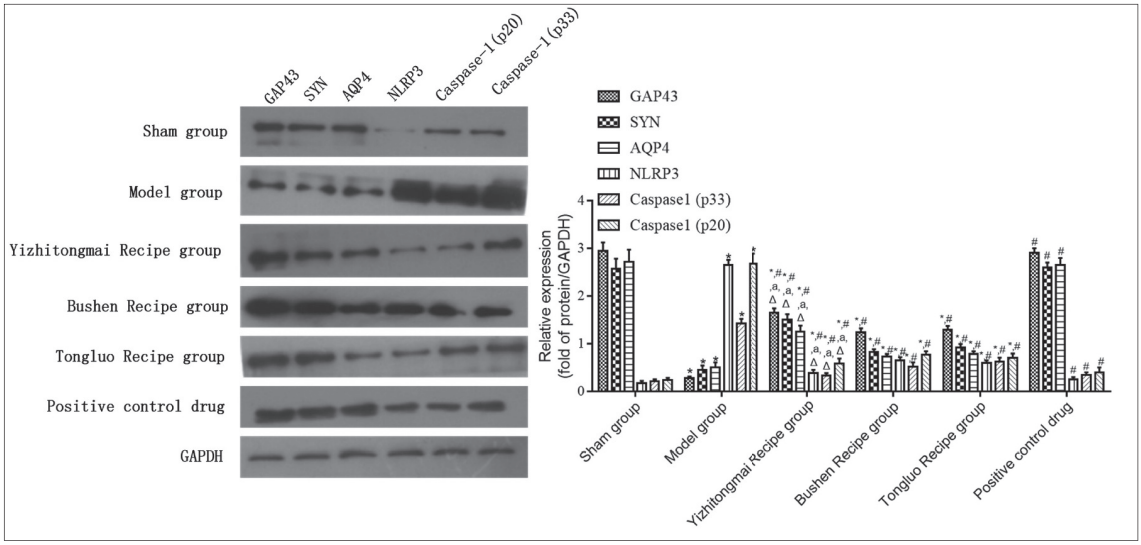


Fig 6. Western blotting results. Compared with Sham group, *P<0.05; compared with Model group, #P<0.05; compared with Bushen Recipe group, ΔP<0.05; compared with Tongluo Recipe group, aP<0.05

Discussion

Vascular dementia belongs to the categories of "dementia" and "forgetfulness" in traditional Chinese medicine, and its major pathogenesis is insufficient kidney essence and blood stasis [16]. In Yizhitongmai recipe, dragon's blood, centipede, Gastrodia elata and Hirudo can resist inflammation, remove blood stasis, promote blood circulation and dredge collaterals [17-20]. Additionally, ginseng, Rhizome of rehmannia and Sharpleaf galangal fruit

can protect hippocampal neurons and resist oxidation [21-23]. Moreover, earthworm can repair cerebral ischemia-induced tissue damage and delay thrombosis [24]. As a result, Yizhitongmai recipe, which combines the effects of various medicinal materials, can invigorate the kidneys and replenish the essence, remove blood stasis and dredge collaterals, effectively relieving the symptoms of VD. In this study, the results of Morris water maze test showed that the learning and memory ability of the VD model rats significantly declined compared with that in Sham group at 7 d after operation, suggesting the successful modeling. After drug administration, the learning and memory ability of rats recovered in Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group, especially in Yizhitongmai Recipe group, compared with that in Model group. It can be seen that a better curative effect can be achieved by tonifying kidney in combination with dredging collaterals.

The hippocampus is an important structure responsible for storage and regulation of learning and memory, with unique vascular architecture and densely arranged microglia, and hypoperfusion brain injury can easily cause hippocampal structural damage [25]. The results of HE and TUNEL staining in this study showed that necrotic hippocampal neurons, arranged disorderly and loosely, could be clearly seen, and the number of apoptotic cells was large in Model group. In Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group, the cellular damage was relieved and the number of apoptotic cells declined, suggesting that Yizhitongmai Granule can alleviate hippocampal tissue injury, and protect hippocampal neurons in VD rats, thereby restoring the learning and memory ability of rats.

The neurovascular unit consists of neurons, glial cells and blood vessels, and its injury is closely related to the pathogenesis of VD [26]. Hypoperfusion brain injury-induced oxidative stress and inflammatory response can affect the dynamic balance of neurovascular unit micro-environment. In this study, Model group had significantly higher levels of ROS and LDH but significantly lower levels of SOD, GAP43, SYN and AQP4 in hippocampal tissues than Sham group. An increased level of ROS can inhibit SOD, reduce the body's antioxidant capacity, and worsen oxidative stress injury. LDH is one of the indexes assessing the degree of cellular oxidative stress injury [27]. GAP43, SYN and AQP4 are proteins associated with neuronal synaptic plasticity and synaptic injury repair, and the changes in their levels can affect learning and memory ability [28]. In this study, the oxidative stress indexes and levels of GAP43, SYN and AQP4 in hippocampal tissues were greatly improved in Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group compared with those in Model group, demonstrating that Yizhitongmai Granule can reduce oxidative stress response, repair neuronal injury and enhance synaptic plasticity.

Inflammasomes are implicated in the body's innate immunity, and the activation of NLRP3 inflammasomes is the basis of a series of inflammatory responses. As pointed out in many studies, the expression level of NLRP3 has close correlations with cognitive dysfunction diseases such as VD and Alzheimer's disease [29-31]. Activated NLRP3 can help activate Caspase-1 through apoptosis-related speckle-like protein, and then IL-18 and IL-1β precursors can be made mature and released outside cells by activated Caspase-1, worsening the inflammatory response and resulting in pyroptosis [32]. In this study, the levels of inflammatory factors and NLRP3/Caspase-1 signaling pathway-related proteins in hippocampal tissues were significantly higher in Model group than those in Sham group, while they declined in Yizhitongmai Recipe group, Bushen Recipe group and Tongluo Recipe group, especially in Yizhitongmai Recipe group, compared with those in Model group, suggesting that Yizhitongmai Granule can lower the levels of inflammatory factors through inhibiting the NLRP3/Caspase-1 signaling pathway, thereby alleviating inflammatory injury in hippocampal tissues.

In conclusion, Yizhitongmai Granule and its decomposed recipes can effectively improve the learning and memory ability of VD rats, protect hippocampal neurons, relieve oxidative stress and inflammatory response caused by hypoperfusion brain injury, and inhibit the NLRP3/Caspase-1 signaling pathway, thereby exerting a cerebro-protective effect on VD rats.

Availability of Data and Materials

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

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Ethical Approval

This study has been approved by the animal ethic committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University (Approval No. 2021120082), and all experiments were carried out as per related guidelines.

Competing Interests

There is no conflict of interest.

Authors' Contributions

MP, GS designed this study; HM prepared this manuscript; YL performed this study; QL analyzed experimental data; HM Writing. All authors read and approved the final version of the manuscript.

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Research Article

Molecular Prevalence, Hematological Biomarker, Associated Risk Factors and Chemotherapeutic Trials of Ehrlichiosis in Dogs in Pakistan

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Abstract: The study was a prospective trial, planned to determine the molecular epidemiology of ehrlichiosis in dogs through blood smear microscopy and Polymerase chain reaction (PCR). A total of 384 cephalic blood samples were collected from domestic (n=288) and stray dogs (n=96) belonging to varied demographics. Molecular detection of *Ehrlichia* spp. was conducted through PCR by targeting 16S rRNA gene using the genus specific primers. Final logistic regression analysis revealed that previous history of tick infestation and housing hygiene were significant (P<0.001) risk factors associated with molecular prevalence of canine Ehrlichiosis. The animals of Group-1 (n=7) received only Minocycline at the rate of 12 mg per kg PO 21 days. The dogs of Group-2 (n=7) received Minocycline at the rate of 12 mg per kg PO with Imidocarb dipropionate at the dose rate of 6.6 mg per kg once. Group-3 (n=7) received Ciprofloxacin 10 mg per kg PO only. Group 4 (n=7) received Ciprofloxacin 10 mg per kg along with Prednisolone 1 mg for 8 days. Success of treatment was evaluated based on PCR tests. The results of the treatment trials revealed 71.42%, 85.71%, 57.14% and 71.42 % recovery rate for Group 1, Group 2, Group 3 and Group 4, respectively. The treatment trial concluded that minocycline along with Imidocarb dipropionate produced highest recovery rates.

Keywords: *Ciprofloxacin, Dogs, Ehrlichiosis, Minocycline, Polymerase chain reaction, PCR*

Pakistan'da Köpeklerde Ehrlichiosis'in Moleküler Prevalansı, Hematolojik Biyobelirteçler, İlgili Risk Faktörleri ve Kemoterapötik Denemeler

Öz: Bu çalışma, kan froti örneklerinin mikroskobik analizi ve Polimeraz zincir reaksiyonu (PCR) yoluyla köpeklerde ehrlichiosis'in moleküler prevalansının belirlenmesi için yapılan prospektif bir çalışmaydı. PCR'de 16S rRNA genini hedefleyen cins-spesifik primerler kullanılarak %9.63 (37/384) oranında genel bir yaygınlık gözlemlendi. Toplam eritrosit sayısı (TEC), Eritrosit sedimentasyon hızı (ESR), Hemoglobin (Hb) düzeyi ve Trombosit sayısı gibi hematolojik biyobelirteçler, hastalıklı bireylerde önemli ölçüde azaldı. Kene istilası öyküsünün varlığı ve kötü barınak hijyeni, hastalıkla önemli ölçüde ilişkili risk faktörleriydi. Enfekte hayvanlar arasında şu şekilde bir kemoterapi denemesi yapıldı; Grup-1 (n=7)'e, Minosiklin uygulandı (21 gün boyunca 12 mg/kg PO); Grup-2 (n=7)'ye, çalışmanın başlangıcında bir kez tek doz (6.6 mg/kg) halinde İmidokarb dipropionat ile birlikte Minosiklin (21 gün boyunca 12 mg/kg PO) uygulandı, Grup-3 (n=7)'e, Siprofloksasin (21 gün süreyle 10 mg/kg PO) uygulandı, Grup 4 (n=7)'e 8 gün süreyle Siprofloksasin (10 mg/kg) ve Prednisolon (1 mg) uygulandı ve Grup 5 (n=7)'teki hayvanlar Kontrol grubu olarak kabul edildi. Tedavinin başarısı, PCR testlerine göre değerlendirildi ve Grup 1, Grup 2, Grup 3 ve Grup 4 için sırasıyla %71.42, %85.71, %57.14 ve %71.42 iyileşme oranlarını elde edildi. Tedavi denemesi sonucu, Minosiklinin, Imidocarb dipropionate ile birlikte uygulamasının en yüksek iyileşme oranı sağladığı sonucuna varılmıştır.

Anahtar sözcükler: *Siprofloksasin, Köpek, Ehrlichiosis, Minosiklin, Polimeraz zincir reaksiyonu, PCR*

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Introduction

Canine vector-borne diseases (CVBDs) are caused by a diverse range of bacteria, viruses, and eukaryotic parasites that are conveyed by arthropod blood-sucking vectors, mostly ticks and mosquitoes [1]. Several critical variables, including a change in global climatic conditions could explain the exponential spread of arthropod vectors and CVBDs [2]. Ticks have emerged as the most pernicious arthropod vector in several different ecological habitats. Some of these CVBDs present a serious zoonotic threat [3]. Dog population has been incrementally rising in the last couple of decades owing to the fact that this trend of keeping pet dogs has gained greater cultural acceptance [4]. This shift has affected global distribution of CVBDs as well. Climate change, ease of international transportation, and rapid rise in human, canine, and other reservoir animal populations have proven to be key factors in this regard [5]. Climate has a significant impact on the survival and dissemination of arthropod vectors, as well as the dispersion of CVBDs [3].

The *Ehrlichia* species belongs to the family *Anaplasmataceae*, capable of infecting canine, bovine and human hosts. Ehrlichiosis is a disease caused by an obligatory intracellular Gram-negative bacterium that replicates in the host's mononuclear cells [3]. The pathogen is mainly transmitted by so called brown dog tick, *Rhipicephalus sanguineus* [5]. High grade fever, anorexia, dullness, enlarged spleen, pancytopenia and spontaneous hemorrhagic tendencies are the most obvious clinical signs [4]. The incidence of ehrlichiosis is steeply elevated during peak tick infestation seasons i.e., spring and autumn [5]. The ehrlichiosis has the zoonotic potential and believed to be common in both rural and urban areas of tropics. Molecular and serological diagnostic tests have validated the existence of *Ehrlichia* in cats, dogs, wild animals, and humans [4]. A Brazilian study revealed that, *Ehrlichia canis* is the most prevalent species found amongst dogs. The global prevalence for *Anaplasma phagocytophilum*, and *E. canis*, were reported to be 1.6% and 6.3% respectively. The development of tick-borne disease has been linked to age and severity of tick infestation [6].

The clinical diagnosis of Ehrlichiosis in animals is difficult because the clinical signs are ambiguous and the serological assays have limited diagnostic application. The isolation of pathogen is difficult and requires a tissue culture medium for its growth [5]. Cytoplasmic inclusion bodies called morulae could be identified during microscopic examination of blood smears but its limited sensitivity as compared to serological or molecular assays impede widescale implementation [6]. Ehrlichiosis has worldwide distribution. Australia was previously believed to be free from Canine Ehrlichiosis but was recently discovered in its northern and western territories [2]. In Pakistan, 24% prevalence of ehrlichiosis has been previously reported in dogs [7]. Minocycline a new drug that belongs to tetracycline antibiotics group is regarded as the cornerstone for chemotherapy and the drug of choice for treatment of Canine Ehrlichiosis [7]. Previous studies have reported considerable efficacy for both minocycline and imidocarb dipropionate [6]. This combination had proved most effective and successful treatment for resolving clinical disease and pathogen eradication. Minocycline, a close relative of doxycycline, is an obvious choice for *E. canis* as well as other members of *Anaplasmataceae* and *Rickettsiales* families [8]. Minocycline may be a better choice for treating these elusive bacteria than doxycycline because of its high lipophilicity, minimal protein binding, and greater penetration into tissues including the brain [2]. In the present study, authors have endeavored to establish the most efficacious drug against canine ehrlichiosis by firstly identifying positive samples, establishing aggravating factors and compare the efficacy of minocycline with treatment regimens that have been proven effective in prior publications.

Material and Methods

Ethical Consideration

Approval of this study was obtained from the University of Veterinary and Animal Sciences, Lahore, Thesis Committee (Approval no: 8226). This research was a prospective trial, therefore only clinical patients were inducted after informed consent was obtained from their respective caretakers. Research design was in complete compliance with the established guidelines stated in Pakistan's Prevention of Cruelty to Animals Act (1890), Punjab Wildlife Protection, Preservation, Conservation and Management Act (1974).

Research Area

Dogs were sampled in district Sheikhupura of Punjab Province, to estimate the seroprevalence, associated risk factors and relative efficacy of various drug regimens against canine ehrlichiosis. The climate of study area was semi-arid and sampling was performed between March and September of 2021. The GIS map shows different locations from where blood samples were collected *(Fig. 1)*.

Collection of Samples

A total of 384 blood samples of clinically infected dogs (domestic, n=288 and stray, n=96) were selected belonging to different breeds, age and gender from different government as well as private kennels to estimate the prevalence, risk factors and efficacy of different drugs used against canine ehrlichiosis. All the dogs were included in sampling frame.

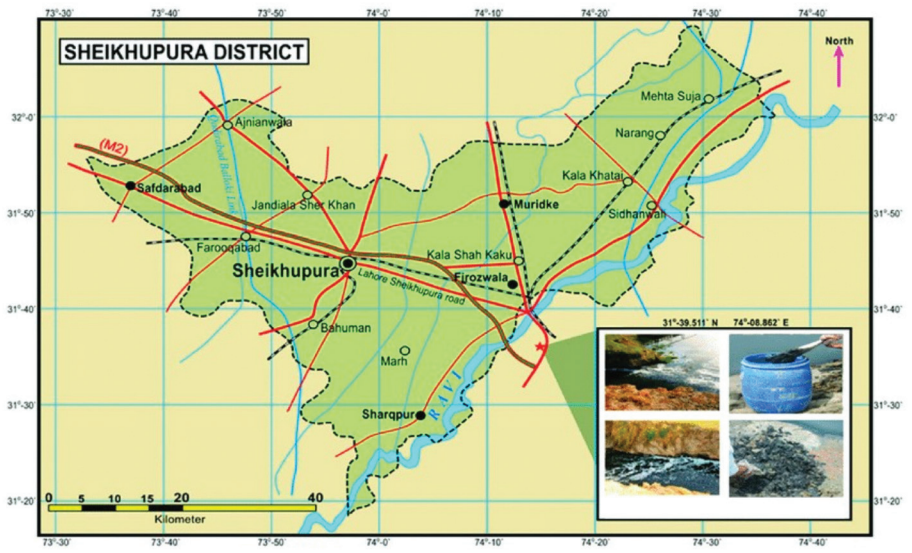

Fig 1. GIS Map showing the study area

The samples were transferred in an ice box containing ice pack and brought to laboratory for further processing.

Inclusion and Exclusion Criteria

Dogs with history of tick infestation, anemia, lymphadenitis, excessive weight loss, fever above 104˚F, spontaneous bleeding disorders and no prior treatment history within last one month were selected for this prospective epidemiological trial. Dogs suffering from any major infectious or non-infectious disease, other than ehrlichiosis were excluded from the study. A questionnaire was used to collect information regarding area, host, breed, gender, age, and number of dogs, extent of veterinary care, health status and periodicity of tick infestation etc.

Methodology

Thin Blood Smears and Phlebotomy

Two methods of sampling were employed for detection of ehrlichiosis from blood. Firstly, thin blood smears (in triplet) were created from an ear vein and air dried on the spot. Secondly, 3 mL blood was taken aseptically from the cephalic vein into EDTA-coated vacutainers (medivac). The samples were delivered to the laboratory where the cold chain was maintained. For each sample animal description, managemental, and environmental determinants were captured on a data collection form [9].

Microscopic Examination of Blood Smears

The blood smears were fixed in a 10% ethanol solution and stained with a Giemsa stain. The stain was wiped away with running tap water after 15 min, and the discolored smears were allowed to dry. The streaks were spotted using a 100x oil immersion lens. The smears were examined for intracytoplasmic inclusion bodies in blood cells that looked similar to the ones observed in case of *Anaplasma* spp.[9]. Detection of such inclusion bodies would support primary animal screening.

Genomic Analysis

The DNA was extracted from the blood samples of dogs by using an Exgene™ (GeneAll®) DNA extraction kit [10]. The sampled blood was prepared for DNA extraction by mixing it with absolute ethanol in 1.5 mL to maintain a final volume of 200 µL. Solution was repeatedly centrifuged and incubated subsequent to addition of 20 µL of Proteinase K, 200 µL of buffer Bl and 200 µL of absolute ethanol respectively. The entirety of the solution was transferred to an SV column and centrifuged at 6000 rpm until all the solution had passed through the membrane and became colorless. The mixture was pipetted to another tube and centrifuged after the addition of 700 µL of buffer TW. Finally, the SV column was transferred to another microcentrifuge tube and 200 µL of buffer AE was added before a final round of centrifugation and incubation.

The DNAs after extraction from the blood samples were taken to purity and concentration measurements with the help of Gel electrophoresis. On average the DNA yield per sample was 40 ng/µL. This showed that the samples had suitable DNA for amplification through PCR. The DNA quantity was assessed by using nano drop [10]. A primer targeting 16S rRNA gene of *Ehrlichia* spp. was utilized. Using the appropriate bioinformatics tool, the forward primer i.e., Ehr-F2: 5-AATAATAATGCTGGTCAAGT ATGGAATCAT-3; and the reverse primer Ehr-R2: 5-AAGCGTGTTCCCATACATCCATAG-3 were used to amplify the 16S rRNA gene [7,11,12]. PCRs were carried out in a final volume of 20 µL with 10 µL of TOP real TM qPCR 2x Pre-Mix, 4 µL of DNA, 2 µL (10 pmol) of each primer, and 2 µL of distilled water. Initial denaturation was conducted at 95°C for 5 min, after which the reaction was cycled 40 times with denaturation at 95°C, annealing at 57°C, and extension at 72°C, each step lasting 30 sec, and the last extension at 72°C lasting 10 min. The amplification of gene was carried out in a thermal cycler

(Scilogex PC1000-G™) according to the guidelines of the manufacturer. The PCR products were seen on a UV illuminator in a 1.5% ethidium bromide-stained agarose gel at 120 volts and 200 amperes following gel electrophoresis with an expected size ranging between 300-400 base pairs [13]. Samples positive for *Ehrlichia canis* were graciously donated by Dr. Muhammad Zia (PhD Scholar) from Department of Parasitology, University of Veterinary and Animal Sciences Lahore. A 100bp DNA ladder (BioShop®, Canada) along with negative controls (PCR mixture without DNA) were amplified during each PCR as well.

Hematological Analysis

Complete blood cell count was carried out using hematology analyzer (BioSystems BTS-350®). A 3 mL sample of blood was taken straight from the cephalic vein into an EDTA vacutainer from 10 positive dogs based on PCR and 10 healthy dogs. Using a hematology analyzer, several hematological parameters such as total erythrocyte count (TEC), total leukocyte count (TLC), hemoglobin (Hb), platelet count, and packed cell volume (PCV) were measured [14].

Chemotherapeutic Clinical Trial

After screening, dogs were divided into five groups; comprising seven dogs in each group. Following treatments were given to each group to determine the efficacy of different drugs against Ehrlichiosis in dogs:

Group-1 (n=7): Minocycline 12 mg/kg PO (21 days); Group-2 (n=7): Minocycline 12 mg/kg PO (21 days) with Imizole (imidocarb dipropionate) 6.6 mg/kg subcutaneously once; Group-3 (n=7): Ciprofloxacin 10 mg/kg PO (21 days); Group 4 (n=7): Ciprofloxacin 10 mg/kg PO with Prednisolone 1 mg (8 days); Group 5 (n=7): Control (non-treated). The efficacy for each drug was determined by percentage of recovery from each drug and consequent improvement in hematological parameters [14].

Statistical Analysis

The data regarding the risk factors were analyzed using logistic regression model. Data regarding comparative therapeutic efficacies during treatment trials was assessed using paired t-Test, keeping level of significance (P<0.05). All the statistical analyses were performed by SPSS version 26.0 (version 26, IBM, Chicago, IL).

Results

Prevalence of Ehrlichiosis

A total of 384 samples were collected and screened for *Ehrlichia* infection by blood smear and molecular examination. The blood smear examination of samples revealed that out of 384 samples, 22 were found positive for inclusion bodies resembling *Ehrlichia* with a positive percentage of 5.73% *(Fig. 2)*.

The PCR analysis of samples revealed an overall prevalence of 9.63% (37/384) in dogs *(Table 1) (Fig. 3)*.

Risk Factors Associated with Ehrlichiosis in Dogs

The relationship of assumed risk factors like breed, sex, age, tick infestation, history of tick infestation, type of acaricide, hygiene condition and acaricide interval were analyzed statistically to find out association with occurrence of disease *(Table 2)*. The risk factors were initially analyzed using the chi-square method and the variables having (P<0.01) were further analyzed using a multivariable logistic regression model *(Table 2)*.

The risk factors like tick infestation, past tick history and house hygiene are considered significant risk factors towards the occurrence of Ehrlichiosis in dogs as the P-value is less than 0.05. These significant risk factors were further analyzed by logistic regression model to find out the association between these factors in causation of Ehrlichiosis in dogs. Based on final regression model, only two risk factors were found potential risk factors towards the incidence of disease (P<0.05). The dogs having history of tick infestation in the past were at 3.103 times more

Table 1. *Overall prevalence of Ehrlichiosis in dogs determined through Blood smear microscopy and PCR test*

Diagnostic Test	Positive	Prevalence (%)
PCR positive	37/384	9.63
Microscopy positive	22/384	5.73

Percentage Prevalence have been calculated by running the same samples from 384 Dogs through both diagnostic tests

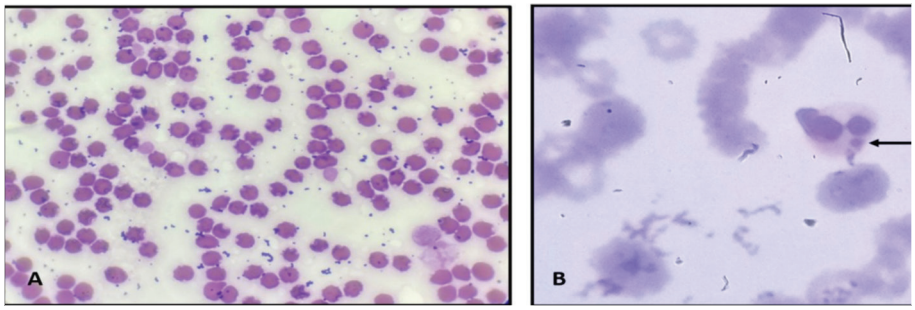

Fig 2. Photomicrographs of a thin blood smear sampled from infected dog: (A) Presence of *Ehrlichia* like intracytoplasmic inclusion bodies in monocytes (Giemsa staining, 200x); (B) An arrow has been used to identify intracytoplasmic morula of *Ehrlichia canis* in a magnified image of dog's monocyte (Giemsa staining, 400x)

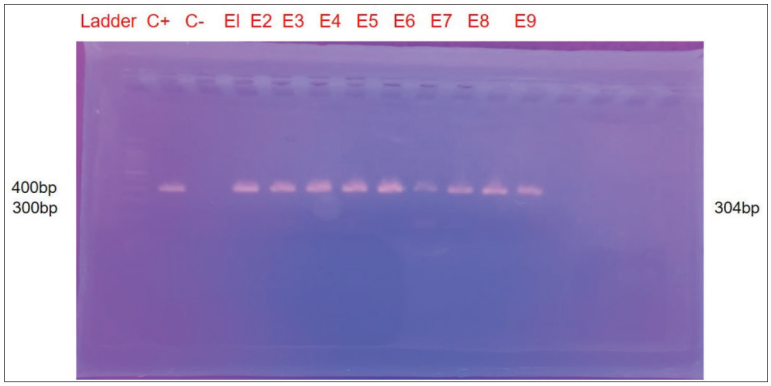

Fig 3. Results for agarose gel electrophoresis of PCR for *Ehrlichia* at 304bp: Lane 1 contains a 100bp DNA ladder (BioShop®, Canada); Lane 2 is Positive Control (C+); Lane 3 is Negative Control (C-); Lane 4 to 12 contain DNA purified from dog samples suspected for Ehrlichiosis and are represented as E1 to E9. Numbers on the left indicate molecular sizes in base pairs.

Table 2. *Summary of risk factors related with Ehrlichiosis in dogs*

Variables	Level of Variable	No. of Samples (n=384)	Positive (%)	Negative	P-Value
Breed	Labrador	45	26 (57.78)	19	0.06
	German Shepherd	135	54 (40.00)	81	
	Bully	115	50 (43.10)	65	
	Non-descript	89	45 (50.56)	44	
Sex	Male	193	91 (47.15)	102	0.64
	Female	191	86 (44.79)	105	
Age	<1 year	244	159 (64.89)	85	0.89
	> 1 year	140	18 (12.85)	122	
Tick Infestation	Absent	265	89 (33.58)	176	<0.001
	Present	119	88 (73.94)	31	
History of tick infestation	Yes	224	126 (56.25)	98	<0.001
	No	160	51 (31.87)	109	
Hygiene condition	Good	147	48 (32.65)	99	<0.001
	Poor	237	129 (54.43)	108	
Type of acaricide	Topical	151	73 (48.34)	78	0.52
	Parenteral	163	76 (46.62)	87	
	Not applied	70	28 (40.00)	42	
Acaricide interval	> 3 months	353	162 (45.89)	191	0.90
	< 3 months	24	12 (50.00)	12	
	Not applied	07	03 (42.85)	04	

Values are represented in terms of positive and negative samples for Ehrlichiosis. A P<0.05 indicated statistical significance amongst corresponding variables

risk at acquiring diseases as compared to animals having no previous tick history. The P-value is also less than 0.05 and it is considered as true risk factor. However, the dogs existing in poor hygienic conditions have 3.095 times more chances of disease occurrence as compared to the dogs living in good hygienic measures and P-value is also less than 0.05 *(Table 3)*.

Effects on Hematological Parameters at Different Time Periods During Chemotherapy

To determine the influence of *Ehrlichia* on various haematological parameters, a comparative hematological analysis was done on *Ehrlichia* positive and healthy animals. The obtained findings were evaluated using an independent T-test, and it was discovered that in dogs infected with *Ehrlichia*, there was a significant (P<0.05) drop in Total Erythrocyte Count (TEC), Erythrocyte Sedimentation Rate (ESR), Hemoglobin (Hb) level and Platelet count. The comparative hematological study revealed a significant (P<0.05) decrease of TLC in acutely diseased dogs compared to the healthy ones *(Fig. 4)*.

Therapeutic Trials Against Canine Ehrlichiosis

The efficacy of three antibiotics was measured on the basis of disappearance of clinical signs and hematological parameters at 7, 14 and 21 days after initiation of therapy *(Table 4)*.

Table 3. *Risk factors included in final logistic regression model*						
Variables	**Variable Levels**	**Odd Ratio**	**95% C.I.**		**S. E**	**P-Value**
			Lower	**Upper**		
Tick Infestation	Absent	1	0.803 - 6.583		0.537	0.121
	Present	2.299				
History of tick infestation	No	1	1.035 - 9.306		0.560	0.043
	Yes	3.103				
Hygiene condition	Good	1	1.102 - 8.694		0.527	0.032
	Poor	3.095				
Relative probability for Ehrlichiosis in presence or absence of a variable has been presented in the table. A P<0.05 indicated statistical significance						

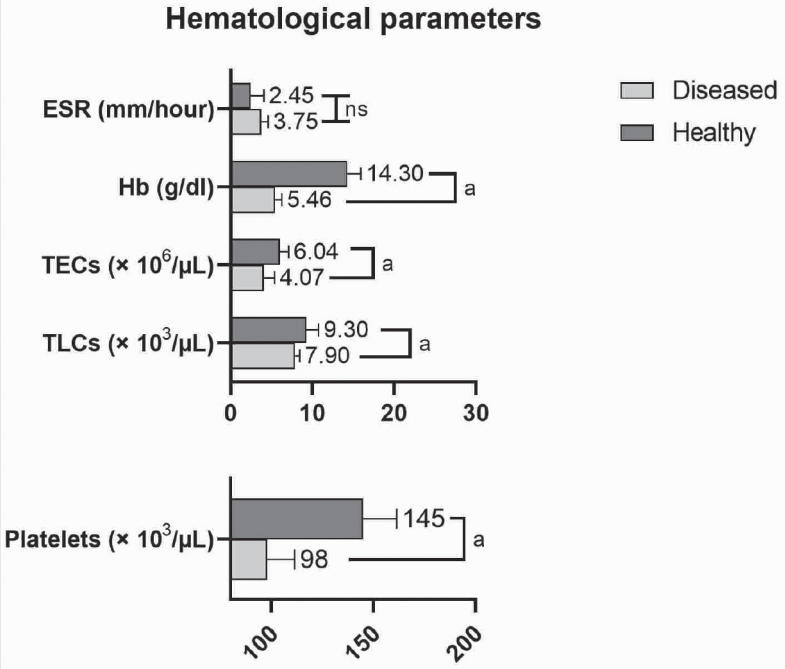

Fig 4. Interleaved Bars graph illustrating a comparison of Erythrocyte sedimentation rates (ESR), Hemoglobin (Hb), Total Erythrocyte counts (TECs), Total Leucocyte counts (TLCs) and Platelets between diseased and healthy dogs. Superscript i.e., (a) represents the statistical significance whereby (P<0.05) while (ns) denotes non-significance amongst variables

Table 4. *Therapeutic trials of different drugs against Canine Ehrlichiosis*					
Days	**Success of Treatment Trial**				
	Group 1 Minocycline (N=7)	**Group 2 Minocycline + Imidocarb Dipropionate (N=7)**	**Group 3 Ciprofloxacin (N=7)**	**Group 4 Ciprofloxacin + Prednisolone (N=7)**	**Group 5 Control Positive (N=7)**
7 days	3/7	4/7	3/7	3/7	0/7
14 days	4/7	5/7	4/7	4/7	0/7
21 days	5/7	6/7	4/7	5/7	0/7

Minocycline alone performed adequately well and there was nominal statistical difference between the treated groups. However, the treatment trial concluded that minocycline along with Imidocarb dipropionate produced highest recovery rates.

Discussion

Tick-borne infections in dogs are becoming more common across the world, making them crucial for small animal practitioners and public health [7]. Ehrlichiosis in dogs is found and reported all over the world [2]. Variety of factors influences the occurrence of disease including host age, vector distribution, habitat, dog survival, climatic conditions and management approaches [5]. The molecular prevalence of tick-borne illnesses in Pakistan like babesiosis has been documented but limited work has been done on Canine Ehrlichiosis [7]. Pérez-Macchi et al.[15] illustrated the prevalence of Ehrlichiosis in Uruguay amongst dogs using polymerase chain reaction as 10.41%.

While an Argentinian study reported the prevalence of 6.7% [16]. Both these findings were corroborated by present outcomes. However, a prior study conducted in three districts of Punjab, Pakistan reported a relatively higher prevalence (28%) of Ehrlichiosis in dogs [7]. A higher rate of prevalence has been reported in India as well [17]. Whereas a much lower prevalence (2%) was reported in Malaysia [18].

In a Nigerian study where light microscopy and polymerase chain reaction were employed in a similar fashion as in the current one, prevalence of 10.25% of this disease was reported, which was much higher than present findings [19]. Area, weather climatic differences in various regions, test methods, vegetation cover prevalent tick species as well as animal husbandry and agronomic practices have been hypothesized to contribute towards variable disease occurrence [5]. Similar to past findings, authors could not validate breed and sex as disease determinants. Researchers have already elucidated that environmental, ecological, and social variable, rather than immunological characteristics, may pose a significant risk in disease development [20-22]. However, authors observed a sharp disparity of infection rates amongst genders as fewer cases of disease in bitches were reported. Whereas, infection rate in our present study were higher in younger animals (<1 year) than the adult ones (>1 year), which contradicted prior findings [15].

The frequency of tick-borne infections in this study were closely related to the extent of tick infestation. Similar studies in the past have concluded that dogs infested with ticks were 3.3 times more at risk to disease as compared to non-tick infested ones [23,24]. Pakistan is located in the tropical and subtropical regions of the world with an unrelenting and humid climate [20]. It makes it quite suitable for tick proliferation and sustenance. Dogs without proper preventive measures have a greater risk of tick infestation [7]. Authors detected that in dogs infected with *Ehrlichia,* there was a significant ($P<0.05$) drop in TEC, ESR, Hb level and MCHC. These findings are important indicators of anemia in diseased animals [4,25]. Previously, it has been reported that dogs suffering from Ehrlichiosis experience anemia and thrombocytopenia [26]. Acute cases ehrlichiosis in dogs were also presented with spontaneous incidence of lowered Hb, TEC, and platelet counts leading to underlying blood coagulopathy [27].

The molecular methods utilized in this investigation were demonstrated to be very sensitive when compared to blood smear microscopic examinations, a fact that has now been reaffirmed by several epidemiological surveys [28]. Examining stained blood smears is less sensitive and requires technical expertise [10]. It is frequently unsatisfactory because the pathogen is either missing or present in very low levels. Intermittent low parasitemia is a characteristic of persistent infection [10]. Nevertheless, during acute infection, blood smear examination remains the easiest and most accessible diagnostic test for clinicians with reasonably sensitivity [9]. Molecular and serological approaches are better at detecting chronic and subclinical illnesses, and they are mostly useful for epidemiological studies. Despite repeated efforts to improve PCR screening, a negative result should be regarded with care due to the cyclic nature of disease [9].

Therapeutic doses of tetracyclines such as doxycycline have traditionally been used against Canine Monocytic Ehrlichiosis (CME) [26]. Several authors have corroborated their efficacy to inhibit binding of bacterial ribosomes with aminoacyl-tRNA in both experimental as well as natural settings [14,25,29,30]. Earlier reports have suggested a minimum inhibition concentration (MIC) of 0.03 mg/ml for doxycycline [31,32] establishing it as a "gold standard" broad spectrum drug for the treatment of Canine Ehrlichiosis. However, several recent studies have deduced that this presumed efficacy was highly subjective and vastly dependent upon several factors namely dosing regimens, degree of infectious load, host's immunological status, sampling methodology and the sensitivity of assays employed for detection [14,25,29,30]. Moreover, risk of doxycycline resistance in *E. canis* owing to its widespread usage in endemic areas could not be underplayed either [33,34]. Additionally, presumed side-effects such as diarrhea, anorexia, vomiting, and elevated hepatic enzymes associated with its prolonged usage have incentivized researchers to investigate clinical efficacy of minocycline [25,30]. Being a pharmacological relative of doxycycline, it was an obvious choice for *E. canis* treatment, as well as other members of *Anaplasmataceae* and *Rickettsiaceae* families [25,35]. Minocycline has been found efficacious against CME in prior studies [25,35]. Nevertheless, risk of re-infection has been associated with all tetracyclines, including Minocycline. Therefore, prior publications have suggested studies to investigate its efficacy in combination with drugs such as Imidocarb dipropionate and Metronidazole to counter the possibility of remission [26].

Considering the cyclical nature of Ehrlichiosis, Imidocarb dipropionate has always been considered a viable option for treatment of clinical remissions of CME [36]. A research group at American College of Veterinary Internal Medicine had even suggested it to be a second line of treatment in CME [37]. However, studies have demonstrated its immense capability in providing hematological recovery amongst the most perniciously acute cases as well [26,37]. In a more recent publication, authors have proposed a combination of Minocycline and Imidocarb dipropionate to be the most effective and successful treatment for resolving the clinical manifestation of CME [25]. Authors have been

able to corroborate these prior guidelines in present settings.

Comparable studies have reported Rifampicin, a DNA-dependent RNA polymerase inhibitor to possess similar efficacy to that of doxycycline against *E. canis* [29] but an inability to clear *Ehrlichia chaffeensis* amongst human hosts has rendered it obsolete. Similarly, Enrofloxacin, a DNA gyrase inhibitor has been diligently researched for its efficacy against *E. canis* infection [38]. Though initially found to be quite promising, it failed to provide hematological recovery or clearing of acute experimental *E. canis* infection [26]. The potential for emergence of enrofloxacin resistant *Ehrlichia* spp. has been alluded to, by several researchers as well [39,40]. However, the efficacy of fluoroquinolones for the mitigation of severe aplastic pancytopenia in dogs cannot be disregarded either [26]. Consequently, drugs such as ciprofloxacin have also been used or proposed for treatment of CME due to their immense therapeutic potential and their efficacy in mitigating *E. canis*-associated aplastic pancytopenia [26,40]. Antibiotic usage in a dog suffering from Ehrlichiosis are heavily predicated upon selective intestinal decontamination, negligible effect on platelet function and minimal toxicity [29]. Most researchers have observed greater efficacies in cases where either a combination of different classes of drugs were used or adjuvants were administered to mitigate drug related side-effects.

The findings of this study suggested that PCR is a more sensitive and specific method for diagnosis of canine ehrlichiosis. Tick infestations, history of tick infestation and hygienic conditions have all been identified as significant risk factors for disease transmission. The hematological parameters of diseased animals, such as TECs, TLCs, and Hb were dramatically reduced. This study concluded that minocycline can remove or decrease circulating *E. canis*, suggesting that it could be a viable alternative to the "gold standard" doxycycline in present local circumstances.

Availability of Data and Materials

The authors declare that the experimental data supporting the present study findings have been made available to the corresponding author (A. H. Rabbani).

Ethical Consideration

Approval of this study was obtained from the University of Veterinary and Animal Sciences, Lahore, Thesis Committee (Approval no: 8226).

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Competing Interests

There was no conflict of interest with respect to authors reporting their research findings.

Author Contributions

Experimental design was conceived by SA, FAA, AZ and SNA. Data were collected by SA, YRK, ON and MS. Statistical analysis was conducted by AA and KH. Original draft was written by SA, and AHR. All authors have contributed to the revision and final proof-reading of the manuscript.

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Research Article

The Characteristic Analysis of Ribosomal Protein L12 in *Haemaphysalis longicornis* (Acari: Ixodidae) Ticks

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Abstract: Ribosomal protein L12 (RpL12) plays an important role in ovarian development and engorgement in vertebrates and invertebrates. However, the functional characteristics of RpL12 in ticks are not clear. Here, an open reading frame of the RpL12 gene was cloned from cDNA of *Haemaphysalis longicornis.* The sequence was analysed, and expression levels were determined in different tissues and developmental stages using qPCR. To assess the immunization and challenge of ticks, the recombinant protein rRpL12+GST was used in immunological experiments. The results showed high conservation of RpL12 among species and comparisons of the amino acid sequence from *H. longicornis.* RpL12 was approximately 60% expressed in the ovary among the examined tissues of unfed adult female *H. longicornis,* and the expression level of RpL12 in unfed ticks was significantly lower than that in *H. longicornis* at the egg and engorged stages. Western blotting showed that rabbit antiserum against *H. longicornis* adult ticks recognized RpL12, with an average egg weight of 49.94% and a 17.22% reduction in the engorged weight of adult ticks, but the mortality increased only 6.00%. These results suggest that RpL12 could be used to generate anti-tick vaccines and provide novel information on the RpL12 gene of ticks, providing a better understanding of its mechanisms in reproduction and oogenesis.

Keywords: *Haemaphysalis longicornis, Recombinant protein, Ribosomal protein, RpL12, Ticks*

Haemaphysalis longicornis (Akar: Ixodidae) Kenelerinde Ribozomal Protein L12'nin Karakteristik Analizi

Öz: Ribozomal protein L12 (RpL12), vertebralı ve vertebrasızlarda ovaryumun gelişiminde ve angorjmanında önemli bir rol oynar. Ancak kenelerde RpL12'nin fonksiyonel özellikleri net değildir. Bu çalışmada, RpL12 geninin bir açık okuma kalıbı *Haemaphysalis longicornis*'in cDNA'sından klonlandı. Sekans analizi gerçekleştirildi ve farklı dokularda ve gelişim aşamalarında ekspresyon seviyeleri qPCR ile belirlendi. İmmünizasyon ve takiben kenelerle enfestasyonu değerlendirmek için, immünolojik deneylerde rekombinant protein rRpL12+GST kullanıldı. Bulgular, kene türleri ve *H. longicornis*'e ait amino asit dizi örnekleri arasında RpL12'nin yüksek düzeyde korunduğunu gösterdi. RpL12, aç erişkin dişi *H. longicornis*'in incelenen dokuları arasında yumurtalıkta yaklaşık %60 oranında eksprese edildi. Aç kenelerdeki RpL12 ekspresyon seviyesi, *H. longicornis*'un yumurta ve doymuş formlarındakilerden önemli ölçüde düşüktü. Western blotlama, yetişkin *H. longicornis* kenelere karşı üretilen tavşan antiserumunun ortalama %49.94 yumurta ağırlığı ve doymuş kene ağırlığında %17.22'lik bir azalma ile RpL12 ile güçlü bir reaktivite sergilediğini ortaya koydu, ancak ölüm oranı %6.00 artmıştı. Bu sonuçlar, RpL12'nin, kenelerde üreme ve oogenez mekanizmalarının daha iyi anlaşılmasını sağlayarak, kene aşısı geliştirmede ve kenelerin bu geni hakkında yeni bilgiler sağlamada kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Haemaphysalis longicornis, Rekombinant protein, Ribozomal protein, RpL12, Kene*

Introduction

Ticks are ectoparasites of veterinary and medical importance with a worldwide distribution and are considered vectors of human and animal pathogens [1]. Ticks not only weaken hosts by sucking their blood but also transmit various pathogens [2]. *Haemaphysalis longicornis* Neumann, 1901 is an Acari, Ixodidae that can exchange three hosts in a

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life cycle (three-host tick), which is a species of tick that is widely distributed in the world, including Australia and East Asia, and recently found in the US [3-6]. It is recognized as the most important vector in the worldwide human and animal transmission of bacterial and viral pathogens, such as *Rickettsia conorii*, *Theileria orientalis* and *Babesia microti* [7-9]. *H. longicornis* has wide hosts, including livestock and wild animals, such as cattle, sheep, rabbits, coyote *(Canis latrans),* eastern cottontail *(Sylvilagus floridanus),* raccoon *(Procyon lotor),* Virginia opossum *(Didelphis virginiana),* white-tailed deer *(Odocoileus virginianus),* woodchuck *(Marmota monax),* and a *Peromyscus sp.* mouse [10]. In view of the threat of the pathogen transmitted by *H. longicornis* to livestock breeding and human public health safety. Therefore, the study of *H. longicornis* is extremely important. The prevention of tick-transmitted diseases and the control of ticks remain important challenges for research [11]. The traditional method of tick control relies mainly on chemical acaricides [12], but the long-term use of insecticides leaves behind pesticide residues and causes environmental pollution [13]. Therefore, it is important to find an environmentally friendly method to kill or delouse ticks. As conserved genes, ribosomal proteins (Rps) are expressed throughout the lifecycle of each organism [14]. Among these proteins, RpL12, RpL24, RpL7/L12, Rpp0 and RpsA belong to the same family in animals. Previous studies have shown that the expression level of RpL24 is higher in the ovary than in other tissues of shrimp, which indirectly shows that RpL24 may play key roles in the reproduction of shrimp [15]. Some new evidence has shown that ribosomal proteins are transported from mitochondria to other organelles and exhibit functions leading to the failure of embryos to form germline progenitors [16]. Recently, ribosomal proteins were studied in ticks, and the results showed that these proteins have significant effects on blood feeding, moulting and reproduction and could be useful in tick control [17]. Additionally, the absence of S-27 reduced the engorgement weight and feeding ability of ticks, and the silencing of S-27 in eggs led to abnormalities in shape and hatching [18]. Ribosomal protein P0 has been demonstrated to be a multifunctional protein in the large subunit of eukaryotic ribosomes, and ticks treated with HlP0 dsRNA obtained a strikingly lower body weight, a lower engorgement rate, and higher mortality after blood sucking than the control groups [19]. Other studies have shown that a synthetic 20 amino acid peptide from the P0 sequence was effective as a vaccine against *Rhipicephalus sanguineus* tick infestations in an immunization and challenge experiment using rabbits [20]. Therefore, these ribosomal proteins could be selected as potential antigens for vaccines against ticks. It would be expected to be active against many species of ticks.

Based on the above, whether RpL12 plays a role in the anti-tick immune response is not clear in ticks. In this study, the objective was to evaluate the anti-tick immune effect of rRpL12 in *H. longicornis*.

Material and Methods

Ethical Statement

The present study was approved by the Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Approval no. LVRIAEC 2021-006). The tick samples were collected in strict accordance with the requirements of the Ethics Procedures and Guidelines of the People's Republic of China.

Ticks and Tissue Collection

Haemaphysalis longicornis ticks were cultured by feeding on rabbits during various developmental stages in the laboratory. All stages of the ticks were maintained at incubator temperature under a relative humidity of 80±5%. Engorged ticks were maintained separately for oviposition. All collected ticks were immediately placed in phosphate-buffered saline (PBS) and washed twice in a solution containing 0.133 M NaCl, 1.11% sodium dodecyl sulfate (SDS) and 0.0088 M ethylenediaminetetraacetic acid (EDTA). Engorged adult *H. longicornis* ticks were cut under a 20-fold dissection light microscope, and the ovary, salivary glands, midgut, and epidermis were separated [21]. Total RNA was extracted with TRIzol RNA extraction reagent (Invitrogen, China) following the manufacturer's instructions and stored at -80°C for later use.

RpL12 Cloning and Sequencing

The RNA was reverse transcribed to cDNA, and then PCR and qPCR were performed. The nucleotide sequence of RpL12 was obtained from the cDNA of *H. longicornis*, *H. punctata* and *R. sanguineus* ticks. The gene-specific primers were designed using Primer Premier 6.0 software (RpL12-No1: 5'-ATG CCT CCC AAG TTT G-3') and a universal primer (RpL12-No2: 5'-CTG TAC AAG CTT GAT CC-3') and were synthesized by TaKaRa (Dalian, China). The PCR products were purified using a TaKaRa Agarose Gel DNA Purification Kit Ver. 2.0 (TaKaRa, Dalian, China), and the products were ligated into the pMD®19-T vector (TaKaRa, Dalian, China). The positive clones were sequenced with vector-specific primers (T7 and SP6) by Sangon (Shanghai, China).

Sequence Analysis

The open reading frames (ORFs) were amplified from *H. punctata* and *R. sanguineus*, and the PCR products were purified and ligated into the pGEM-T vector and transformed into the JM109 strain of *E. coli*. The positive clones were used for the sequencing of the nucleotide sequences of the inserts by TaKaRa (Dalian, China). The amino acid sequence of RpL12 was deduced using the Expert Protein Analysis System *(http://us.expasy.org/)*. The

similarities of the amino acid sequences were assessed with the BLAST algorithm of NCBI *(http://www.ncbi.nlm.nih.gov/BLAST/)*. The potential phosphorylation sites were predicted with the NetPhos 3.1 algorithm *(http://www.cbs.dtu.dk/services/NetPhos/)*.

qPCR Analysis of RpL12 in Different Tissues and Developmental Stages

Total cDNA was reverse transcribed from total RNA for eggs, unfed larvae, unfed nymphs, unfed adults and engorged adult ticks and from salivary glands, midguts, ovaries, epidermis and muscle dissected from engorged adult ticks. These samples were used to detect the expression levels of RpL12 in *H. longicornis* by qPCR. In the process, these tissues were thoroughly rinsed with PBS to remove haemocytes. All the samples were subjected to SYBR Green qPCR to determine the expression of RpL12. Two gene-specific primers, qRpL12-No3 (5'-AAG TGG GTG CCA CAT CTG C-3') and qRpL12-No4 (5'- ATC TTC AGC CCT TTC CAG TCC-3'), were designed to amplify a 112-bp segment of the RpL12 gene. β-actin F and R were used as internal references, and a 69-bp fragment was produced. PCR was performed using SYBR Premix Ex Taq (TaKaRa, Dalian, China) with the temperature profile and reaction conditions recommended by the manufacturer and the ABI two-step real-time PCR system (Applied Biosystems, USA). The expression level of RpL12 was analysed by the comparative cycle threshold (CT) method. The normalized CT values (ΔCT) were determined by one-way analysis of variance, and the significance of the differences in RpL12 expression in different tissues and developmental stages was determined using Student's t test with GraphPad InStat version 4.00 (GraphPad Software, USA). Differences were considered significant if $p<0.05$ (two-tailed test).

In vitro Recombinant RpL12 Production and SDS-PAGE Analysis

Cleavage sites for the restriction enzymes BamHI and EcoRI were added to the RpL12-No1 and RpL12-No2 primers for PCR amplification. The purified PCR product was inserted into the pGEX-4T-1 vector and then expressed in *E. coli* BL21 (DE3) pLysE (Novagen, USA) competent cells. The recombinant RpL12 protein (rRpL12) was induced in 2xYT medium (20 mL) with a 1/1000 volume of isopropyl-β-D-thiogalactoside (IPTG) (at a concentration of 0.8 mM) for 8 h at 37°C with shaking at 180 rpm. rRpL12 was purified using the MagneGST™ Protein Purification System according to the manufacturer's instructions (Promega, USA). The pGEX-4T-1 empty vector was also induced as a control under the same conditions. The purified protein and GST control were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and were detected/identified by staining the gel with Coomassie Brilliant Blue.

Immunization and Challenge Infestation

Six 2-month-old rabbits purchased from a tick-free area were divided into two groups of three rabbits each for immunization and challenge experiments. The rabbits belonging to the first group were immunized with rRpL12, and the rabbits of the second group served as controls. The fusion protein (1 mg) was emulsified in Freund's complete adjuvant (1:1) and subcutaneously inoculated into the experimental group of rabbits. In the protocols for rabbit immunization, the established textbook protocols suggest the second injection with antigen after 14 days and the third immune after 21 days, and the 3rd injection usually contains much less antigen to challenge infestation. For the challenge, 50 unfed adult ticks were introduced into bags that were adhered to the backs of the rabbits belonging to the first group and maintained for 30 days after the last booster injection. Various parameters, such as the feeding duration, engorged weight, degree of oviposition, and egg weight of each engorged tick, were determined to evaluate the effects of rRpL12-induced anti-tick immunity. Furthermore, the ovarian development of the fed female ticks was observed after they became engorged.

Results

Here, the RpL12 protein of *H. longicornis* was analysed using the Basic Local Alignment Search Tool for proteins (BLASTp) and EditSeq. The results revealed an ORF encoding a polypeptide with 165 amino acids, including 25 strongly basic, 19 strongly acidic, 58 hydrophobic and 33 polar amino acids, and in its amino acid sequence, sites 38 and 124, which are serines, are phosphorylation sites *(Table 1, Fig. 1)*. The amino acid sequences were conserved among the ticks and Drosophila *(Fig. 2)*. The identity of RpL12 showed 98.76% for *H. longicornis* between the cultured strain and Shandong strain (KAH9366394), and the identity was approximately 90% similar with Dermacentor, Rhipicephalus and Ixodes. The identity with Drosophila was approximately 82% similar *(Table 2)*, which further confirms the conservation of RpL12 among ticks.

The expression levels of RpL12 in various tissues and developmental stages of ticks were evaluated. Total cDNA was extracted from the samples obtained at different developmental stages and from the salivary glands, midguts, epidermis, and ovaries dissected from engorged female ticks and then subjected to qPCR analysis. The results showed that RpL12 was mainly expressed in the ovary, and it was also expressed in other tissues but in relatively low abundance *(Fig. 3-a)*. Among the tested developmental stages, the expression level of RpL12 in unfed ticks was significantly lower than that in females at the engorged stage *(Fig. 3-b)*.

rRpL12 was fused with pGEX-4T-1, and the expressed rRpL12+GST fusion protein was analysed by SDS–PAGE

Table 1. *Characteristics analysed for amino acids of RpL12 with Haemaphysalis longicornis*

Character Description	Number	Character Description	Number
Molecular Weight	17.67 KD	Amino Acids	165
Strongly Basic (+)	25	Strongly Acidic (-)	19
Hydrophobic Amino Acids	58	Polar Amino Acids	33
Isolectric Point	9.239	Charge at PH 7.0	6.328
Adenine (A)	24.10% (n=120)	Guanine (G)	29.52% (n=147)
Thymine (T)	17.67% (n=88)	Cytosine (C)	28.71% (n=143)

(+) represents strongly basic; (-) represents strongly acidic; (A) represents adenine; (G) represents guanine; (T) represents thymine; (C) represents cytosine; (%) indicates the percentage of bases A, T, C, and G to all bases

atg	cct	ccc	aag	ttt	gat	ccg	acg	gaa	att	aaa	gtt	gtg	tgc	ctt	cga	gcc	gtt
M	P	P	K	F	D	P	T	E	I	K	V	V	C	L	R	A	V
ggc	ggt	gaa	gtg	ggt	gcc	aca	tct	gcc	ttg	gct	ccc	aag	att	ggt	ccc	ctt	ggt
G	G	E	V	G	A	T	S	A	L	A	P	K	I	G	P	L	G
ctg	tcg	ccg	aag	aag	gtc	ggt	gat	gac	atc	gcc	aag	gcg	acg	cag	gac	tgg	aaa
L	S	P	K	K	V	G	D	D	I	A	K	A	T	Q	D	W	K
ggg	ctg	aag	atc	acc	gtc	aag	ctc	atc	atc	caa	aac	agg	cag	gct	acc	atc	gaa
G	L	K	I	T	V	K	L	I	I	Q	N	R	Q	A	T	I	E
gtg	gtg	ccc	agc	gct	gca	tcg	ctc	atc	atc	aag	gcg	ctc	aag	gag	ccg	cca	cgc
V	V	P	S	A	A	S	L	I	I	K	A	L	K	E	P	P	R
gac	cgc	aag	aag	gtc	aag	aac	gtg	aag	cac	agc	ggg	aac	ctg	acc	ttc	gac	gag
D	R	K	K	V	K	N	V	K	H	S	G	N	L	T	F	D	E
atc	ctc	acg	atc	gca	cgg	acg	atg	cgg	gcc	cgc	tct	atg	gcg	agg	agc	ctt	tct
I	L	T	I	A	R	T	M	R	A	R	S	M	A	R	S	L	S
ggc	acc	gtc	aag	gag	atc	ctg	ggt	aca	tgc	cag	tcc	gtc	gga	tgc	act	gtc	gat
G	T	V	K	E	I	L	G	T	C	Q	S	V	G	C	T	V	D
ggc	aac	cac	cca	cac	gac	gtc	att	gac	aag	gtc	aac	agc	ggt	gac	gtc	gag	gtg
G	N	H	P	H	D	V	I	D	K	V	N	S	G	D	V	E	V
ccc	gaa	gag	taa														
P	E	E	-														

Fig 1. Analysed amino acid and nucleic acid sequences of the RpL12 gene. Shaded amino acids represent phosphorylation sites

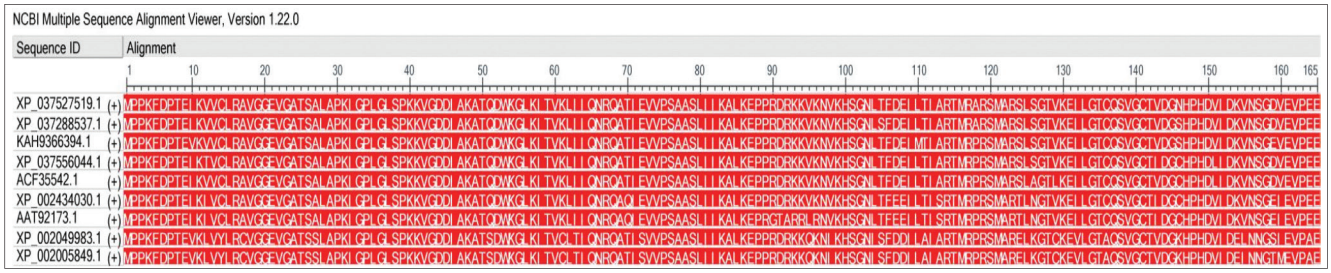

Fig 2. Multiple sequence alignments of RpL12 amino acid sequences in major arthropods (NCBI Multiple Sequence Alignment Viewer, Version 1.22.0)

on a 12% w/v polyacrylamide gel. The molecular weight of the fusion protein was observed to be approximately 44 kDa, close to its calculated MW (43.68 kDa). Western blotting revealed that rabbit anti-*H. longicornis* adult serum exhibited strong reactivity with rRpL12+GST proteins, whereas negative rabbit serum did not react with the fusion proteins *(Fig. 4)*.

The effects of rRpL12 on the examined physiological and behavioural parameters of tick feeding are shown in *Table 3*. No apparent differences were observed in the duration of feeding or percentage of mortality, whereas significant differences ($0.01<P<0.05$) in the spawning rate (62.45 vs. 80.12, respectively) and oviposition rate (77.32 vs. 82.68, respectively) were observed between

Table 2. RpL12 amino acid sequence alignment from ticks and Drosophila					
Species	**Accession NO.**	**Identity (%)**	**Species**	**Accession NO.**	**Identity (%)**
H. longicornis	No accession	Query	*Dermacentor silvarum*	XP_037556044	97.43
H. longicornis	KAH9366394	98.76	*Rhipicephalus sanguineus*	XP_037527519	96.97
Ixodes scapularis	XP_002434030	94.55	*Ixodes pacificus*	AAT92173	89.70
Dermacentor variabilis	ACF35542	97.58	*Rhipicephalus microplus*	XP_037288537	96.36
Drosophila mojavensis	XP_002005849	82.42	*Drosophila busckii*	XP_017838361	82.42
Drosophila navojoa	XP_017966042	82.42	*H. longicornis* (4D8 gene)	DQ159972	-

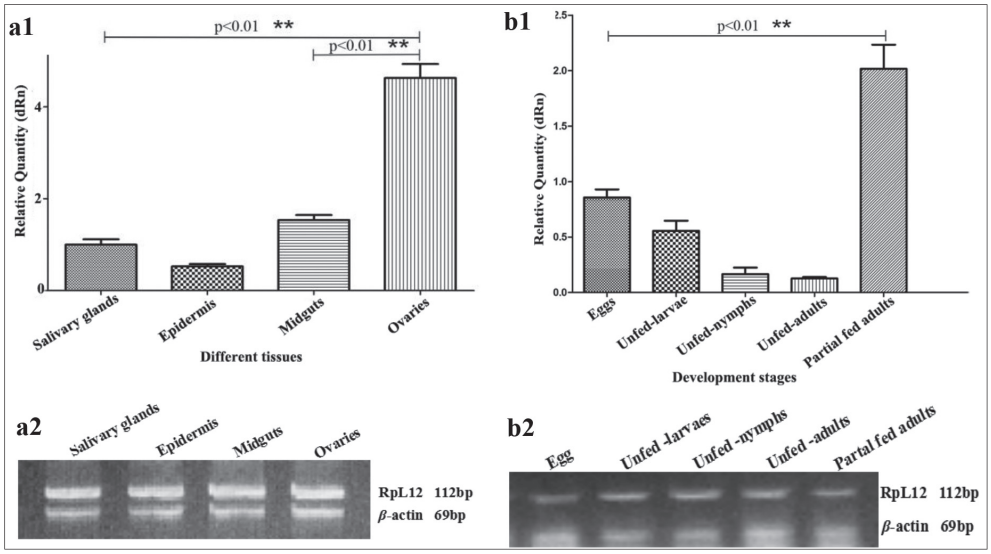

Fig 3. Expression of RpL12 in various tissues and at different developmental stages of ticks. **a1**- Column chart showing the expression level of RpL12 in different tissues of *Haemaphysalis longicornis*, **a2**- The corresponding PCR products were analysed in agarose gels, **b1**- Expression level of RpL12 at different developmental stages of *Haemaphysalis longicornis*, **b2**- The corresponding PCR products of different developmental stages. The results are shown as the means ± SEMss from three separate repeats. Asterisks indicate significant differences identified from the comparison of the various tissues and developmental stages **P<0.01 (Student's t test)

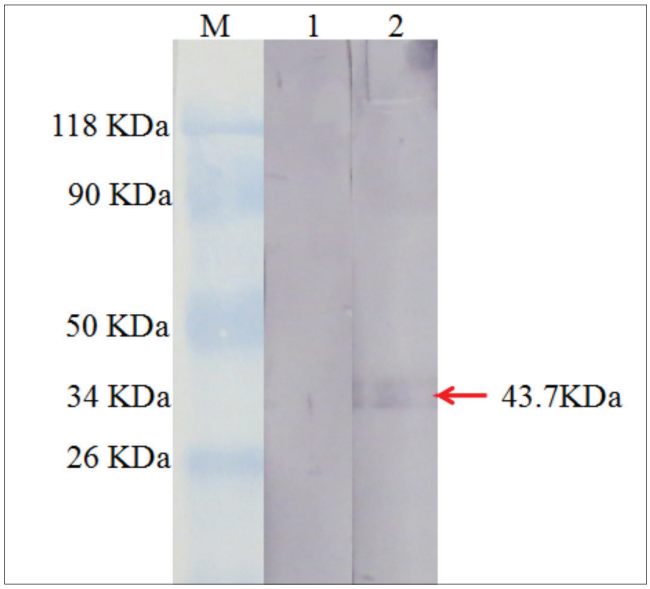

Fig 4. Western blot analysis of rRpL12. Rabbit-negative serum from *Haemaphysalis longicornis* adults was used as the primary antibody. M: Protein molecular weight marker; Lane 1, GST tag protein product with negative serum; lane 2, pGEX-4T-1+ RpL12-expressing product reacted with rabbit anti-*H. longicornis* adult serum

the vaccinated and control groups. Engorged female ticks were dissected, and their ovaries and midguts were examined. The evaluation of ovarian morphology showed that the ovary length of the experimental group was less than that of the control group. Although the density of nodules was high in the experimental group, this density was not conducive to the acquisition of nutrients for eggs, thus affecting the development of eggs. Therefore, in this study, ovarian development was significantly affected in ticks feeding on rabbits that were immunized with RpL12. In contrast, the morphology of the midgut was unchanged in the control group *(Fig. 5)*.

Discussion

L12, a ribosomal protein found in eukaryotes and archaea, is located near a translation factor-binding site on the surface of the large ribosomal subunit. It plays a role in the kinetics of peptide synthesis and might be involved in the interactions between the proteins L14 and L3 through other factors [22]. Another study showed that ribosomal proteins have some functions in animal reproduction

Table 3. *Effect of vaccination with rRpL12 on tick feeding*			
Parameter	**Immunization Treatment**		**Percentage (%)**
	rRpL12	**PBS**	
Duration of feeding (days)	10-13	11-14	-
Engorged weight, mean (mg)[a]	260±10*	294±8	17.22 (down)
Mortality (%)[b]	15±2.3	13±1.7	6.00 (up)
Oviposition rate (%)	77.32	82.68	5.36 (down)
Average egg weight (mg)	20.26±4.2**	28.56 ± 3.6	49.94 (down)
Spawning rate (%)	62.45*	80.12	17.67 (down)

**Significance (0.01<P<0.05) was calculated using Student's t test; [a] Dead ticks were excluded; the values are expressed as the means ± standard deviations; [b] The mortality and oviposition rates of the ticks after the feeding period were statistically analysed*

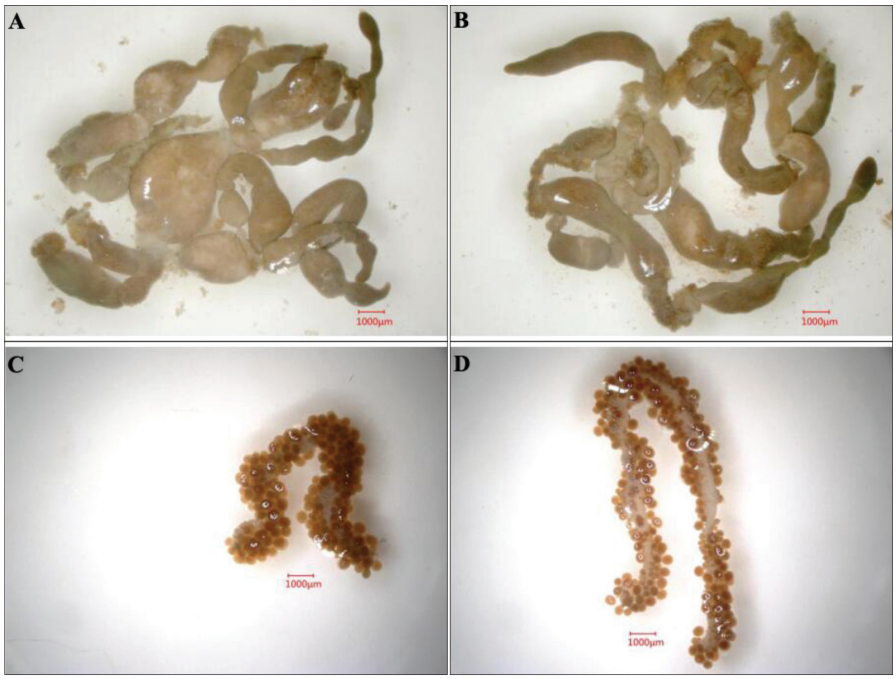

Fig 5. rRpL12 inhibition affects ovarian development. Engorged ticks were dissected to obtain their ovaries and midgut, and morphological development was compared between the experimental and control groups. **A** indicates the midguts of the immune group; **B** indicates the midguts of the control group; **C** indicates the ovaries of the immune group; **D** indicates the ovaries of the control group. In addition, the ruler was 1000 μm

and oogenesis [23,24]. Therefore, we were interested in the gene features of RpL12 in different tissues and at various developmental stages that might be related to physiological functions in ticks.

Here, the ORF sequence of RpL12 was cloned from *H. longicornis* ticks. The analysis of the pI of RpL12 showed that it is a strongly basic ribosomal protein with a pI of 9.239. The RpL12 nucleic acid sequence showed that the A+T content was much higher than the C+G content, indicating that the gene had high activity. It may play an important role in animal immunity *(Table 1)*. Two typical phosphorylation sites were predicted in RpL12 *(Fig. 1)*. Phosphorylation and dephosphorylation play a fundamental role in directly regulating various aspects of protein function, for example, cell growth, differentiation, apoptosis and cell signalling, under healthy conditions [25]. Multiple sequence alignment of amino acids from different species showed that RpL12 was highly conserved *(Fig. 2)*. The conserved features make it possible for the vaccine to be broad-spectrum.

The expression level of RpL12 was determined in different developmental stages and various tissues of *H. longicornis* ticks *(Fig. 3-a,b)*. The results indicated that RpL12 is required for feeding and ovary development in ticks. Although RpL12 is expressed in the salivary glands and midgut, its expression level is lower than that in the ovaries. Arguably, this could mean that there is no secondary function of RpL12 in these two tissues for blood digestion. In addition, RpL12 expression is relatively high in the egg stage and decreases during starvation along with tick development and maturity. The results also confirmed that the expression of RpL12 showed no relationship with unfed status in ticks. Again, the function of RpL12 has an impact on ovarian development. With the progression of tick development and increases in tick weight, the expression level of RpL12 decreased. However, the expression of RpL12 increased immediately after the unfed ticks were fed. It was also found that the expression level of RpL12 was higher in the ovary, indicating that feeding on blood by adult ticks plays an important role in

the maturation of ovarian cells; the putative physiological effect of L12 is in accordance with that of RpL12 of the same family [26]. These results have also been demonstrated in *Drosophila melanogaster*. Ribosomal proteins are associated with ribosomal subunits after fertilization of *Drosophila melanogaster* embryos. This event substantially precedes the blastoderm stage of embryonic development. At the preblastoderm stage, embryos synthesize many and possibly all of the r-proteins, and these subsets are incorporated into ribosomes. After blastoderm formation, all of the newly synthesized r-proteins along with newly synthesized rRNA are incorporated into ribosomal subunits [27]. This contributes to the normal development of the ovaries in arthropods.

Ribosomal proteins have also been shown by several studies to participate in the innate immune response. One of the prominent examples is RpL13A, which was reported to engage the interferon-γ (IFN-γ)-mediated inflammatory response by selectively modulating gene expression [28]. The inflammatory response is a double-edged sword that kills both pathogens and host cells. Identification of RpL13A as a negative regulator of inflammatory proteins suggests that this Rps could be a repressor of inflammatory signalling. Another example of Rps involvement in immune signalling is RpS3, which selectively modulates NF-κB target gene expression. NF-κB was originally identified to regulate genes crucial for the immune response but was later shown to also regulate genes implicated in cell survival or proliferation [29]. Together, these lines of evidence indicate that some Rps play diverse roles in the host immune response by either boosting immune signalling or facilitating pathogen production under different circumstances [30]. To investigate the immune effects of RpL12 on ticks, this putative function will be confirmed by monitoring the anti-tick immune effect of rRpL12. Based on the immune reaction between antisera against unfed adults and rRpL12 determined by Western blotting, the recombinant protein was well recognized by rabbit anti-*H. longicornis* tick serum. The cross-reactivity of rRpL12 from different tick species was analysed, and the results showed strong immunoreactivity *(Fig. 4)*. Because the RpL12 gene is highly conserved in arthropods, the protein might be developed into a universal immunogen. The results of the rRpL12 vaccination experiment showed that the physiological behaviour of the ticks had a significant effect in the rabbit model, and this finding was obtained for the effects on the duration of feeding, mortality, and average egg weight per tick. However, a significant effect was found on the engorged weight and the oviposition and spawning rates of the ticks, particularly the oviposition rate, which was decreased *(Table 3)*. As a ribosomal protein, RpL12 is a suitable candidate vaccine for antisera against rRpL12 that recognizes the target site because the expression of RpL12 *in vivo* was neutralized by antiserum against rRpL12. Another possible explanation is that RpL12 plays structural and functional roles in the eggs of ticks and is thus not essential for their survival and blood feeding. Morphological analysis showed that rRpL12 affected ovary development, which was a key reason for the observed decrease in the spawning rate (62.45% in the experimental group and 80.12% in the control group) observed in the immunization experiment. The evaluation of ovarian morphology indicated that the development of the ovaries was significantly inhibited in the immune group *(Fig. 5)*. These findings suggested that in the process of egg maturation in the ovary, abnormal ovarian development was induced by rRpL12, and egg development was consequently affected; therefore, the oviposition rate was also significantly decreased *(Table 3)*.

In conclusion, RpL12 is a conserved gene of *H. longicornis*. The expression level of the gene was significantly increased during tick feeding and in ovaries, while its expression levels were different in other developmental stages and among tissues. These findings demonstrated that RpL12 has important functions in feeding and ovarian development mortality and egg weight, although it has no effect on feeding time. Therefore, it might induce a strong protective effect against *H. longicornis* in rabbits. However, the processes of feeding and ovary development in ticks should be further investigated in further studies.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding authors.

Ethical Statement

The present study was approved by the Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Approval no. LVRIAEC 2021-006).

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

XRL and GYL: the hypothesis of this study; JL, GYL and XRL: work management, article writing; JL, WGL and QYR: experimental procedure follow-up; XKS and RFY: literature review, review of results, final decision.

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RESEARCH ARTICLE

Development of Lateral Flow Test for Serological Diagnosis of Tularemia [1]

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Abstract: Tularemia is a highly contagious zoonotic infection caused by *Francisella tularensis*. Bacterial culture, serology and molecular methods are used in the diagnosis of tularemia. The agent is a dangerous pathogen, and the importance of serological tests in diagnosis has increased because of the difficulty in culturing the organism. In this study, a practical, fast and reliable lateral flow-based immunochromatographic test was planned to detect *F. tularensis* specific antibodies in the field. Partially purified lipopolysaccharide antigen obtained from the live vaccine strain of *F. tularensis* was used as antigen. To determine the sensitivity of the test, 17 true positive tularemia serum samples with known Microagglutination test results, and to determine of specificity of the test, 30 true negative serum samples were used. In this study, *Brucella*-positive patient sera of various titers from our laboratory's serum bank to determine possible cross-reactivity with *Brucella* antibodies were also tested. The sensitivity and specificity of the Lateral Flow Test (LFT) rapid diagnostic kit were evaluated in comparison to the standard microagglutination test. The sensitivity and specificity of the newly developed Lateral Flow Test (LFT) were found at 100% and 93.5%, respectively. LFT for tularemia revealed 5% cross-reaction with positive sera for brucellosis. Cross-reactions were observed at antibody titers of 1:20 and below. In conclusion, it was concluded that the newly developed lateral flow test is a fast, reliable, and practical alternative test for the serological diagnosis of tularemia and cross-reaction in the serological tests conducted for brucellosis and tularemia should always be considered.

Keywords: *Francisella tularensis, Lateral Flow Test, Serology*

Tulareminin Serolojik Teşhisi için Lateral Flow Testinin Geliştirilmesi

Öz: Tularemi, *Francisella tularensis* tarafından oluşturulan son derece bulaşıcı, infeksiyöz zoonotik bir hastalıktır. Tulareminin teşhisinde bakteriyel kültür, seroloji ve moleküler metodlar kullanılmaktadır. Etken tehlikeli bir patojen olup, kültürünün yapılmasında karşılaşılan zorluklardan dolayı teşhiste serolojik testlerin önemi artmıştır. Bu çalışmada *F. tularensis* spesifik antikorları saptamak için sahada uygulaması kolay, güvenli hızlı sonuç veren lateral akış temelli bir immunokromatografik test geliştirilmesi amaçlandı. Antijen olarak *F. tularensis* canlı aşı suşundan elde edilen kısmen purifiye lipopolisakkarit antijeni kullanıldı. Testin duyarlılığının saptanmasında Mikroaglutinasyon testi sonuçları belli olan tularemi yönünden 17 gerçek pozitif ve özgüllüğünün saptanmasında 30 gerçek negatif serum örnekleri kullanıldı. Çalışmada ayrıca *Brucella* antikorları ile olası bir çapraz reaksiyonu değerlendirmek için laboratuvarımız serum bankasında bulunan çeşitli titrelerdeki Brusella pozitif hasta serumları da test edildi. Lateral Flow Testi (LFT) hızlı tanı kitinin sensitivite ve spesifitesi standart olarak kabul edilen mikroaglütinasyon testi ile karşılaştırmalı olarak değerlendirildi. Yeni geliştirilen testin duyarlılığı ve özgüllüğü sırasıyla %100 ve %93,5 olarak bulunmuştur. Tularemi için geliştirilen testin bruselloz yönünden olası bir çapraz reaksiyonunun değerlendirilmesi amacı ile yapılan testlerde, bruselloz ile %5 oranında çapraz reaksiyon saptanmıştır. Çapraz reaksiyonlar 1:20 ve altındaki antikor titrelerinde gözlemlenmiştir. Sonuçta geliştirilen lateral flow test prototipinin hastalığın tanısında güven ile kullanılabilecek, hızlı ve pratik bir serolojik test alternatifi olduğuna ve bruselloz ve tulareminin serolojik testlerinde bu hastalıklar için çapraz reaksiyonun olabileceğinin daima göz önünde bulundurulması gerektiği sonucuna varılmıştır.

Anahtar sözcükler: *Francisella tularensis, Lateral Flow Test, Seroloji*

INTRODUCTION

Although the route of transmission of tularemia caused by *Francisella tularensis* to humans is mai nly via rabbit-like animals and rodents, it can occur in many other direct or indirect ways besides insects such as ticks and flies.

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Effective treatment of tularemia cases depends on the actual diagnosis. This situation shows the importance of early diagnosis of the disease.

Culture of bacteria, serologic diagnosis (microagglutination test (MAT), Enzyme-Linked ImmunoSorbent Assay (ELISA) and molecular methods can be used successfully to diagnose tularemia [1]. However, culture requires a high-security laboratory (Biosafety Level 3) and experienced personnel [2]. Antibodies against *F. tularensis* can be detected by agglutination and the ELISA [3-6]. The tube agglutination test (TA) has been used to detect antibodies to the pathogen for many years [3,4,7]. However, since the 1970s, the microagglutination test (MAT) has been used because the TA test is time-consuming, is not suitable for testing many specimens, and requires an excessive amount of antigen [8-10]. The antigen used in MAT is usually produced in-house by laboratories and there is no national or international standardization. There is also no standard for the dyes used to detect the MAT test antigen and reaction better. It has been reported that the ELISA in which lipopolysaccharide derived from *F. tularensis* as antigen is ten times more sensitive than the tube agglutination test for the diagnosis of tularemia in humans [11].

Rapid diagnostic tests have successfully saved time in human and veterinary medicine diagnoses. The history of biosensors began in 1962 with the development of enzyme electrodes by scientist Clark and Lyons [12]. These rapid diagnostic tests are widely used and further developed in agricultural production, food processing, environmental monitoring, clinical diagnostics, drug testing, biotechnology, and the determination of biological and chemical warfare agents [13]. The researchers note that the Lateral Flow Test (LFT) method is superior to other serological tests because it provides results in a short time, is easy to perform, interpretable and sensitive [14].

Lateral Flow Test can be described by some procedures that include precise interaction of Abs and Ags. LFT system consists of four main components: the sample pad that carries the analyte to the absorbent pad, the conjugate pad on which the tagged analytes are attached to the biocomponents, the Nitrocellulose membrane that contains the control and test line, and the absorbent pad that stores the waste. The principle of an LFT is based on the movement of a liquid sample through a polymer strip with attached molecules that interact with the analyte to provide a signal that can be detected visually [15]. The most critical elements of the assay are the antibodies and the membrane, but all other materials used should also be considered to ensure compatibility and consistency of the product. An LFT is a rapid, inexpensive, portable, and user-friendly assay. However, the results are mostly qualitative (on/off) or semi-quantitative [16].

This study aims to develop a rapid diagnostic test based on immunochromatography using the LPS antigen for the detection of antibodies to tularemia.

Material and Methods

Ethical Statement

Approval was received from Harran University Animal Experiments Local Ethics Committee (HU/HADYEK: 2018/003/02).

Reference Bacterial Strain

The *F. tularensis* LVS vaccine strain (NCTC 10857) was obtained from Prof. Dr. Aynur Karadenizli at Kocaeli University. The supplied strain was grown on Cystine Heart Agar (Difco) containing 2% hemoglobin solution (Oxoid) in a 5% CO_2 environment. Homemade standardized MAT antigen from previous study was used.

Positive and Negative Control Sera

Standard sera from tularemia positive individuals with known (MAT) results were obtained from the Turkish Health Authority, the National Tularemia Reference Laboratory and the Kocaeli University Department of Medical Microbiology. The reference titers MAT of these sera were 1:20, 1:80, 1:160, 1:640 and 1:1280. These sera were used as positive control sera for MAT, ELISA and LFT during the project. To calculate the specificity and sensitivity of the LFT prototype produced in the study, 17 tularemia antibody positive and 30 negative human serum samples from the HÜBAP (Harran University Experimental Research Project No: 18072) project were used. This study tested *Brucella* positive patient sera of various titers from our laboratory's serum bank with LFT to evaluate possible cross-reactivity with *Brucella* antibodies.

In the serological tests used in the study, the reference and test sera were tested ten times. The means and standard deviations of the optical densities (OD) detected by ELISA were determined. The sera from patients diagnosed with brucellosis were also tested ten times by MAT, ELISA, and LFT to evaluate cross-reactivity with *Brucella*. The mean and standard deviation of the OD values obtained by ELISA were determined *(Table 1)*.

ELISA

Partially purified LPS layer by Trizol treatment was used as the solid-phase antigen in ELISA [17]. After checkerboard analysis of the isolated antigen with positive and negative sera, the most appropriate antigen dilution was prepared with carbonate bicarbonate buffer (pH 9.6) and 100 µL of the antigen dilution determined by checkerboard analysis was added to each well of the 96-well plate (NUNC, 269620, Denmark). After washing

Table 1. *Test results of MAT, LFT and, ELISA with reference MAT antigen*

Serum Titers Tested with the Reference MAT Antigen	In House MAT	In House ELISA Mean OD±Standard Deviation	LFT
F. tularensis MAT 1:20	1:20	Positive (0.546±0.111)	Negative
F. tularensis MAT 1:80	1:80	Positive (0.747±0.094)	Positive
F. tularensis MAT 1:160	1:160	Positive (1.346±0.089)	Positive
F. tularensis MAT 1:640	1:640	Positive (2.47±0.091)	Positive
F. tularensis MAT 1:1280	1:1280	Positive (3.09±0.102)	Positive
F. tularensis MAT Negative	Negative	Positive (0.145±0.061)	Positive
Brucella positive serum 1:80 Reference MAT	Negative	Positive (0.190±0.087)	Negative
Brucella positive SAT 1:320 Reference MAT	1:10	Negative (0.201±0.08)	Negative
Brucella positive SAT 1:1280 Reference MAT	1:20	Negative (0.225±0.072)	Negative

MAT: *Microagglutination test,* ***SAT:*** *Serum agglutination test*

and blocking, the positive and negative sera were diluted at 1:100 and added. After washing, recombinant A/G conjugate (Pierce 32490) labeled with HRPO was added. After washing, 100 µL of a chromogenic substrate (2 µg ortho-phenylenediamine and 0.03% H_2O_2 in 0.1 M citrate buffer (pH: 5.5) was added. After the plates were kept at room temperature for 10 to 15 min, 100 µL of 4 N H_2SO_4 was added to each well to stop the reaction and the absorbance values of the plates were read at 490 nm using an automated ELISA reader (VERSAmax 3.13/B2573). The mean of the negative serum OD's plus three standard deviations (SD) was determined as the ELISA cutoff value [18].

Lateral Flow Test Strips

In the preparing test strips for the diagnosis of tularemia, LPS antigen was obtained from the *F. tularensis* strain and prepared according to the method Eugene and Hackett [17] used for the test line. Strips prepared from all inactive bacterial solutions and LPS antigen were used for comparison.

Preparation of Colloidal Gold

Five mL of a 1% Hydrogen tetrachloroaurate trihydrate stock solution was mixed with 500 mL of distilled water and heated to boiling. Then 5 mL of a 1% sodium citrate solution was added to the gold solution and boiled until the color turned red. After boiling for another 5 min, the solution was ready and was stored in a black bottle at +4°C until use [19].

Preparation of Colloidal Gold Probes

After determining the optimal concentration, protein A/G diluted in the indicated ratio was added to the pH-adjusted colloidal gold solution, mixed and incubated at room temperature for 25 min. Then, a 10% (w/v) Bovine serum albumin (BSA)(Sigma) solution was added at a ratio of 1/10 and kept at room temperature for 10 to 15 min. The mixture was centrifuged at 15000 g and +4°C for 30 min. After centrifugation, the pellet was suspended in PBS and used as conjugate [20].

Preparation of Immunochromatographic Test Strips

Francisella tularensis LPS and purified polyclonal human IgG (Merck, Germany) were applied to a nitrocellulose membrane using a lateral flow dispenser as test and control, respectively and dried at 37°C for 2 h. The prepared colloidal gold probes were sprayed onto the glass fiber membrane using a lateral flow dispenser and dried entirely at 37°C. After the pads were dried, they were combined and cut using a cutter. The resulting strips were placed in plastic cassettes [20].

Measurement of Specificity and Sensitivity of Test Strips

Positive and negative reference serum panels were used to measure the sensitivity and specificity of the test. The sensitivity and specificity of the LFT rapid diagnostic kit were evaluated in comparison to the MAT test, which is the accepted standard. For this purpose, the following formula was used.

Sensitivity = *Francisella* Positive Sera/*Francisella* Positive Sera+ False Negative

Specificity = *Francisella* Negative Sera/*Francisella* Negative Sera+ False Positive

Results

Each reference and test sera were tested ten times to evaluate the reliability and reproducibility of the serological tests used in the study. The means and standard deviations of the optical densities determined by ELISA were calculated. Sera from patients positive for brucellosis were tested ten times by MAT, ELISA and LFT to evaluate possible cross-reactivity with *Brucella*. The mean and standard deviation of the OD values obtained by ELISA were determined. All the results in the present tables were prepared considering the values obtained from these

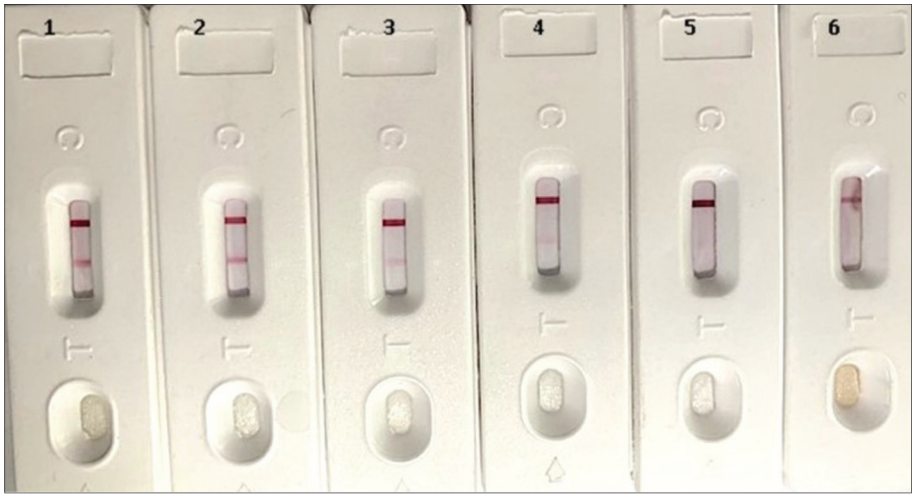
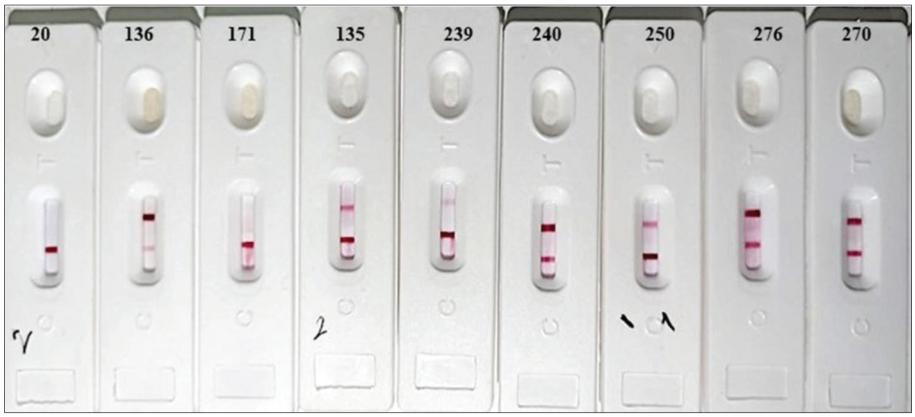


Fig 1. LFT results of sera tested with Reference *F. tularensis* MAT antigen and Reference *B. abortus* SAT antigen. 1. MAT 1:1280 2. MAT 1:640 3. MAT 1:160 4. MAT: 1:80 5. MAT 1:20 6. Negative serum

Fig 2. LFT results of *Francisella* antibody positive MAT sera



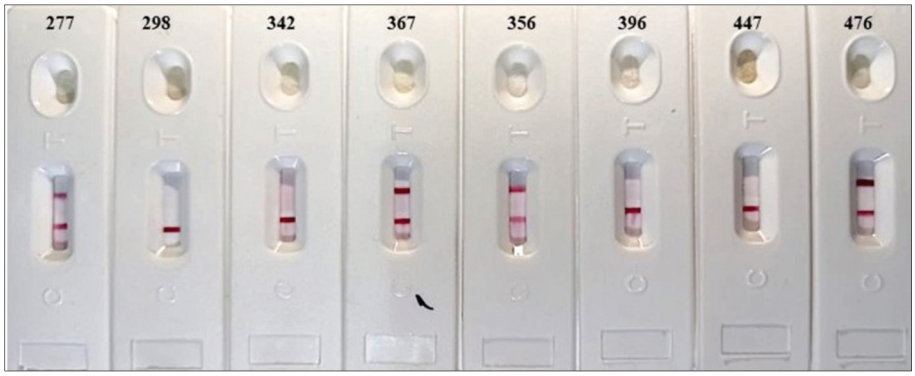


Fig 3. LFT results of *Francisella* antibody positive MAT sera

averages. When the positive reference sera MAT were tested with the LFT developed at the end of the study, all positive sera gave positive results with the LFT, except for the MAT 1:20 positive serum *(Fig 1)*.

Positive reference sera for *F. tularensis* and positive sera for brucellosis in various titers were tested with the LFT developed in the study MAT and ELISA. The results are shown in *Table 1*. It was found that all tests gave the same results when the positivity criterion for tularemia was assumed to be 1:20 and higher. However, while ELISA and MAT could detect a titer as low as 1:20, LFT could not detect this titer *(Table 1)*.

To calculate the specificity and sensitivity of the LFT prototype, 17 positive and 30 negative human serum samples, also used in the previous HÜBAP material (18072), were tested for tularemia with LFT. The results are shown in *Fig. 2* and *Fig. 3*.

A comparison of these sera with the results from MAT is shown in *(Table 2)*. Accordingly, the sensitivity of LFT was 100%, while the specificity was calculated to be 93.5%. In our study, the intensity of the LFT test line in sera 136 and 476 with a titer of 1:1280 was observed quite intensively with MAT and the results showed complete agreement with MAT *(Table 2)*.

Table 2. *LFT test results of F. tularensis positive MAT positive sera*

***F. tularensis* Positive Serum No**	**LFT Result**	**MAT Titer**
20	Negative	1:20
136	Positive	1:1280
171	Negative	1:20
135	Positive	1:320
239	Positive	1:80
240	Positive	1:640
250	Positive	1:160
276	Positive	1:640
270	Positive	1:640
277	Positive	1:320
298	Negative	1:20
342	Negative	1:20
367	Positive	1:640
356	Positive	1:320
396	Negative	1:20
447	Negative	1:20
476	Positive	1:1280
30 Tularemia negative serum (1:20 and above negative)	29 Negative	30 Negative
Total=17 positives (1:20 and above positive)	----- 11 Positive	17 Positive 11 Positive
F. tularensis LFT sensitivity	100%	100%
F. tularensis LFT specificity	93.5%	100%

Table 3. *Results of Brucella positive sera with different titers by Francisella LFT*

Number of *Brucella* Positive Sera	***Francisella* LFT Negative**	***Francisella* LFT Positive**
40	38 (1:80-1:1280 arası)	2 (1:1280 ve 1:2560)
Result	95%	5%

In addition, to determine a possible cross-reaction with brucellosis, various titers of *Brucella* antibodies of positive human sera from the serum bank of our laboratory were tested by LFT. The result was that 38 of 40 positive sera were negative (95%), while two serum samples were positive (5%) *(Table 3)*.

Discussion

Francisella tularensis is a pathogen that causes severe lethal infections in humans and some mammals. Studies have shown that successful antibiotic therapy for tularemia depends on timely diagnosis [21]. Because of this, an early and reliable diagnosis of the disease is essential. Diagnosis of the disease is based mainly on serological tests, as the pathogen is a dangerous and highly contagious microorganism [22]. However, there are no standardized commercial antigens and test kits that can be used for the serological diagnosis of the endemic diseases in Turkey. Therefore, the LFT prototype with LPS antigen was developed in the study.

It is known that ELISA and MAT are susceptible tests for diagnosing the disease [1,2,11,23]. A recent study conducted in our country showed that seropositivity rates for 72 human sera were found as 4.2% for ELISA and MAT. Seropositivity for 190 serum samples from sheep were found as 3.2% for MAT and 4.7% for ELISA. These figures show that tularemia cannot be considered as an insignificant disease in human and sheep. Therefore, rapid tests can be important in diagnosis and epidemiology of the disease [24].

This study, obtained positive results with the LFT from all positive sera except the MAT 1:20 positive serum. Because a single serum sample with a titer of 1:160 or greater is considered positive in the final diagnostic criteria for the disease [2], it is believed that failure to detect a titer of 1:20 in the LFT is not thought to reduce the sensitivity of the test. Titers of 1:10 to 1:80 are already widely associated with cross-reactivity [10].

In this study, the sensitivity of the LFT was found to be 100%, whereas the specificity was calculated to be 93.5%. Splettstoesser et al.[25] in their study on the development of the LFT, determined a sensitivity of the test of 98.3% and a specificity of 96.5% and reported that this test is a reliable test for detecting the disease in the field. Kilic et al.[6] have used a commercial LFT kit and found that the method had a sensitivity of 99.3% and a specificity of 94.6%. The values for sensitivity and specificity obtained in our study using the LFT method show significant similarity with the results obtained by all these researchers.

In the study, brucellosis positive human sera of different titers from the serum bank of our laboratory were also tested with LFT to evaluate possible cross-reactivity with brucellosis. The result was that 38 of 40 positive sera were negative (95%), whereas two serum samples were positive (5%) *(Table 3)*. Kilic et al.[6]. reported positive results in 5 of 50 brucellosis positive sera using the LFT kit. Cross-reactivity was observed in brucellosis and tularemia, albeit at low levels. It is known that the similarity of LPS in the cell wall structure of these two pathogens is responsible for these cross-reactions [22]. These findings are consistent with the results of our study and the possibility of cross-reactivity between these two diseases should always be considered in serological testing for brucellosis and tularemia.

It is concluded that the LFT is a valuable serologic test that can be used to diagnose of tularemia. It is safe, practical and provides rapid results. It is expected that using this test in the field will contribute to the control of disease and the timely measures to be taken.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (O.Y. Tel).

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author's Contributions

Experimental design and correction of the manuscript; OYT, data collection, lab work, first draft of the manuscript; SEG, AGY, OK, AK fieldwork; OK, SEG, AGY, OYT. All authors have read and agreed to the published version of the manuscript.

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Research Article

Potential of *Glycyrrhiza glabr*a (Licorice) Extract an Alternative Biochemical and Therapeutic Agent Against Coccidiosis in Broiler Chickens

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Abstract: To control coccidiosis, anticoccidials are generally used as feed additives. However, the frequent usage has given rise to the occurrence of resistant strains to available anticoccidial drugs. Botanicals may work as substitutes to anticoccidial drugs. The current research was designed to evaluate the efficacy of aqueous methanol extracts of *Glycyrrhiza glabra* (licorice) (roots) as anticoccidial in different concentrations i.e. (100, 200 and 300 mg/kg of body weight). For *in vivo* trial, 105-day-old broiler birds were grouped into seven equal groups (A, B, C, D, E, F and G). At the age of one week, groups A, B and C were orally treated with three doses of the *G. glabra* extracts (100, 200 and 300 mg/kg of body weight, respectively). Group D was medicated with Vitamin E and Group E worked as the infected medicated control group. Group F served as the infected non-medicated control group (PBS treated, negative control) and Group G was designated as the normal control group (non-infected and non-medicated group). At the age of the 14th day, all groups were infected orally with 60.000 sporulated oocysts of different *Eimeria* species. Though, comparable with reference drug G. glabra showed comparable anticoccidial efficacy against following parameters. i.e., feed conversion ratio, lesion score, fecal score and oocyst score, serum profile and hematological values showed no adversative effects (P<0.05) than infected non-medicated (negative control) group of aqueous methanol extract of *G. glabra* on the trial broiler birds. So, in this study the biochemical and therapeutic property of G. glabra extract found dose dependent manner against coccidiosis in broiler chickens.

Keywords: *Phytomedicine, Poultry, Alternatives, Disease*

Etlik Piliçlerde Koksidiyoza Karşı Alternatif Bir Biyokimyasal ve Terapötik Ajan Olarak *Glycyrrhiza glabra* (Meyan Kökü) Ekstraktının Potansiyeli

Öz: Koksidiyozun kontrolünde genellikle yem katkı maddesi olarak antikoksidiyaller kullanılır. Fakat, bunların sık kullanımı mevcut antikoksidiyal ilaçlara dirençli suşların ortaya çıkmasına neden olmuştur. Bitkisel ilaçlar antikoksidiyal ilaçların yerini alabilir. Bu çalışma, *Glycyrrhiza glabra* (meyan kökü)'nın (kökleri) sulu metanol ekstraktlarının farklı konsantrasyonlarda (örneğin; 100, 200 ve 300 mg/kg vücut ağırlığı) antikoksidiyal etkinliğinin değerlendirilmesi için tasarlandı. *In vivo* denemeler için, 105 günlük etlik piliçler yedi eşit gruba (A, B, C, D, E, F ve G) ayrıldı. Bir haftalıkken A, B ve C gruplarına oral olarak üç doz şeklinde (sırasıyla 100, 200 ve 300 mg/kg vücut ağırlığı) ekstrakt uygulandı. Grup D'ye Vitamin E uygulandı ve Grup E, ilaç uygulanmış enfekte kontrol grubu olarak yer aldı. Grup F, ilaçsız enfekte kontrol grubu (PBS ile tedavi edilen, negatif kontrol) ve Grup G, enfekte olmayan ve ilaçsız normal kontrol grubu olarak yer aldı. 14. günde, tüm gruplar farklı *Eimeria* türlerine ait 60.000 sporlu ookist ile oral yolla enfekte edildi. Referans ilaç ile kıyaslandığında *G. glabra*, yemden yararlanma oranı, lezyon skoru, dışkı skoru ve ookist skoru gibi parametreler yönünden karşılaştırılabilir antikoksidiyal etkinlik göstermesinin yanı sıra, serum profili ve hematolojik değerler de, *G. glabra*'nın metanol ektraktının deneme gruplarında, enfekte ilaçsız kontrol grubundan (negatif kontrol) daha olumsuz bir etki sergilemediğini ortaya koydu (P<0.05). Böylece bu çalışmada, *G. glabra* kök ekstraktının etlik piliçlerde koksidiyoza karşı doza bağlı olarak biyokimyasal ve tedavi edici özelliği saptanmıştır.

Anahtar sözcükler: *Bitkisel ilaç, Kümes hayvanları, Alternatifler, Hastalık*

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Introduction

Avian coccidiosis is mostly produced with single cell parasitic protozoa of genus *Eimeria* which belongs to phylum *Apicomplexa* having complex life cycle [1]. *Eimeria* species affected birds mostly show symptoms of enteric damage resulting into bloody diarrhea, reduced weight gain and mortality which lead to the economic losses in industry [2]. Additionally, links have been reported between Emirian infection and higher intestinal colonization with bacterial foodborne pathogens such as *Clostridium perfringens* and *Salmonella enterica* serovars Typhimurium and Enteritidis. Thus, increasing risk of zoonotic food borne diseases and to food security. There are seven species of *Eimeria* which have been recognized which cause coccidiosis in chickens by residing in different sections of intestine. The most virulent species is *E. tenella* that causes cecal coccidiosis in chickens. Infection starts with the ingestion of sporulated oocyst [3]. According to an estimation in US poultry industry, coccidiosis causes a loss of $127 million annually and this can be expected to cause likewise loses world-wide [4,5]. Due to ubiquitous nature of coccidian oocysts, they sporulate rapidly and millions of new oocysts emerge from each single sporulated oocyst of *Eimeria* which make very difficult to keep birds protected from coccidiosis [6].

Synthetic chemicals and anticoccidials are generally added in feed and water to control coccidiosis. Sulfanilamide was the first anticoccidial used as a treatment against coccidiosis in poultry birds, and a variety of anticoccidial feed additives and antibiotics, have also been developed and used. However, with frequent use of these drugs, anticoccidial drug resistance has emerged in *Eimeria* species by the passage of time [7]. Efficacy of these chemical agents is becoming questionable with the advent of these drug-resistant parasite strains. Adverse effects of these drugs are also reported on the health status of birds and humans, due to the drug residual effects in meat leading to the reduced consumption by humans [8,9].

It's the need of hour to look in indigenous, cost effective and potent resources for eradication and control of coccidiosis. Therefore, we must select alternatives for the operative and designated control of coccidiosis [10,11]. The experimental work showed that probiotics could be a good hope due to its antioxidant, immunomodulatory and growth-promoting effect against poultry coccidiosis alone or in combination with vaccines, including IMP1C based vaccine [12-14]. In the last few years, some botanicals gave us promising hope for their use as anticoccidials [15,16] and immunomodulatory effects [17-20]. Studies also evaluate that plants methanolic extracts possess best anticoccidial, anthelminthic and antioxidant activities due to presence of medicinally important phytochemicals [21,22].

Some botanicals have been described as promising anti-coccidials and immunomodulators [23,24]. Because of the restrictions on the synthetic anticoccidials due to their resistance against *Eimeria* species in poultry, antioxidant-rich plant extracts have attained promising future importance [25]. Botanicals high in antioxidant chemicals such phenols, flavonoids, tannins, and saponins are being used to treat coccidiosis as an alternate method [26]. *Glycyrrhiza glabra* plant is well renowned for its therapeutic effects like anti-allergic, anti-inflammatory, spasmolytic, laxative, antistress, antidepressant, antiulcer, estrogenic, immunomodulator, and antidiabetic substance in livestock and public health. It is also used to treat cough, throat infections, mouth and stomach infections [27]. Therefore, the present research was designed to evaluate the anti-coccidial benefits of aqueous methanolic extract of *G. glabra* (roots) against a simulated mixed *Eimeria* infection in chickens based on the available literature, which included antioxidant capabilities of botanicals.

Material and Methods

Ethical Statement

This research was conducted with approval from ethical committee of University of Agriculture Faisalabad (Approval No: 144) Faisalabad, Pakistan.

Plant Material

Glycyrrhiza glabra roots were purchased from a local market. A botanist from the Botany Department (University of Agriculture, Faisalabad-Pakistan) recognized and confirmed the plant material. In an electric mill, dried plant material was ground into powder. Using Soxhlet's equipment at 80°C, an aqueous methanolic (70%) extract of *G. glabra* was obtained by using rotary evaporator at 35°C under decreased pressure. The extracted material was freeze dried and stored at 4°C until used.

Parasite

Natural *Eimeria* infected chicken guts were obtained from several poultry selling sites and different poultry farms of district Faisalabad, Pakistan. Microscopically, the GIT material was observed using the method described by [28]. Sporulation of oocysts was achieved by incubating them in a 2.5 percent potassium dichromate solution for 48 hours at 24-30°C with 60-80 percent humidity. The sporulation was tracked using a light microscope at a magnification of 40x to examine sporocysts.

Management

A total of 105-day-old broiler chicks were purchased from a local market in Faisalabad and kept on floor pens using standard management procedures. For the first two weeks, all chicks were fed broiler starter diet, then finisher ration used till last day. Anticoccidial ingredients were not included in the standard feed. Feed and water were freely

available, and chicks were vaccinated against Newcastle Disease, Infectious Bronchitis, Infectious Bursitis [29]. During the first week of life, the temperature was kept at 85-90°F; however, it was dropped by 5°F on a weekly basis. Throughout the experiment, light was delivered for 24 h at a time. The experiment was carried out for 42 days.

Experimental Design

Chicks (n=105) were separated into seven equal (n=15) groups for the anticoccidial experiment. On day 14 of the experiment, all groups except G were orally infected with 60.000 sporulated oocysts of mixed *Eimeria* species. At the same day, Groups A, B and C were orally given *G. glabra* extract at 100, 200 and 300 mg/kg of body weight. Group D was treated with Vitamin E at the dose rate of 87 mg/kg of body weight. Group E was treated with Toltrazuril (Baycox® Bayer, Leverkusen, Germany) at the dose rate of 1 mL/ liter of water. Group F was treated with PBS (negative control) and Group G remained (Normal control). This procedure continued for day 14, 15 and 16 of experiment.

Feed Conversion Ratio

FCR was calculated on the 30th day of the experiment through total feed consumed/total weight gain.

Oocyst, Lesion and Fecal Score

After 7 days of post infection, 6 birds from each group were slaughtered and score of oocyst (0-5), lesions (0-4) and fecal were observed by [30-32].

Hematological Analysis

Packed cell volume determinations (PCV), red and white blood cell count and hemoglobin (Hb%) level were assessed by using hematology analyzer FMI- 6180 (Jiangsu, China) by following the standard method as reported.

Serum Chemistry

Toxicity level of *G. glabra* roots in infected chickens was observed by measuring different levels of serum enzymes like aspartate aminotransferase (AST), alanine transferase (ALT), lactate dehydrogenase (LDH), urea, and creatinine. All these procedures were performed by given guideline of Kits producing company (Diagnostic Ltd. UK).

Statistical Analysis

Statistical significance was determined using SAS statistical analysis software utilizing Duncan's multiple range and analysis of variance [33].

Results

The better FCR was observed in groups treated with GGE at different doses but, excellent result was observed at higher dose. Feed conversion ratio of groups treated with GGE at the dose rate of 300 mg/kg of body weight, was comparable to Toltrazuril and Vitamin E *(Table 1)*.

The oocyst score in all treated groups was minimum ($P<0.05$) compared to infected un-medicated (negative) control group. Among GGE treated groups, minimum oocyst score was recorded in chickens treated with GGE at 300 mg/kg of body weight followed in increasing order by groups treated with 200 and 100 mg/kg of body weight. Oocyst score of groups treated with GGE at the dose rate of 300 mg/kg of body weight, Toltrazuril and Vitamin E treated groups was significantly comparable *(Table 2)*.

The fecal score in all treated groups was minimum ($P<0.05$) compared to infected un-medicated (negative control) control group. Among GGE treated groups, minimum fecal score was recorded in chickens treated with GGE at 300 mg/kg of body weight followed in increasing order by groups treated with 200 and 100 mg/kg of body weight. Fecal score of groups treated with GGE at the dose rate of 300 mg/kg of body weight, Toltrazuril and Vitamin E treated groups was almost similar *(Table 3)*.

The lesion score in all treated groups was minimum ($P<0.05$) compared to infected un-medicated control group. Among GGE treated groups, minimum lesion score was recorded in chickens treated with GGE at 300 mg/kg of body weight followed in increasing order by

Table 1. *Feed conversion ratio (FCR) in different treatment groups*

Treated Groups	Feed Consumed	Final Weight	Feed Conversion Ratio
GGE 100 mg/kg	1698.20	651.9	2.60
GGE 200 mg/kg	1730.10	706.5	2.44
GGE 300 mg/kg	1783.7	778.2	2.29
Vitamin E, 87 mg/kg	1764.6	803.25	2.19
Toltrazuril, 1 mL/L	1750.18	805.17	2.17
Infected Group	1679.94	588.5	2.85
Normal Group	1836.71	939.4	1.95

GGE: *Glycyrrhiza glabra extract; * Statistical analysis was not possible because of group feeding of chicken*

Table 2. *Oocyst score (n=6) in different treatment groups*

Treated Groups	Oocyst Score						Mean±SD
	0	+1	+2	+3	+4	+5	
GGE 100 mg/kg	2	2	0	2	-	-	2.01±0.74^{b}
GGE 200 mg/kg	2	1	1	2	-	-	1.4±0.72^{bc}
GGE 300 mg/kg	0	3	2	1	-	-	1.49±0.74^{b}
Vitamin E, 87 mg/kg	1	3	1	1	-	-	1.30±0.75^{c}
Toltrazuril, 1 mL/L	2	1	3	-	-	-	1.16±0.85^{c}
Infected Group	-	-	1	1	2	2	2.73±0.31^{a}
Normal Group	-	-	-	-	-	-	-

GGE: *Glycyrrhiza glabra extract;* ***0:*** *No oocysts;* ***+1:*** *1-10 oocysts per field of microscope;* ***+2:*** *11-20 oocysts per field of microscope;* ***+3:*** *21-50 oocysts per field of microscope;* ***+4:*** *51-100 oocysts per field of microscope;* ***+5:*** *more than 100 oocysts per field of microscope; Means with different letters are significantly different (P<0.05)*

Table 3. *Fecal score (n=6) in different treatment groups*

Treated Groups	4th Day	5th Day	6th Day
GGE 100 mg/kg	1.79±0.74^{b}	2.60±0.54^{b}	0.97±0.13^{b}
GGE 200 mg/kg	1.60±0.56^{b}	2.55±0.52^{b}	0.59±0.51^{b}
GGE 300 mg/kg	1.43±0.52^{bc}	1.63±0.52^{b}	0.52±0.54^{bc}
Vitamin E, 87 mg/kg	1.29±0.52^{bc}	1.31± 0.52^{c}	0.41±0.52^{bc}
Toltrazuril, 1 mL/L	1.23±0.50^{bc}	1.29±0.52^{c}	0.36±0.52^{bc}
Infected Group	3.95±0.41^{a}	3.43±0.50^{a}	2.63±0.62^{a}
Normal Group	0.01±0.0^{a}	0.00±0.0^{e}	0.00±0.0^{e}

GGE: *Glycyrrhiza glabra extract; Means with different letters are significantly different (P<0.05)*

Table 4. *Lesion score (n=6) in different treatment groups*

Treated Groups	Lesion Score					Mean±SD
	0	+1	+2	+3	+4	
GGE 100 mg/kg	-	2	2	2	-	1.54±0.53^{b}
GGE 200 mg/kg	1	2	2	1	-	1.32±0.51^{bc}
GGE 300 mg/kg	2	3	1	-	-	1.33±0.53^{bc}
Vitamin E, 87 mg/kg	2	3	1	-	-	1.15±0.53^{c}
Toltrazuril, 1 mL/L	3	1	2	-	-	1.10±0.21^{c}
Infected Group	-	-	1	1	4	3.79±0.50^{e}
Normal Group	-	-	-	-	-	-

GGE: *Glycyrrhiza glabra extract;* ***0:*** *No gross lesion;* ***+1:*** *Very few;* ***+2:*** *More numerous;* ***+3:*** *Large amount;* ***+4:*** *Blood; Means with different letters are significantly different (P<0.05)*

groups treated with 200 and 100 mg/kg of body weight. Lesion score of groups treated with GGE at the dose rate of 300 mg/kg of body weight, Toltrazuril and Vitamin E treated groups was comparable *(Table 4)*.

The PCV percentages, RBCs and WBCs counts in all treated groups were significantly higher (P<0.05) than infected un-medicated control group. Among GGE treated groups, maximum hematological values were recorded in chickens treated with GGE at 300 mg/kg of body weight followed in decreasing order by groups treated with 200 and 100 mg/kg of body weight. The PCV percentages, RBCs and WBCs counts of groups treated with GGE at the dose rate of 300 mg/kg of body weight, Toltrazuril and Vitamin E treated groups were comparable *(Table 5)*.

These serum enzyme (ALT, LDH, Urea and Creatinine) values in all treated groups were minimum (P<0.05) compared to infected un-medicated control group. Among GGE treated groups, minimum serum enzyme values were recorded in chickens treated with GGE at 300 mg/kg of body weight followed in increasing order by groups

Table 5. Hematological Values in different treatment groups

Treated Groups	PCV%	Hb g/dL	RBC10^6/µL	WBC 10^3/µL
GGE 100 mg/kg	22.61±1.68^b	11.99±1.06^b	2.99±0.76^b	19.82±2.84^c
GGE 200 mg/kg	24.11±1.41^b	12.30±1.44^b	3.13±0.86^b	22.51±2.61^{bc}
GGE 300 mg/kg	25.50±1.76^a	13.22±1.28^a	3.96±0.71^a	23.56±1.53^b
Vitamin E, 87 mg/kg	26.00±2.21^a	12.10±0.73^a	4.10±0.71^a	24.89±2.78^b
Toltrazuril,1 mL/L	27.12±2.23^a	12.88±0.64^a	4.28±0.71^a	25.51±2.79^b
Infected Group	19.15±1.15^b	9.45±0.82^c	1.95±0.21^c	15.00±5.04^d
Normal Group	29.32±1.03^a	13.39±1.23^a	4.89±0.58^a	25.51±3.26^a

***GGE:** Glycyrrhiza glabra extract; **PCV:** Packed cell volume; **Hb:** Hemoglobin level; **RCB:** Red Blood Cell; **WBC:** White Blood Cell; Means with different letters are significantly different (P<0.05)*

Table 6. Serum Enzyme Values in different treatment groups

Treated Groups	ALT	LDH	Urea	Creatinine
GGE 100 mg/kg	12.91±0.92^a	549.01±17.82^b	12.90±0.81^b	0.33±0.04^b
GGE 200 mg/kg	11.88±0.97^b	519.93±22.43^b	11.99±0.95^b	0.21±0.03^b
GGE 300 mg/kg	11.70±1.27^b	493.21±21.12^b	8.32±1.02^b	0.15±0.03^c
Vitamin E, 87 mg/kg	10.90±1.27^b	512.23±20.22^b	9.20±1.02^c	0.17±0.03^c
Toltrazuril, 1 mL/L	11.21±1.13^b	495.43±20.13^b	7.30±0.48^c	0.15±0.03^c
Infected Group	24.72±2.32^a	865.94±23.16^a	20.18±1.12^a	0.45±0.05^a
Normal Group	8.90±1.86^c	475.43±16.67^c	5.29±0.48^c	0.11±0.02^d

***GGE:** Glycyrrhiza glabra extract; **ALT:** Alanine transaminase; **LDH:** Lactate dehydrogenase; Means with different letters are significantly different (P<0.05)*

treated with 200 and 100 mg/kg of body weight. Serum enzyme values of groups treated with GGE at the dose rate of 300 mg/kg of bodyweight, Toltrazuril and Vitamin E treated groups were significantly comparable *(Table 6)*.

Discussion

Botanicals high in antioxidant chemicals such as phenols, flavonoids, tannins, and saponins are being employed as an alternate technique to treat coccidiosis [25-27]. The current study found that GGE has anticoccidial potential in terms of improved feed conversion ratio, reduced oocyst, lesion scores, and fecal score in a dose-dependent manner. The Toltrazuril and Vitamin E treated groups had similar results (P>0.05) on these metrics. Previous studies evaluating the anticoccidial potential of several plant extracts have also revealed similar dose-dependent results [34-36].

The beneficial effect of GGE on these parameters could be attributed to the antioxidant chemicals found in this plant, which may help to alleviate the oxidative stress caused by coccidiosis. It is also suggested that supplementation of herbs mixture at the dose 2 mL/L to the coccidiosis challenged broiler chickens overall improved the health and immunity by regulating the mRNA expression of immunity-related toll-like [37]. Furthermore, plant extract inhibited the development of the *Eimeria* life cycle in the host cell before oocysts were discharged in chicken faces, resulting in lower *Eimeria* oocyst excretion and infection severity [38].

Glycyrrhiza glabra is a famous plant with peeled or unpeeled roots and stolon that is generally known as licorice (English) and mulethi (Hindi) is enriched with olatile oil, amino acids, amines (glucose and sucrose 5-15 percent sugars), starch, saponins and flavonoids, tannins. It has a long history of use as a demulcent, expectorant, anti-allergy, anti-inflammatory, spasmolytic, moderate laxative, anti-stress, anti-depressive, antiulcer, liver protector, estrogenic, emmenagogue, and antidiabetic. Bronchitis, dry cough, respiratory infections, catarrh, tuberculosis, genitourinary illnesses, urinary tract infections, abdominal pain, gastric and duodenal ulcers, stomach inflammation, and mouth ulcers are all treated with it. Licorice has been shown in recent investigations to have steroid-like properties. Eczema, peptic ulcers, duodenal and stomach ulcers, and dental plaque have all been found to benefit from it [39]. The ancient civilizations of India, Rome, Greece, Egypt, and China all used this plant in their pharmacopoeia. Glycyrrhizin, glabranin A&B, glycyrrhetol, glabrolide, isoglabrolide, glabridin, formononetin, glabrone, neoliquiritin, hispaglabridin A&B, herianin, umbelliferone, onocerin, p-amyrin, stigmasterol are the primary bioactive elements of *G. glabra* [40]. The substance "glycyrrhizin," produced from *Glycyrrhiza glabra*, and its derivatives have anti-oxidant and anti-inflammatory properties. CD4+ T-cell

and tumour necrosis factor-mediated cytotoxicity are both inhibited by glycyrrhizin. Glycyrrhizin acts as a membrane stabilizer [41].

All results point to the possibility of testing its immunomodulatory and anticoccidial properties. The anticoccidial and liver protective properties of a methanolic extract of *Azadirachta indica* and *Carica papaya* leaf extract against *Eimeria* infection in mice and chicken were investigated in another study. Infected mice and chicken were given a methanolic extract showed an anticoccidial effect and stabilized blood enzyme values at different dose rates (ALT, AST), indicating hepatoprotective properties [42]. Infected chickens were given aqueous extracts of *A. indica* and *Khaya senegalensis* at 400 mg/kg, which had a favorable effect on blood enzyme levels [24]. All of this research imply that plant-derived extracts may be less hazardous when used to treat coccidiosis in chickens.

The anticoccidial effects of *G. glabra* aqueous methanolic extract in broiler chickens were discovered in this study. *G. glabra* showed anticoccidial effectiveness against coccidiosis in a dose-dependent manner. It suggests that extracts obtained from *G. glabra* may be less harmful in managing coccidiosis in chickens. Characterizing the active components of *G. glabra* that are involved in increasing the anticoccidial potential against avian coccidiosis will require more research.

Availability of Data and Materials

Research and Supporting data will be available from the author (Kashif Hussain) on request.

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Competing Interest

The author declared that there is no conflict of interest.

Ethical Statement

This research was conducted with approval from ethical committee of University of Agriculture Faisalabad (Approval No: 144) Faisalabad, Pakistan.

Author Contributions

KH and AFA conceived and designed the experiments; AA and RZA analyzed the data and drafted the manuscript; AR and WZ performed experiments and acquired data. TUR and SM search the data and help in *Eimeria* identification. All authors read and approved the final manuscript.

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Research Article

Clinical and Radiological Evaluation of Tie-in Osteosynthesis with Intramedullary Threaded Pin in Diaphyseal Humeral, Tibial, and Femoral Fractures in Dogs

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Abstract: This study aimed to perform the "tie-in" osteosynthesis technique using an intramedullary threaded pin for the treatment of diaphyseal humeral, tibial, and femoral fractures in dogs, thus minimizing rotational movements and strengthening stabilization, particularly in distal diaphyseal fractures. The study included 16 fracture cases involving 14 dogs of various breeds and sexes, aged 3-12 months, and diagnosed with diaphyseal humeral, tibial, and femoral fractures. Depending on the size of the case, Ø2-4 mm-threaded Steinmann pins were preferred for use in intramedullary pinning and Ø2-4 mm-threadless Steinmann pins were placed transversal for fixation. Acrylic, rod, or fiberglass plaster was used to attach the inserted pins. Fusion was formed in all except two cases. Consequently, the animals could use their relevant extremities without issues during the first 3 days following the operation, but from day 3 until day 15, the animals were reluctant to use their relevant extremities. After day 15, the animals could use their extremities without difficulty. The external fixator components used in fracture fixation were entirely removed after 5-7 (mean 6) weeks. In the functional evaluation, the conditions were very good in nine cases, good in four, moderate in one, and poor in two. The results reveal that the use of threaded pins in intramedullary pinning, the first step of the tie-in method, provides good stabilization in fracture treatment, especially in distal diaphyseal fractures.

Keywords: *Diaphyseal fracture, Dog, Femur, Humerus, Tibia, Tie-in osteosynthesis*

Köpeklerde Diyafizer Humerus, Tibia ve Femur Kırıklarında İntramedüller Yivli Pin İle Tie-in Osteosentezin Klinik ve Radyolojik Olarak Değerlendirilmesi

Öz: Bu çalışma ile köpeklerde diyafizer humerus, tibia ve femur kırıklarının sağaltımı için "tie-in" osteosentez tekniğinin intramedüller yivli pin ile gerçekleştirilmesi, bu sayede rotasyonel hareketlerin minimuma indirilmesi, özellikle distal diyafizer kırıklarda stabilizasyonun daha güçlü hale getirilmesi amaçlanmıştır. Diyafizer humerus, tibia ve femur kırığı tanısı konulan, yaşları 3 aylık ile 12 aylık arasında değişen farklı ırk ve cinsiyete sahip 14 köpeğe ait 16 kırık olgusu çalışmaya dahil edildi. Fiksasyonda olgunun büyüklüğüne göre intramedüller pinleme için Ø2-4 mm yivli Steinmann pinler ile transversal olarak yerleştirilecek olan Ø2-4 mm yivsiz Steinmann pinler tercih edildi. Yerleştirilen pinleri birleştirmek için akrilik, rot veya fiberglas alçı kullanıldı. Olguların 2'si hariç tüm olgularda kaynama şekillendi. Olguların tamamında operasyon sonrası ilk 3 gün ilgili ekstremitelerini kullanmada herhangi bir problem olmadığı fakat, 3. günden sonra 15. güne kadar hastaların ilgili ekstremitelerini kullanmada isteksiz olduğu, 15. günden sonra ise hayvanların ekstremitelerini sorunsuz olarak kullandıkları öğrenildi. Kırık fiksasyonunda kullanılan eksternal fiksatör bileşenleri 5-7 (ort. 6) hafta sonra tamamen uzaklaştırıldı. Fonksiyonel değerlendirmede olguların 9'unda çok iyi, 4'ünde iyi, 1'inde orta ve 2'sinde ise zayıf olarak değerlendirildi. Sonuç olarak, kırık sağaltımında iyi bir stabilizasyon sağlayan tie-in yönteminin ilk aşaması olan intramedüller pinlemede yivli pin kullanımının stabilizasyonu güçlendirdiği ve özellikle distal diyafizer kırıklarda iyi bir stabilizasyon sağladığı ortaya konulmuştur.

Anahtar sözcükler: *Diyafizer kırık, Köpek, Femur, Humerus, Tibia, Tie-in osteosentez*

Introduction

Although humeral fractures in dogs are uncommon [1,2], tibial and femoral fractures are frequently encountered [3,4]. Fractures resulting from various traumas occur most frequently following traffic accidents [3]. Implants such as intramedullary pins, bone plates, screws, external fixators, and cerclage wire are widely used to treat long-bone

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fractures [4,5]. Fracture treatment may vary according to factors such as the animals' characteristics, fracture type, lesions associated with the fracture, operator ability, and cost. Thus, the advantages of the chosen technique should be maximized and the disadvantages kept at a minimum [4,6].

Intramedullary pining is the fixation technique most often utilized for treating long-bone fractures. While it is a simple and practical technique, it does have some disadvantages, including pin migration, infection, and an inability to resist the bone's rotational strength. Thus, combining a unilateral external fixator with an intramedullary pin is a widely employed procedure in preventing these complications [7,8].

The "tie-in" technique, which allows the early use of the extremity with good stabilization, is an easy-to-apply minimally invasive technique that causes minimal damage to growth plates and endosteal vascularization owing to the use of intramedullary small diameter pins and is compatible with bone growth and is an easy-to-apply minimally invasive technique that causes minimal damage to growth plates and endosteal vascularization [9]. However, drawbacks have been observed, such as pin migration, rotational movement of bone fragments following intramedullary pinning, which is the first stage of the procedure, and appearance of serosanguineous discharge in the proximal part of the pin as a result of these movements [2,3,9-13].

This study aimed to use an intramedullary threaded pin to conduct the "tie-in" osteosynthesis approach for the treatment of diaphyseal humeral, tibial, and femoral fractures in dogs, reducing rotational motions and improving stabilization, especially in distal diaphyseal fractures.

Material and Methods

Ethical Approval

This study obtained approval from the Animal Experiments Local Ethics Committee of Kafkas University (Approval number: KAÜ-HADYEK/2021-168). In addition, an "informed consent form" was obtained from the owner of each animal.

Case Selection

The study included 16 fracture cases involving 14 dogs of various breeds and sexes, aged 3-12 months, who were brought to the Animal Hospital of the Faculty of Veterinary Medicine of Kafkas University, with the complaint of lameness because of a traffic accident or other traumas and diagnosed with fractures in the diaphyseal humerus, tibia, and femur *(Table 1)*.

Animals that presented with complaints of lameness because of traffic accidents or other traumas were subjected to systematic general examinations. Animals with acute trauma were assessed for bleeding and diaphragm rupture. The fracture was then inspected and palpated. For a definitive diagnosis, mediolateral and craniocaudal radiographs of the affected extremity of each dog were taken. After determining the fracture's anatomical location and shape, it was prepared for operation.

Surgical Equipment

Depending on the size of the case, Ø2-4 mm-threaded Steinmann pins (Safir®, Antalya/Turkey) were preferred for use in intramedullary pinning, and Ø2-4 mm non-threaded Steinmann pins (Safir®, Antalya/Turkey) were placed transversely for fixation. Acrylic, rod, or fiberglass plaster (Optima Cast®, Coin Enterprise Co. Ltd, ABD) was used to attach the inserted pins.

Surgical Procedure

Cefazolin, 30 mg/kg, IM (Cezol, Deva®, Istanbul), was administered to the animals 0.5-1 h before surgery. Following the shaving and cleaning of the relevant extremities, the area was prepared for aseptic surgery. Following sedation with 0.2 mg/kg xylazine HCl (Rompun® 2%, Bayer, Istanbul) intramuscularly and induction with 5 mg/kg ketamine HCl (Ketakontrol®, Doğa İlaç, Istanbul) intravenously, the procedure was performed under inhalation anesthesia with a 2% concentration of isoflurane (Forane, AbbVie®, Istanbul).

To access the fracture site, the lateral approach to the femur, craniolateral approach to the humerus, and medial approach to the tibia were preferred. Following the exposure of the fracture fragments, a threaded Steinman pin with a diameter of 2-4 mm and a length of 25-30 cm was introduced retrogradely into the intramedullary cavity, not exceeding 40%-50% of the bone diameter. After the anatomical bone alignment, 1 or 2 Steinman pins in diameters suitable for the proximal and distal fracture fragments were placed unilaterally (type I) enough to pass the opposing cortex and perpendicular to the long axis of the bone, taking into account the shape of the fracture, anatomical location, and animal's weight. Control radiographs were taken thereafter, and the operation opening was closed using the standard manner. The exterior pin ends were connected with acrylic, rod systems, or fiberglass plaster, considering the weight of the dogs. The pin tips were secured with the chosen fixation material, and the process was completed by securing the pin's root with 10% povidone-iodine *(Fig. 1)*.

Radioulnar fractures were found in two animals with femoral fractures. Apart from the tie-in configuration technique, various fixation methods (plate osteosynthesis) were used to treat the radioulnar fractures.

Table 1. *Preoperative and postoperative results of 14 patients which underwent intramedullary threaded pin tie-in configuration*

Case No	Signalment	Description of the Fracture	Configuration	Concomitant Injury /Treatment	Complications	Functional Outcome
1	5-months-old, Male, Mix Breed, 8 kg	Diaphyseal, oblique, femoral fracture	1 IM threaded pin (2 mm), 1 (PF) (2 mm), 1 (DF) (2 mm)	None	None	Excellent
2	3.5-months-old, Male, Mix breed, 6 kg	Diaphyseal, oblique, humeral fracture	1 IM threaded pin (2 mm), 1 (PF) (2 mm), 1 (DF) (2 mm)	Radial paralysis/Recovered after osteocentesis	None	Excellent
3	3-months-old, Female, Mix breed, 8 kg	Diaphyseal, transversal, bilateral, femoral fracture	1 IM threaded pin (2 mm), 2 (PF) (2 mm), 1 (DF) (2 mm)	None	None	Excellent/Good
4	4.5-months-old, Male, Mix breed, 7 kg	Diaphyseal, transversal, femoral fracture	1 IM threaded pin (2 mm), 1 (PF) (2 mm), 1 (DF) (2 mm)	None	None	Excellent
5	12-months-old, Male, Mix breed, 25 kg	Diaphyseal, oblique, femoral fracture	1 IM threaded pin (3 mm), 2 (PF) (3 mm), 2 (DF) (3 mm)	None	Transversal pin migration	Poor
6	3-months-old, Male, Mix breed, 6 kg	Diaphyseal, oblique, femoral fracture	1 IM threaded pin (3 mm), 2 (PF) (2 mm), 2 (DF) (2 mm)	None	None	Excellent
7	5-months-old, Male, Mix breed, 16 kg	Diaphyseal, transversal, tibial fracture	1 IM threaded pin (3 mm), 1 (PF) (3 mm), 2 (DF) (3 mm)	Radius-Ulna Fracture/ Plate osteosynthesis	Hypertrophic callus	Fair
8	6-months-old, Male, Turkish shepherd dog, 20 kg	Diaphyseal, transversal, femoral fracture	1 IM threaded pin (4 mm), 2 (PF) (3 mm), 2 (DF) (3 mm)	Radius-Ulna Fracture/ Plate osteosynthesis	None	Good
9	8.5-months-old, Male, Mix breed, 15 kg	Distal diaphyseal, transversal, tibial fracture	1 IM threaded pin (3 mm), 2 (PF) (2 mm), 1 (DF) (3 mm)	None	None	Excellent
10	5-months-old, Male, Zerdava,12 kg	Diaphyseal, oblique, humeral fracture	1 IM threaded pin (3 mm), 1 (PF) (3 mm), 2 (DF) (2 mm)	None	None	Excellent
11	5.5-months-old, Female, Kangal dog,24 kg	Diaphyseal, segmental, femoral fracture	1 IM threaded pin (4 mm), 2 (PF) (4 mm), 2 (DF) (3 mm)	None	Transversal pin migration	Poor
12	9-months-old, Female, Kangal dog, 33 kg	Diaphyseal, oblique, tibial fracture	1 IM threaded pin (4 mm), 2 (PF) (4 mm), 2 (DF) (4 mm)	None	None	Good
13	8-months-old, Male, Mix Breed, 32 kg	Diaphyseal, oblique, humeral fracture	1 IM threaded pin (4 mm), 2 (PF) (4 mm), 2 (DF) (3 mm)	None	None	Excellent
14	11-months-old, Female, Kangal dog, 34 kg	Diaphyseal, oblique, bilateral femoral fracture	Right femur: 1 IM threaded pin (4 mm), 2 (PF) (3 mm), 1 (DF) (3 mm), Left femur: 1 IM threaded pin (4 mm), 1 (PF) (3 mm), 2 (DF) (3 mm)	None	None	Excellent/Good

PF: *Proksimal fragment,* ***DF:*** *Distal fragment,* ***IM:*** *Intramedullary*

Fig 1. Fixation of postoperative "tie-in" external fixator components

Postoperative Care and Follow-up of Cases

Animals were admitted to the hospital for 48-72 h and received daily antibiotic treatment, pain management, and postoperative care. The animals' owners were informed about keeping the animals in a narrow area before discharge and daily cleaning of the pin bottoms with povidone-iodine (10%). In addition, antibiotics (cefazolin, 30 mg/kg, IM, Deva, Istanbul) were administered for 7 days and meloxicam (0.2 mg/kg/day, SC, Bavet Meloxicam, Bavet®, Istanbul) for 3 days for pain control. Clinical and radiological evaluations were made for control at intervals of 2 or 3 weeks postoperatively.

Taking into mind the healing tables, the pins were totally removed after 5-7 (average 6) weeks.

According to Yardımcı et al.[3], recovery was graded based on relative measurements such as the animal's readiness to use the operated limb, degree of weight-bearing, and presence of resistance to flexion/extension of the shoulder, elbow, hip, and knee joints. Accordingly, the findings were marked as excellent (no visible lameness, full weight bearing, functional use of the operated limb, and no

palpable pain), good (obvious full weight bearing, no obvious lameness during gait but mild lameness following strenuous exercise, and no palpable pain), moderate (marked lameness but consistent weight bearing, and apparent resistance to flexion and extension), and weak (no limb use, non-weight-bearing lameness, resistance, and pain in flexion and extension). The last clinical evaluation was conducted for control within 1-2 months after pin removal. All evaluations were completed by an academic who was not a member of the research team.

Results

In this study, the dogs were 3-12 months old (6.36±2.89 months). Femoral fractures were found in eight of the animals (unilateral in 6 cases and bilateral in 2), humeral fractures in 3, and tibial fractures in 3 *(Table 1)*. Acrylic plaster for dogs weighing <20 kg (n = 8), rod system for dogs weighing 20-30 kg (n = 3), and fiberglass plaster for dogs weighing >30 kg (n = 3) gave sufficient stabilization in pin fixation to join the outside pin ends, without any complications. In only one case, the owner reported that the animal broke the acrylic on postoperative day 2 (case 4). The animal was immediately brought to the clinic, and the fixation procedure was renewed with fiberglass plaster. In all cases, the use of the relevant extremities was not difficult in the first 3 days after the operation. However, from days 3-15, the animals were cautious to use their respective extremities when pressing the ground, either reluctantly or not at all, and after day 15, the animals used their extremities without any difficulty. The recovery of animals with humeral fractures (n = 3) was uneventful and without any complications. One of these animals (case 2) had radial paralysis in addition to the fracture in clinical examination. In this case, the paralysis resolved spontaneously during the fracture healing process, and the animal exhibited a normal gait. It was learned from the owner that no clinical problem occurred in this case until day 15, while 2 of 3 cases with tibial fractures were fully healed. However, when X-ray images were taken on days 21 and 35, a hypertrophic callus had formed on the fracture line *(Fig. 2)*. When the owner was asked whether they followed the recommendations for confinement, they permitted the animal to move freely because it has no walking difficulties. Despite the formation of overflow callus, the animal could use the relevant extremity with ease. Moreover, 6 of the 8 dogs with femoral fractures (6 unilateral and 2 bilateral fractures) had an uncomplicated recovery, while in two dogs (cases 5 and 11,) a complete recovery was not achieved because the owners were following the confinement recommendations, which results in the protrusion of the transversal pins. The external fixator was removed, and stabilization was accomplished with the plate in these two dogs that could not fully heal.

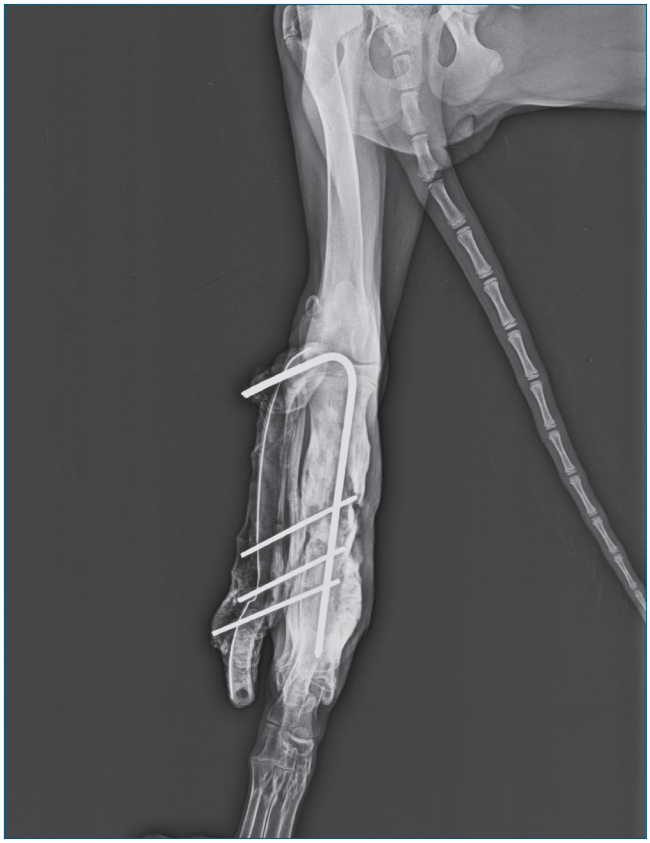

Fig 2. Radiograph on postoperative day 35 of case 7. Hypertrophic callus formed in the tibia

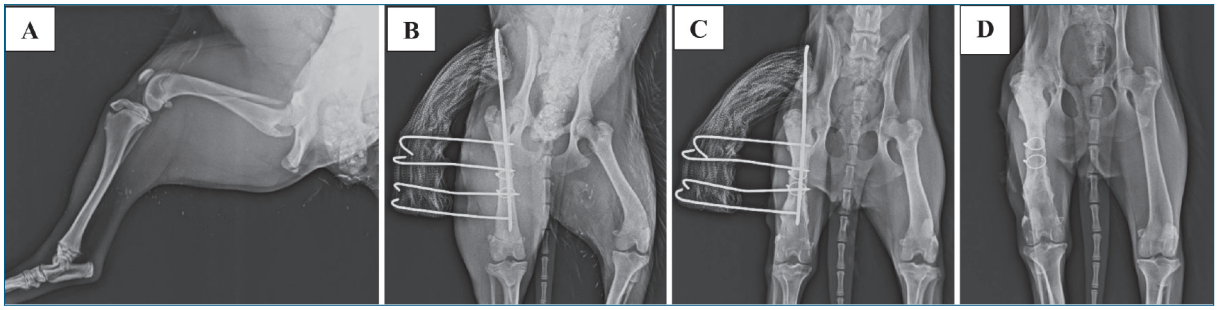

Fig 3. A- Preoperative radiograph of a diaphyseal long oblique femoral fracture, **B**- Immediate postoperative radiograph, **C**- Radiograph on postoperative day 35, **D**- Radiograph on postoperative day 40

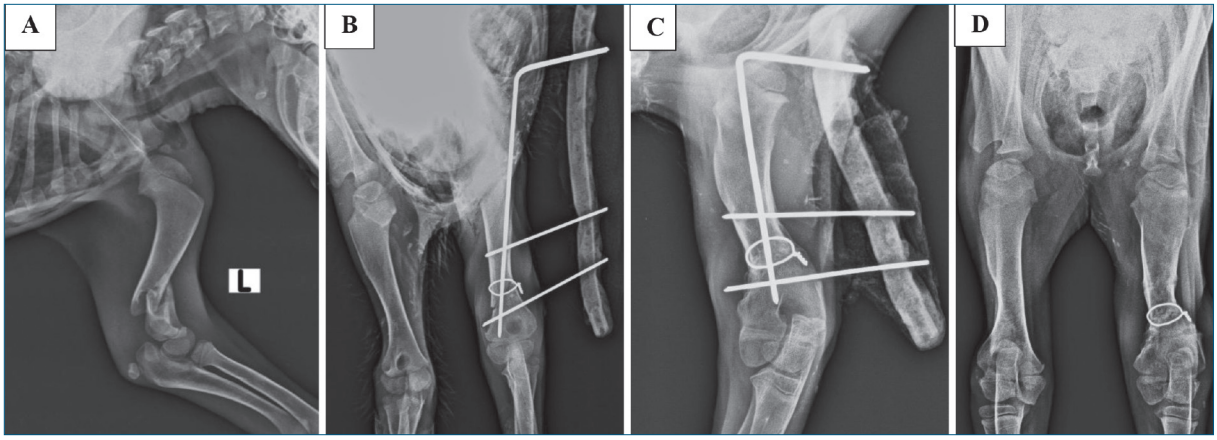

Fig 4. A- Preoperative radiograph of a case of distal diaphyseal short oblique humeral fracture, **B**- Immediate postoperative radiograph, **C**- Radiograph on postoperative day 35, **D**- Radiograph on day 35 after the removal of the postoperative tie-in components

Table 1 summarizes the clinical findings and recovery status of the cases.

In many cases, seroma formation at the entrance of the intramedullary pin was noted to be limited or insignificant. No major pin infection was observed in the transversally placed pins. Except for three cases that developed complications, all other cases healed without issues *(Fig. 3, Fig. 4)*. Following the pin removal, the owners were followed up for 1-2 months to obtain information about the condition of the animals, and they did not have any complaints.

Discussion

Although femoral and tibial fractures are prevalent, especially in dogs in the developmental period [9,13,14], humeral fractures are the least common long-bone fractures [1]. The majority of fractures occur following trauma sustained in traffic accidents. Especially, in animals brought due to a complaint of trauma-related fractures, conditions such as nerve damage, internal bleeding, rupture of the diaphragm, and fractures in more than one bone may occur [1,2]. In this study, the fractures occurred in dogs aged <12 months. This may be related to the fact that animals are in a more active stage of their lives and in the early phases of bone development. Although various traumas effectively result in fractures, fractures in the majority of our cases were formed following traffic accidents. Given the extent to which the body is damaged in motor vehicle accidents, lesion formation in more than one bone is possible. While bilateral femoral fractures were observed in two of the cases, one involved an ipsilateral radioulnar fracture in tandem with a tibial fracture and another involved a femoral fracture. Radial paralysis was observed in the clinical examination of only one animal with a humeral fracture, but no signs of paralysis were found following the operation. One could argue that paralysis occurs as a result of the pressure exerted by fracture fragments, which subsides with fracture fixation.

Many fixation methods are used in the treatment of humeral, femoral, and tibial fractures, including Thomas splint, bandage, intramedullary pin, plate osteosynthesis, and external fixator components [2,15-18]. However, each of these strategies has several advantages and disadvantages over one another. Because closed reduction is not possible due to the strong muscles around the femur and humerus, closed reduction (bandage, etc.) is not very suitable for fractures of these bones [1,3]. Intramedullary pining is the most commonly used fixation technique for the treatment of long-bone fractures. Although it is a simple and practical technique, it has many disadvantages, such as pin migration, infection, and inability to resist the rotational force of the bone [7,8]. To avoid these problems, fixation with plates is more preferred today [1,19]. However, one of the disadvantages of bone plates is that they must be removed by an invasive surgical operation [4,19]. Although external fixator systems (semicircular-circular) provide adequate stabilization in fracture treatment, the unsuitable anatomical structure of the relevant regions prevents their use in fractures of these bones, especially in the approach to humeral and femoral fractures. As a result, a unilateral (type I) exoskeleton fixator is an easy approach to the fracture, minimally invasive, easily intervenable in emergencies, does not require an operation to remove it from the implanted bone after healing, can resist rotational forces, and is an intramedullary pin [3,7,9,13]. After weighing the advantages and disadvantages of each fixation method, our study determined that the tie-in method, which has some advantages in humeral, femoral, and tibial fractures, was preferred. In our study, a threaded pin was used in intramedullary pinning, the first step of the tie-in method; thus, the fragment rotation that may result from the pin was neutralized.

Intramedullary and externally implanted pins are commonly

combined using acrylic or metallic rod and a rod system consisting of connection clips when employing the tie-in configuration technique [4,8,20]. However, clip loosening, shape of the intramedullary pin, and migration have been reported as problems resulting in the loss of stability [4,12,20]. In our investigation, fiberglass plaster was employed to combine intramedullary and externally implanted pins in addition to acrylic and rod systems. One of the animals shattered the acrylic, and no further complications were found. The animal has a nervous temperament, and stress factors have a role in this situation. Although a few issues were associated with the fixation apparatus, the most appropriate equipment should be chosen based on the animal's movement, temperament, and weight during the joining process. According to our experience, fiberglass plaster provides good stabilization among the materials used for joining pins because of its low cost and mechanical strength that can withstand excessive stress, especially in large animals. On the contrary, acrylic is a better choice for small animals, as a lighter material.

In fracture fixation, the tie-in method has several advantages over alternative methods. The interlocking of the connection apparatus and pins is the most essential characteristic of this system, as it prevents the loosening and migration of the transversal and intramedullary pins [9]. However, difficulties have been documented in several investigations, particularly in intramedullary pin migration and discharge at the proximal ends of the intramedullary pin [2-4,12,21]. In this investigation, no loosening, pin migration, or a significant infection that could be precipitated by these issues were observed in any of the cases because the intramedullary pin is threaded, unlike the traditional tie-in procedure. However, in many animals, this investigation showed limited or insignificant seroma production at the pin insertion site, as reported by all investigators. Furthermore, the intramedullary threaded pins used have screw quality and provide greater stabilization in distal diaphyseal or proximal diaphyseal fractures. As the diameter of the intramedullary pin in the tie-in method should not be more than 40%-50% of the bone diameter, it is reasonable to conclude that the use of threaded pins will eliminate many issues and contribute significantly to the advancement of the tie-in system.

The primary purpose of fracture fixation is to promote rapid bone healing and early postoperative ambulation to encourage the animals to use functional extremities [2]. The animals did not have any difficulty using the relevant extremities in the first 3 days after the surgeries are performed using the tie-in technique, and they could easily perform their usual activities [2,3]. In our study, the animals could use their respective extremities within the first 3 days after surgery. However, after day 3, some of the animals were not using the relevant extremity and were hesitant to bear weight. After postoperative day 15, they were able to use their extremities without difficulty. During this time, the animals were monitored to observe the occurrence of muscular function loss, and no complications were found. Because of the medicines given for the first 3 days, we can claim that the animals could utilize their extremities with ease. Furthermore, in this scenario, pain stimulation by the influence of numerous mediators in the biological process in the early phases of soft tissue and fracture repair is also effective.

After appropriate stabilization, careful care (particularly at the root of the pin) and feeding, and restriction of motions, fracture healing is usually completed within 28-35 days at the earliest. However, animal size, fractured bone, fracture shape, and other factors can influence the duration of fracture healing [4,13]. According to McCartney et al.[13], the apparatus should be removed between 6 and 8 weeks in animals aged <1 year and between 8 and 10 weeks in animals aged >1 year. Popkov et al.[5] used Ilizarov ESF and intramedullary pins in dogs with experimental fractures and closed growth plates aged 1-5 years, and they found that on postoperative day 28, all fractures were entirely consolidated. Excessive mobility and restriction recommendations in young animals during the recovery phase can result in complications such as transversal pin migration, overflow callus, and late union [2,4]. In our study, the connecting devices were removed after an average of 5-7 (mean 6) weeks. In the cases, no evidence of substantial pin-to-bottom infection occurred. One animal with a tibial fracture had hypertrophic callus, while two animals with femoral fractures experienced transversal pin migration. Excessive straining of the muscles in the fracture area during movement produces aberrant callus production, which is a well-known condition in dogs. According to the experts, the inability to follow the motion restriction rules and excessive activity are the key factors in these cases.

Many treatment options are available for long-bone fractures in dogs. However, it may not be always clear as to which option the operator will implement. Factors such as the anatomical location of the fractured bone, fracture shape, animal weight, physician skill, and cost are effective in determining the appropriate technique. Therefore, obviously, the low-cost minimally invasive tie-in configuration technique, which is an easy approach to fractures, can be adapted to any fracture shape, puts equal load on the fracture line, prevents rotation, and is extremely useful.

In this study on the fixation of long-bone fractures in dogs, the tie-in configuration technique using intramedullary threaded pins had a lower complication rate and clinically satisfactory results.

Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author (U. Aydın) on reasonable request.

Ethical Approval

This study obtained approval from the Animal Experiments Local Ethics Committee of Kafkas University (Approval number: KAÜ-HADYEK/2021-168). In addition, an "informed consent form" was obtained from the owner of each animal.

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Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

Author Contributions

UA, İÖ, and ÖA conceived and supervised the study. UA, İÖ, ÖA, CŞE, EK, UY, and ET collected and analyzed data. UA, UY, and ET performed the operation and radiographic examinations. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

Rapid Visual Detection of *Streptococcus suis* and *Actinobacillus pleuropneumoniae* Through Duplex Recombinase Polymerase Amplification Combined with Lateral Flow Dipsticks

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Abstract: Primers and corresponding probes were designed for the glutamate dehydrogenase *(gdh)* gene of *Streptococcus suis* and the *Apx*IV gene of *Actinobacillus pleuropneumoniae* to establish a dual recombinant enzyme polymerase amplification (RPA)-lateral flow dipstick (LFD) detection method for the simultaneous rapid identification of *S. suis* and *A. pleuropneumoniae.* The specificity test showed that the amplification results for other pathogens were all negative, indicating that the method exhibited good specificity. The sensitivity test showed that the lowest nucleic acid concentration detectable with this method was 10^{-5} ng/μL, which was significantly higher than that observed with PCR and basic RPA. The results showed that this method detected all reference strains and clinical isolates, which was consistent with the PCR detection results. Among the 45 clinical samples, 19 cases of *S. suis,* 1 case of *A. pleuropneumoniae* and no mixed infections were detected. The detection rate was higher than that observed with bacterial isolation and the conventional PCR method, which indicated that this method is very practical and suitable for the rapid clinical detection of *S. suis* and *A. pleuropneumoniae.* Compared with the traditional method, the dual RPA-LFD method has several advantages, including high specificity, high sensitivity, fast speed and minimal requirement of instruments and equipment. In addition, the method can achieve the synchronous and rapid detection of *S. suis* and *A. pleuropneumoniae* and is helpful for the preliminary screening of clinical diseases.

Keywords: *Streptococcus suis, Actinobacillus pleuropneumoniae, Recombinase polymerase amplification, Lateral flow dipstick, Rapid detection*

Streptococcus suis ve *Actinobacillus pleuropneumoniae*'nin Lateral Flow Dipstick İle Kombine Edilmiş Dubleks Rekombinaz Polimeraz Amplifikasyonu Yoluyla Hızlı Görsel Tespiti

Öz: Primerler ve karşılık gelen problar, *Streptococcus suis* ve *Actinobacillus pleuropneumoniae*'nin eş zamanlı hızlı identifikasyonu sağlayan bir dual rekombinant enzim polimeraz amplifikasyon (RPA)-lateral flow dipstick (LFD) teşhis metodunun geliştirilmesi için ilgili bakterilerin sırasıyla glutamat dehidrojenaz *(gdh)* geni ve ApxIV geni için tasarlanmıştır. Yapılan özgüllük testinde, diğer patojenler için amplifikasyon sonuçlarının hepsinin negatif saptanması bu yöntemin iyi bir özgüllük sergilediğini gösterdi. Duyarlılık testi, bu yöntemle saptanabilen en düşük nükleik asit konsantrasyonunun10-5 ng/μL olduğunu gösterdi ve bu değer, PCR ve temel RPA ile saptananlardan önemli ölçüde daha yüksekti. Sonuçlar, bu yöntemin, PCR ile saptananlarla tutarlı olarak tüm referans suşları ve klinik izolatları belirlediğini gösterdi. 45 klinik örnek arasından 19'u *S. suis* ve 1'i *A. pleuropneumoniae* olarak saptandı ve miks enfeksiyon tespit edilmedi. Tespit oranı, bakteri izolasyonu ve geleneksel PCR yöntemine göre daha yüksekti, bu da bu yöntemin oldukça pratik olduğunu ve *S. suis* ve *A. pleuropneumoniae*'nin hızlı klinik tespiti için uygun olduğunu gösterdi. Geleneksel yöntemle karşılaştırıldığında, dual RPA-LFD yönteminin yüksek özgüllük, yüksek hassasiyet, hız ve minimum alet ve ekipman gereksinimi gibi birçok avantajı vardır. Ayrıca bu yöntem, *S. suis* ve *A. pleuropneumoniae*'nin eşzamanlı ve hızlı tanısını sağlayabilecek ve klinik enfeksiyonların ön taramasına yardımcı olabilecek niteliktedir.

Anahtar sözcükler: *Streptococcus suis, Actinobacillus pleuropneumoniae, Recombinase polymerase amplification, Lateral flow dipstick, Rapid detection*

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Introduction

Streptococcus suis is one of the main pathogens that causes significant economic losses in the swine industry and is present in almost 100% of large-scale pig farms worldwide [1]. The main route of infection in pigs is considered the respiratory tract, and the resulting infection mainly causes sepsis, meningitis, arthritis, pneumonia, endocarditis, and even sudden death in severe cases [1-3]. SS serotype 2 (SS2), the most virulent serotype, is the most commonly isolated in human infection cases (74.7% of cases) [1,4]. Furthermore, *S. suis* is a zoonotic pathogen that can infect humans through contact with carrier pigs or pork products and thereby causes septicemia, meningitis, shock-like syndrome, or even death [1]. Over the past 50 years, most *S. suis* infections in humans have involved sporadic cases of occupational contact, such as with farmers, veterinarians, butchers, food processors and other occupational groups [5]. However, the number of cases involving *S. suis* infection in humans has increased in recent years, exhibiting a trend toward the general population. Two large-scale outbreaks of *S. suis* infection, which included a total of 229 infections and 52 deaths, have occurred in Jiangsu Province and Sichuan Province, China; these outbreaks have attracted great attention in the field of public health and scientific research and have seriously threatened public health and safety [5-7].

Actinobacillus pleuropneumoniae infection can cause severe irreversible damage to the lungs [8]. In acute disease outbreaks, morbidity can range from 10% to 100%, and mortality of 1%-10%. The peracute form is characterized by a high mortality rate and sudden death. It can also show subacute and chronic course. *A. pleuropneumoniae* can infect pigs of all ages, and its clinical manifestations are characterized by fibrinous hemorrhagic and necrotizing pleuropneumonia [8]. The bacteria have many serotypes, and there is no cross-protection between serotypes during immunization with inactivated vaccines, which poses a great challenge in the prevention and control of the disease [9]. Currently, the disease is distributed worldwide [8]. *A. pleuropneumoniae* can be detected in all major swine-producing countries, but the virulence of local strains may vary [8].

Respiratory disease is the most important health problem that affects the swine industry worldwide and is commonly known as porcine respiratory disease syndrome (PRDC) [10]. Clinically, *A. pleuropneumoniae* and *S. suis* are common and important pathogens of the respiratory tract in pigs, and their mixed infection triggers PRDC that leads to lung lesions, which in turn results in large economic losses and impaired animal welfare [10,11]. In recent years, the number of cases of mixed infections with *S. suis* and *A. pleuropneumoniae* has gradually increased [10]. These infections are challenging to detect through traditional bacteriological diagnosis; thus, the establishment of a detection method for the rapid diagnosis and identification of these two bacteria is necessary. Such a method is very important for preventing and controlling the spread of the disease in a timely manner. At present, the combination of bacterial isolation and culture and serological typing remains the gold standard for detecting these two pathogens, but this method is time-consuming, demonstrates low sensitivity and has high operational requirements; thus, this method is unfavorable for the promotion of epidemic monitoring [12,13]. In the past two decades, polymerase chain reaction (PCR)-based molecular detection technology has been widely used in the molecular diagnosis of *A. pleuropneumoniae* and *S. suis*. Conventional PCR, PCR-restriction fragment length polymorphism (PCR-RFLP), PCR-sequence characterized amplified region (PCR-SCAR), double PCR, real-time fluorescence quantitative PCR and other technologies are used for the amplification of either conserved genes or the capsular polysaccharide genes specific to different serotypes to achieve the rapid detection and identification of these two pathogens [13-15]. PCR-based molecular technology is an established and reliable method for the isolation and identification of *A. pleuropneumoniae* and *S. suis* but also has various characteristics, including extensive requirements for instruments and equipment, demanding experimental conditions and skilled laboratory personnel.

In recent years, isothermal amplification technology has received increasing attention for the detection of pathogens, and among the related techniques, loop-mediated isothermal amplification (LAMP) is a commonly used method [16]. However, LAMP technology has many problems, including the need for complex primer designs along with multiple pairs of primers; the occurrence of nonspecific amplification, which is due to the ease of interaction between products; and the inability to verify the accuracy of the amplification results by sequencing [17,18]. Therefore, there remains a clinical need for a simple, rapid, and sensitive method that can be used for the early diagnosis of *S. suis* infection. As an isothermal DNA amplification technology, recombinase polymerase amplification (RPA) has undergone rapid development in the field of molecular diagnostics [19]. Due to its simplified instrument requirements and shorter reaction time compared with those of other methods, RPA is considered the most applicable method for on-site diagnosis [19]. A lateral flow dipstick (LFD) is a simple detection device that can be used for the qualitative or semiquantitative detection of target nucleotides and can be used in the field or small regional laboratories without the help of instruments [20]. By labeling with fluorophores, the RPA amplification products can be combined with a LFD (i.e., RPA-LFD) to visually detect amplification products. RPA-

LFD technology has the following characteristics: simple operation, high sensitivity, strong specificity, and suitability for rapid on-site diagnosis [19]. Thus, this technology is suitable for clinical and field detection.

Glutamate dehydrogenase (Gdh), which is a bridge that connects carbon and nitrogen metabolism, plays an important role in the process of bacterial energy metabolism and thereby directly affects the pathogenicity of bacteria [21,22]. The Gdh protein of *S. suis* is expressed at the surface of bacterial cells and is an antigenic, extremely evolutionarily conserved and antigenic component; therefore, Gdh can be used as a diagnostic antigen for *S. suis* to establish a universal detection method for all *S. suis* serovars [22]. *Apx*IV is a species-specific toxin of *A. pleuropneumoniae*, and the *apx*IV sequence is highly conserved among all serotypes and is absent in other species of *Actinobacillus*, *Haemophilus*, and *Pasteurella multocida*; therefore, *apx*IV is often used as a specific clinical diagnostic gene for *A. pleuropneumoniae* [13]. In this study, we designed specific RPA primers targeting the *gdh* gene of *S. suis* and the *apx*IV gene of the *A. pleuropneumoniae* toxin to establish a dual RPA-LFD detection method for the simultaneous detection of *S. suis* and *A. pleuropneumoniae*.

Material and Methods

Strains

Streptococcus. suis serotype 1 (SS1) strain JZLQ036, SS2 strains CVCC606, CVCC1941, JZLQ022, ZY05719, 05ZYH33, and JZLQ019, SS7 strain JZLQ034, SS9 strain JZLQ035, *A. pleuropneumoniae* serotype 1 4074 strain (APP1, CVCC259) and serotype 5b L20 strain (APP5b, CVCC263) were kindly provided by Professor Shen Zhiqiang and Professor Lei Liancheng. Enteropathogenic *Escherichia coli*, *Glaesserella parasuis*, gen. nov., comb. nov., *Pasteurella*, *Salmonella* (ATCC 25922), *Staphylococcus aureus* (ATCC 49525), and *Aeromonas hydrophila* AH-1) were sourced from our Institute. The pMD-18T-gdh plasmid was preserved by our laboratory [23].

Primer Design and Synthesis

The *S. suis gdh* gene sequence (GenBank accession number: AF229683) and *A. pleuropneumoniae Apx*IV gene sequence (GenBank accession number: HM021153) were downloaded from GenBank and compared and analyzed using DNAStar software. Five pairs of *S. suis*-specific RPA primers and five pairs of *A. pleuropneumoniae*-specific RPA primers were designed, and these primers were then synthesized by Sangon Biological Engineering Co., Ltd.

Bacterial Genomic DNA Extraction

The genomic DNA templates of all strains used in the experiment were extracted from overnight cultures of bacteria using a bacterial DNA extraction kit. The concentration of the extracted DNA templates was uniformly diluted to 10 ng/μL for use.

To obtain bacterial DNA from tissues, 1 mL of PBS was added to 2 g of infected pig lung tissue, and a suspension produced by grinding. The suspension was centrifuged at 2000 g/min for 5 min, and a 500-μL aliquot of supernatant was collected and centrifuged at 12000 g/min for 5 min. The supernatant was discarded, and 100 μL of bacterial lysis buffer was added for resuspension. The suspension was maintained at 57°C for 1 h and then at 100°C for 10 min. The suspension was then centrifuged (12000 g/min, 5 min), and the supernatant was stored at -80°C until use for the RPA reaction.

RPA-Basic

For screening of the primers, the RPA reaction was performed using 2 μL of the template and the conditions provided in the instructions for the TwistAmp® Basic kit (TwistDX, UK). The screening ensured that the primer reaction conditions for each *S. suis* or *A. pleuropneumoniae* pair were the same, and templates for positive and negative controls were set for each pair of primers.

After selection of the optimal primer pairs, the RPA reaction was performed using a 50-μL preliminary reaction system according to the instructions for the TwistAmp® Basic kit (TwistDX, UK). The single-plex reaction fraction consisted of 29.5 μL of reaction buffer, 2.4 μL of each of the *S. suis* and *A. pleuropneumoniae* forward and reverse primers, 2 μL of the template (DNA) and 6.4 μL of nuclease-free water. The above mixture was gently mixed, added to the lyophilized RPA reaction tube, shaken and mixed well to rehydrate the solid reactant in the lyophilized state. Afterward, 2.5 μL of 280 mM MgAc was added and incubated at 39°C for 20 min using a thermocycler, and the amplified products were detected by 2% agarose gel electrophoresis. To determine the optimal reaction conditions of dual RPA for RPA-Basic reactions, the RPA primer concentration ratio, temperature and time in the reaction system were optimized. First, the reaction system was used for the screening of different primer volume ratios, and the determined primer ratios were then used for the stepwise screening of different temperatures (30°C, 35°C, 37°C, 39°C, and 45°C) and durations (5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, and 40 min).

The dual RPA-Basic assay was evaluated using genomic DNA of six nontarget bacteria, namely, enteropathogenic *E. coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *S. aureus*, *Pasteurella*, and *A. hydrophila*, to assess the specificity of the RPA-LFD. The template was subjected to 10-fold sequential dilutions to obtain 9 different concentrations (10^{0}-10^{-8} ng/μL). The sensitivity experiment

was performed using the established dual RPA-Basic assay to evaluate the lowest limit of detection of the method.

RPA-LFD

The RPA-nfo amplification reaction was performed according to the conditions provided with the DNA constant-temperature rapid amplification kit (TwistAmp RPA nfo kit; TwistDX), and after the reaction was completed, the results were interpreted using the Milenia GenLine HybriDetect kit. Specifically, 5 μL was added to a centrifuge tube containing 195 μL of HybriDetect Assay Buffer, and after even mixing, the sample end of the colloidal gold test strip was inserted into the centrifuge tube and allowed to reach equilibrium; the result was interpreted within 10 min. The same controlled reaction conditions were used for each pair of *S. suis* or *A. pleuropneumoniae* RPA-nfo reactions, and positive and negative controls were established for each pair of primers.

To improve the sensitivity of RPA-LFD, different primer and probe concentration ratios were evaluated. The reaction components included buffer A, template, primers and probes (the *S. suis* and *A. pleuropneumoniae* forward and reverse primers and probes were changed to obtain a ratio of 1:1:0.3, and a total of 7 groups of experimental groups and 1 group of negative controls were established). The above composition premix was added to a 0.2-mL RPA-nfo reaction tube containing lyophilized enzyme powder. Finally, 2.5 μL of buffer B was added to the cap of the reaction tube and fully mixed. After mixing well, the reaction solution was shaken to the bottom of the tube, and the reaction tube was then immediately incubated at 39°C for 20 min. At the end of the reaction, 5 μL of product was added to a centrifuge tube containing 195 μL of MGCBB and mixed well. The sample end of the colloidal gold strip was then inserted into the centrifuge tube and allowed to reach equilibrium. The results were interpreted by observing the control line and the test line within 10 min.

The specificity of this dual RPA-LFD method was assessed through experiments using the optimized dual RPA-LFD system with genomic DNA from a mixture of *S. suis* and *A. pleuropneumoniae*, *A. pleuropneumoniae*, *S. suis*, *E. coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *S. aureus*, *Pasteurella*, and *A. hydrophila*. The template was diluted to 9 concentrations ranging from 10^{0} ng/μL to 10^{-8} ng/μL. A negative control that lacked any added DNA template was used. Sensitivity experiments were performed using the established dual RPA-LFD system to evaluate the minimum limit of detection of the method.

Sample Collection and Bacterial Isolation

A 40-day-old nursery piglet group in a large-scale pig farm in Henan was suspected of having an infectious disease. The body temperature of the sick pigs rose above 40°C, accompanied by obvious respiratory symptoms. The lung samples of 45 affected piglets were collected aseptically and inoculated with THB agar plates containing 5% (v/v) sheep blood and/or TSA agar plates containing 10% (v/v) newborn calf serum and 0.01% (v/v) NAD by streaking with an inoculation ring. After culturing for 24 h at 37°C in a CO_2 incubator, suspected colonies were picked for Gram stain microscopy.

Detection by Conventional PCR

A pair of primers was designed to specifically amplify a 650-bp fragment of the *Apx*IV gene of *A. pleuropneumoniae* [24]. A multiplex PCR was developed to detect all serotypes of *S. suis* [25].

Clinical Application Test

To further confirm the effect of using the dual RPA-LFD system in clinical application, 45 clinical lung tissues of pigs suspected of being infected with *S. suis* and/or *A. pleuropneumoniae* from Shandong and Henan provinces were collected in this study, and RPA-LFD was performed under the optimal reaction conditions using pMD-18T-gdh recombinant plasmid as a positive control, ddH_2O as a blank control, and total DNA from healthy pig lung tissues as a negative control. Additionally, the abovementioned lung tissue samples were subjected to traditional bacterial isolation and culture and conventional PCR detection [24,25], and the test results were compared and analyzed.

Results

Screening of the RPA-Basic Primers

The screening revealed that the *A. pleuropneumoniae* ApxIV2698F/ApxIV3020R primer pair and *S. suis* *gdh*1166F/GDH1346R primer pair yielded a clear and single band (at the 323-bp and 180-bp positions, respectively) after the RPA amplification reaction, and the controls yielded no bands *(Table 1, Fig. 1)*. Therefore, the primer pairs ApxIV2698F/ApxIV3020R and GDH1166F/GDH1346R were used for the subsequent experiments.

Optimization of the Dual RPA-Basic Amplification System

The optimization work showed that the two specific bands could be detected with volume ratio of *S. suis* and *A. pleuropneumoniae* primers equal to 2.4 μL:0.6 μL, 2.4 μL:1.2 μL, 2.4 μL:1.8 μL, 2.4 μL:2.4 μL, 1.8 μL:2.4 μL, and 1.2 μL:2.4 μL *(Fig. 2-A)*, and the volume ratio of the *S. suis* and *A. pleuropneumoniae* primers of 2.4 μL:1.2 μL was the preferred ratio for the subsequent assays. The optimal primer ratio was then used to screen the reaction time. The results showed that electrophoresis bands could be detected with RPA reaction durations of 15-35 min *(Fig. 2-B)*, but

Table 1. Primer pairs and probes for RPA-Basic

Primer/Probe Name	Primer sequence (5'→3')	Target Fragment Length (bp)
ApxIV2698F	AGCAGTGCTTCTGTCGTTAGAGTCACGCCTTC	323 bp
ApxIV3020R	CGAGAATAATCGGCTACCCAT TTCCCTTCG	
ApxIV323Pn	FAM-CAATTAAGTAGTATACGCAATGTAAAGCAT[THF]ATCCTACCGTTATGC-C3spacer	
gdh1166F	TTCGCTTGTCATGGACTCGTGAAGAAGTAG	180 bp
gdh1346R	TATACCAAACCTTGGGCAATCATGCTATCC	
gdh1225Pn	FAM-AAATACGACCTTGGTACAGACTACCTTGCAGG[THF]GCTAACATCGCAGCCT-C3spacer	

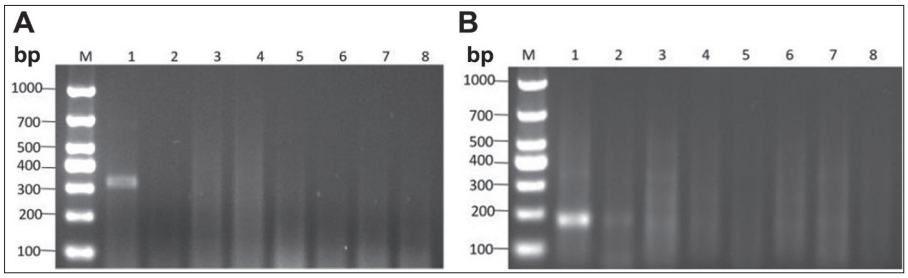
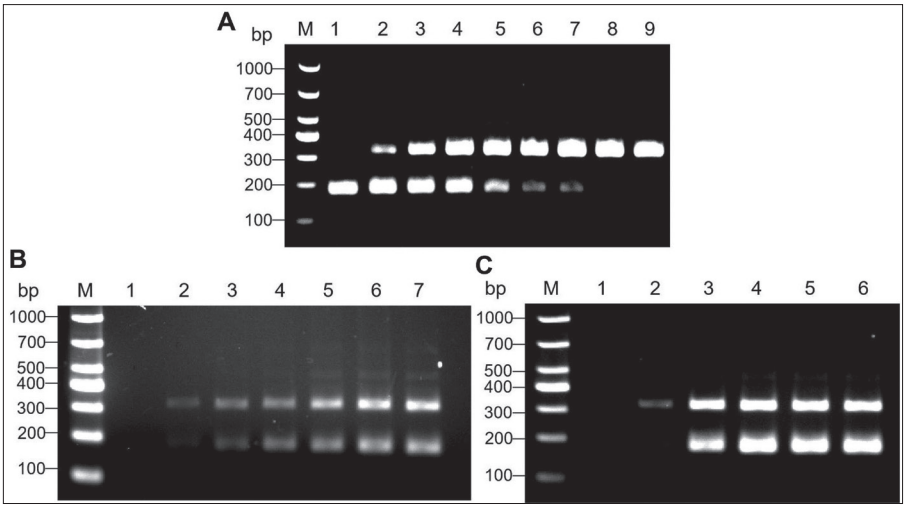


Fig 1. Results of the different primers detected by RPA-Basic. **A.** The results of APP primers detected by RPA-Basic. M. 1000 DNA Ladder, lanes 1-8 are: APP, negative control, enteropathogenic *E. coli*, *Salmonella*, *H. parasuis*, *S. aureus*, *Pasteurella*, *A. hydrophila*. **B.** The results of SS primers detected by RPA-Basic. M. 1000 DNA Ladder; lanes 1-8 are: SS, negative control, enteropathogenic *E. coli*, *Salmonella*, *H. parasuis*, *S. aureus*, *Pasteurella*, *A. hydrophila*

Fig 2. Results from the optimization of the dual RPA-Basic amplification system. **A.** Optimization of the RPA-Basic SS-APP primer volume ratio (μL). M, 1000 DNA Ladder; lanes 1-9, 2.4:0, 2.4:0.6, 2.4:1.2, 2.4:1.8, 2.4:2.4, 1.8:2.4, 1.2:2.4, 0.6:2.4, and 0:2.4, respectively; **B.** Optimization of the RPA-Basic reaction time. M,1000 DNA Ladder; lanes 1-7, 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, and 35 min, respectively; **C.** Optimization of the RPA-Basic reaction temperature. M, 1000 DNA Ladder; lanes 1-6, 25°C, 30°C, 35°C, 37°C, 39°C, and 45°C, respectively



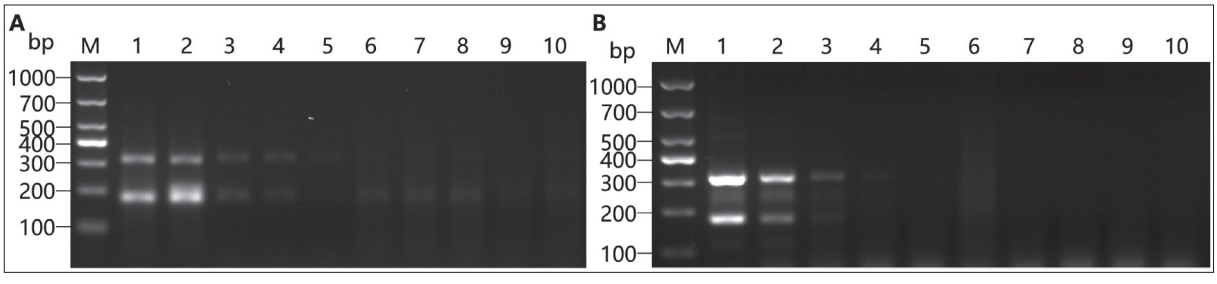


Fig 3. Comparison of the specificity and sensitivity of the dual RPA-Basic system and PCR. **A.** Results from the analysis of the sensitivity of Basic-RPA. M, 1000 DNA Ladder; lanes 1-10, nucleic acid concentrations of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} ng/μL, and negative control, respectively; **B.** Results from the analysis of the sensitivity of PCR. M, 1000 DNA Ladder; lanes 1-10, nucleic acid concentrations of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} ng/μL, and negative control, respectively

no significant difference in the brightness of the test line was observed after 20 min. Therefore, 20 min was selected as the optimal reaction time for the subsequent tests. The reaction temperature was then optimized by determining the optimal primer concentration and reaction time. The results showed that when the temperature was between 30°C and 45°C, the agarose gel could specifically detect RPA amplification products *(Fig. 2-C)*. Considering the detection application, 37°C was selected as the reaction temperature for the subsequent experiments.

Specificity and Sensitivity of the Dual RPA-Basic System

The optimized dual RPA-Basic reaction system yielded amplification products only with the *S. suis* and/or *A. pleuropneumoniae* groups, and no amplification products were observed with the other 6 groups or the negative control group *(Fig. 3)*, confirming the specificity of the dual RPA-Basic detection method. To determine the sensitivity of the dual RPA-Basic system, the template was sequentially diluted 10-fold to obtain 10 concentrations ranging from 10^{0} to 10^{-9} ng/μL. The results showed that the nucleic acid detection limit of RPA-Basic was 10^{-3} ng/μL, which was higher than that of PCR (10^{-2} ng/μL) *(Fig. 3)*.

Screening of Probes for the Dual RPA-LFD System

The selected reverse primers of *S. suis* and *A. pleuropneumoniae* were labeled with biotin, and the designed probes were labeled with 6-Carboxyfluorescein under these conditions. This labeling was only applied to detect a single colloidal gold test strip under this condition. To screen the optimal probe for the dual RPA-LFD system, the same template and reaction conditions were used for each *S. suis* and *A. pleuropneumoniae* RPA-nfo reaction; additionally, each pair of primers was tested using a positive control, negative control, and enteropathogenic *E. coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *S. aureus*, *Pasteurella*, and *A. hydrophila*. The final screening combination is shown in *Table 1*, and the respective reaction results are shown in *Fig. 4*. The test and control lines of the *S. suis* and *A. pleuropneumoniae* RPA-LFD detection groups both appeared, whereas the other groups yielded only a control line, indicating that this primer-probe combination could be used for the subsequent experiments.

Optimization of the Dual RPA-LFD Detection Reaction System

The RPA-LFD primer and probe concentration ratio in the reaction system was optimized using the selected primers and probes listed in *Table 1*. The selected *S. suis* reverse primer was labeled with biotin, the *A. pleuropneumoniae* reverse primer was labeled with digoxigenin digoxin, and both probes were labeled with FAM fluorescein, corresponding to the double colloidal gold detection test strip. The results showed that the colors of the *A. pleuropneumoniae* test line and *S. suis* test line were most similar when the *A. pleuropneumoniae* and *S. suis* upstream and downstream primers and probes were added to the systems containing *A. pleuropneumoniae* volumes of 2, 2, or 0.6 μL and *S. suis* volumes of 0.5, 0.5, or 0.15 μL in the seven experimental groups *(Fig. 5)*; notably, the negative control only yielded the control line; thus, the reaction systems consisting of 2, 2, or 0.6 μL of *A. pleuropneumoniae* and 0.5, 0.5, or 0.15 μL of *S. suis* were selected for the subsequent experiments.

Specificity and Sensitivity of Dual RPA-LFD Detection

The optimized dual RPA-LFD system was used for specificity experiments using genomic DNA from a mixture of *S. suis* and *A. pleuropneumoniae*, *A. pleuropneumoniae*, *S. suis*, the negative control, *E. coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *S. aureus*, *Pasteurella*, and *A. hydrophila*. As shown in *Fig. 6-A*, the results showed that the double-positive and single-positive results for *A. pleuropneumoniae* and/or *S. suis* all displayed both the appropriate test line(s) and the control line. For the controls and the nontarget bacteria, only the control line was displayed; thus, the specificity of the method was demonstrated. To determine the sensitivity of the dual RPA-LFD system, the template was sequentially diluted 10-fold to obtain 10 concentrations (10^{0}-10^{-9} ng/μL). The results showed that the colors of the test lines of *A. pleuropneumoniae* and *S. suis* gradually faded with decrease in the concentrations from 10^{0} to 10^{-5} ng/μL, and the test line completely disappeared with lower concentrations (10^{-6} ng/μL and less) *(Fig. 6-B)*. In addition, only the control line was observed with the negative control. Therefore, the limit of detection of the dual RPA-LFD established in this study was 10^{-5} ng/μL, which is 100 times that of the constructed dual RPA-Basic system and 1.000 times that of PCR.

Isolation and Identification of *S. suis* and *A. pleuropneumoniae*

A total of 10 strains of *S. suis* were isolated from different pig tissue samples at different time points in the affected

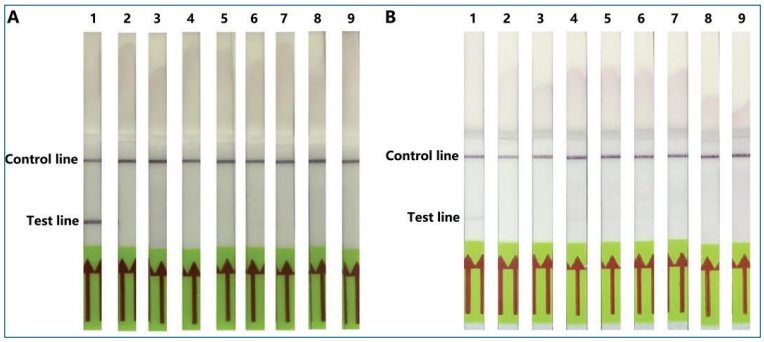


Fig 4. Screening of detection probes for the dual RPA-LFD system. **A.** Screening of probe for SS. Lanes 1-9, *Streptococcus suis*, negative control, enteropathogenic *Escherichia coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *Staphylococcus aureus*, *Pasteurella*, *Bacillus*, *Aeromonas hydrophila*, and *Actinobacillus pleurisy*, respectively; **B.** Screening of probe for APP. Lanes 1-9, *Actinobacillus pleurisy*, negative control, enteropathogenic *Escherichia coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *Staphylococcus aureus*, *Pasteurella*, *Aeromonas hydrophila*, and *Streptococcus suis*, respectively

pig farm, and the bacteria were small round, gray-white and translucent colonies on the THB agar plates. The isolated bacterial smear was Gram-stained and observed under an oil microscope with a light microscope, and it was a blue-purple brevis. *A. pleuropneumoniae* was not isolated from any lung tissue samples *(Table 2)*.

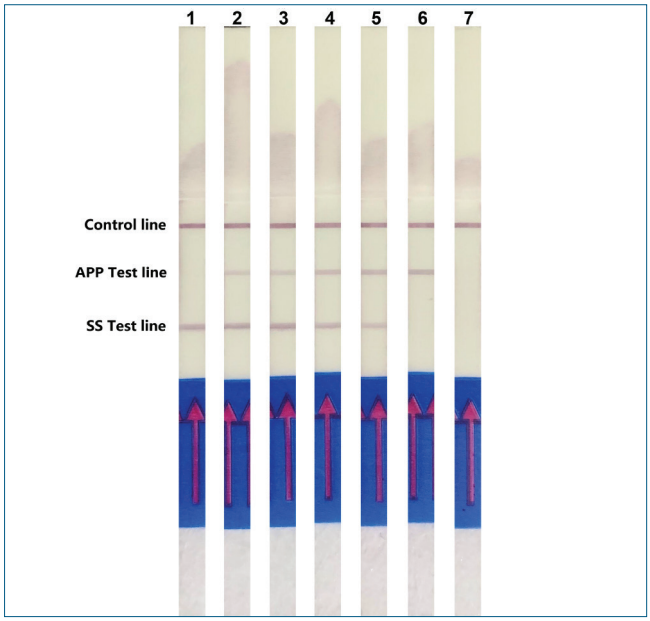


Fig 5. Optimization results of the dual LFD-RPA reaction system. 1. APP (0, 0, 0), SS (2, 2, 0.6). 2. APP (2, 2, 0.6), SS (2, 2, 0.6). 3. APP (2, 2, 0.6), SS (1.5, 1.5, 0.45). 4. APP (2, 2, 0.6), SS (1, 1, 0.3). 5. APP (2, 2, 0.6), SS (0.5, 0.5, 0.15). 6. APP (2, 2, 0.6), SS (0, 0, 0). unit (μL). 7. negative control

Conventional PCR Detection

Among 45 clinical samples suspected of *S. suis* or *A. pleuropneumoniae* infection, a total of 18 *S. suis*-positive samples were detected by conventional PCR detection, including 7 strains of serotype 2, 3 strains of serotype 7 and 2 strains of serotype 9, accounting for 15.2%, 6.7% and 4.4% of the total samples, respectively *(Table 2)*. One strain of *A. pleuropneumoniae* was detected.

Clinical Application of Dual RPA-LFD Detection

Using the established dual LFD-RPA method to detect each serotype strain of *S. suis* and *A. pleuropneumoniae*, the test lines could be clearly observed *(Fig. 7)*. Among 45 clinical samples suspected to involve *S. suis* or *A. pleuropneumoniae* infection, a total of 19 *S. suis*-positive samples and 1 *A. pleuropneumoniae*-positive sample were detected by LFD-RPA (without mixed infection). The dual RPA-LFD method resulted in a higher detection rate of *S. suis* compared with the conventional PCR method *(Table 2)*.

Discussion

A. pleuropneumoniae and *S. suis* are important pathogenic bacteria that endanger the pig industry [9]. Both bacteria

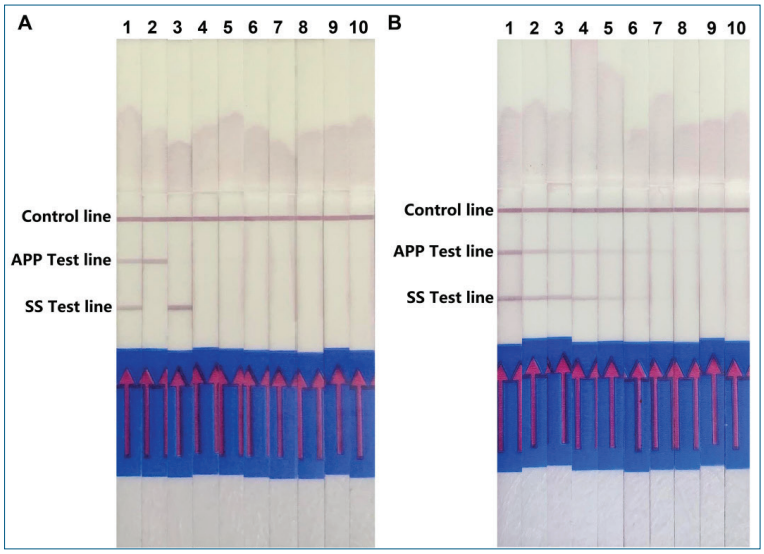


Fig 6. Specificity and sensitivity of the dual RPA-LFD detection method. **A.** Specificity of RPA-LFD. Lanes 1-10, SS and APP positive control, APP positive control, SS positive control, negative control, *Escherichia coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *Staphylococcus aureus*, *Pasteurella*, and *Aeromonas hydrophila*, respectively. **B.** Sensitivity of RPA-LFD. Lanes 1-9, nucleic acid concentrations of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} ng μL, respectively; lane 10, negative control

Fig 7. Clinical sample results with the dual PRA-LFD system. Lanes 1-4, SS and APP positive control, SS positive control, APP positive control, and negative control, respectively; Lanes 5-13, clinical test samples

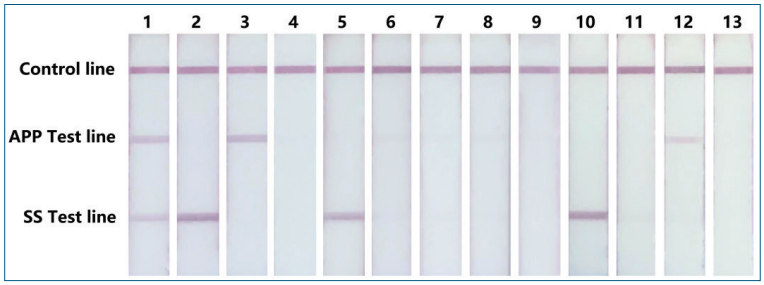


Table 2. *Application of different methods for the detection of actual samples*

Isolates	*S. suis*						*A. pleuropneumoniae*		
	Bacterial Isolates	Serotype-specific PCR				Dual RPA-LFD	Bacterial Isolates	PCR	Dual RPA-LFD
		2	7	9	Other Serotype				
XXHX001									
XXHX002									
XXHX003		+				+			
XXHX004					+	+			
XXHX005	+	+				+			
XXHX006									
XXHX007				+		+			
XXHX008									
XXHX009	+	+				+			
XXHX010					+	+			
XXHX011	+		+			+			
XXHX012	+	+				+			
XXHX013									
XXHX014	+	+				+			
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XXHX024	+				+	+			
XXHX025									
XXHX026									
XXHX027									
XXHX028	+	+				+		+	+
XXHX029									
XXHX030			+			+			
XXHX031									
XXHX032									
XXHX033	+			+		+			
XXHX034									
XXHX035					+	+			
XXHX036									
XXHX037						+			
XXHX038									
XXHX039									
XXHX040	+		+			+			
XXHX041									
XXHX042									
XXHX043					+	+			
XXHX044									
XXHX045									

often concurrently infect pig herds and can cause respiratory symptoms in pigs, which makes the condition complex and results in great difficulty in obtaining clinical differential diagnoses [9]. Therefore, to improve the efficiency and accuracy of detection, this study selected the highly conserved *S. suis gdh* gene and *A. pleuropneumoniae Apx*IV gene as diagnostic antigens and developed a dual RPA-LFD visual rapid detection method that resulted in the highly sensitive, highly specific and simultaneous detection of these two bacteria. Therefore, this method can be conveniently applied in clinical laboratories and/or used for field diagnosis.

In molecular biology detection methods, the accurate selection of target genes is crucial and can directly affect the reliability of the reaction results. *S. suis* strains exhibit extensive genetic heterogeneity within and among serotypes, which increases the difficulty of establishing genetic diagnostic methods [21]. The *gdh* gene of *S. suis* is highly conserved in all capsular serotypes and can be used as a diagnostic antigen [21]. In our previous study, Gdh was selected as a diagnostic antigen for developing PPA-ELISA and dot-PPA-ELISA diagnostic methods, which have been demonstrated to be suitable for the large-scale and on-site/field diagnosis of *S. suis* infections [2,26]. *Apx*IV is one of the virulence factors of *A. pleuropneumoniae* and belongs to the RTX toxin family. *Apx*IV is present in all serotype strains of *A. pleuropneumoniae* but not in other closely related species of bacteria and is a good target gene for the detection of *A. pleuropneumoniae* species [27,28]. Based on these observations, in this study, we selected the *gdh* and *Apx*IV genes as targets for the establishment of a dual RPA-LFD visualization rapid detection method.

Nucleic acid isothermal amplification technology provides simplified nucleic acid artificial replication conditions that require only a constant temperature without thermal cycling. Nucleic acid isothermal amplification technology not only reduces the time of amplification by eliminating repeated heating and cooling steps but also can achieve multiple molecular reactions that proceed asynchronously, which greatly improves the efficiency of nucleic acid amplification [19]. At present, the widely used nucleic acid isothermal amplification methods include nucleic acid sequence-based amplification (NASBA), LAMP, strand displacement amplification (SDA) and RPA [29-32]. Compared with traditional PCR detection methods and other DNA isothermal amplification technologies, RPA can amplify target DNA to detectable levels without special equipment in a shorter time and at lower temperatures [33]. In addition, it has been shown that RPA has a sensitivity similar to that of PCR detection, and the sensitivity of some RPA systems is even 10 or 1.000 times greater than that of PCR detection [34,35]. Detection methods such as real-time fluorescence quantitative RPA, RPA-LFD, and RPA-ELISA that were developed in combination with this technology have been widely used in diagnosis, medical treatment, agriculture, food safety and other fields [7,36,37]. Among these methods, LFD technology is a visual detection tool that can be performed without expensive and complex instruments and trained personnel, and the results can be observed within 5-10 min. This study combined RPA and LFD technology to establish a method for the simultaneous detection of *A. pleuropneumoniae* and *S. suis*. This rapid RPA-LFD visual detection method can be used in resource-constrained laboratories or for on-site/field diagnosis and can be considered a convenient method for the detection of distant, less portable samples. In addition, the limit of detection of the RPA-LFD detection method established in this study was as low as 10^{-5} ng/μL, which is 100 times that of the constructed dual RPA-Basic detection method and 1.000 times that of conventional PCR detection technology.

The practicability of RPA-LFD detection technology is an important indicator of its application and promotion [38]. On the one hand, in this study, RPA-LFD was applied for the analysis of collected disease materials, and the detection rate was higher than that of conventional PCR. On the other hand, experimental screening revealed that the expected RPA amplification bands could appear at the optimal reaction temperature of RPA (between 30°C and 45°C), which indicated that the reaction conditions needed for RPA are relatively flexible and that the reaction can be completed by heating in a water bath. These results indicate that RPA-LFD technology is very practical for the rapid dual detection of *S. suis* and *A. pleuropneumoniae*, which is conducive to the achievement of rapid on-site detection or field detection. In terms of detection efficiency, RPA-LFD detection can be completed within 30 min, whereas conventional PCR and LAMP technology usually take 2.5 h and 1 h, respectively. Therefore, the detection efficiency of RPA-LFD technology is significantly higher than that of PCR or LAMP technology [36]. In addition, RPA-LFD amplification requires only two primers, which greatly reduces the difficulty and complexity of primer design compared with that needed for LAMP technology, which requires six primers [33]. The reliability of the detection technology depends on its specificity, and the analysis of the specificity of the dual RPA-LFD visualization rapid detection system established in this study showed that the detection method did not cross-react with six other common pathogenic microorganisms, which demonstrated that the method exhibits good specificity.

In summary, the dual RPA-LFD detection method for *A. pleuropneumoniae* and *S. suis* established in this study has several advantages, including high sensitivity, high specificity and fast speed. Additionally, using this method, the results can be directly observed by the naked eye,

and large-scale precision instruments are not necessary. Therefore, this method is very suitable for on-site or field detection and has broad application potential.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding authors (X. Xia and H. Zhang).

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Authors' Contributions

XX and HZ designed the research and project outline. SZ, HX, ML and AZ carried out the experiments and analysis the data. HY, MD, XW, ZT, JH, SZ and HX drafted the manuscript. XX, HZ and JH revised the manuscript. All authors have read and approved the final manuscript.

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Research Article

Associations Between c.2832A > G Polymorphism of *CAST* Gene and Meat Tenderness in Cattle: A Meta-Analysis

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Abstract: Genes or genetic markers related to meat quality have been studied for many years. The *CAST* gene is one candidate gene affecting meat tenderness in cattle. This meta-analysis aimed to examine the association of c.2832A>G polymorphism of the *CAST* gene and meat tenderness in cattle. According to the determined criteria, 17 studies were included in the meta-analysis, and pooled ratios of allele and genotype frequencies were calculated. In addition, the combined Warner-Braztler Shear Force values were calculated for three studies that reported these values according to genotypes for the meat tenderness feature. Heterogeneity between studies and publication bias were also tested. As a result of the meta-analysis, pooled ratios of AA, AG, and GG genotypes are 0.578, 0.342, and 0.080; pooled ratios of A and G alleles are 0.777 and 0.223. The highest pooled ratios were obtained for AA genotype and A allele. The combined Warner-Braztler shear force values were calculated as 3.707, 3.893, and 5.137 kilogram-force for AA, AG, and GG genotypes. The highest mean shear force value was obtained for the GG genotype. In conclusion, the meta-analysis results examined the relationship between c.2832A>G polymorphism of the *CAST* gene and meat tenderness were closer to the population parameter. This study may improve genetic selection and provide new strategies to increase meat quality in cattle.

Keywords: *CAST gene, Cattle, Meta-analysis, Meat tenderness, Polymorphism*

CAST Geninin c.2832A > G Polimorfizmi ile Sığırlarda Et Gevrekliği Arasındaki İlişki: Bir Meta-Analizi

Öz: Et kalitesiyle ilgili genler veya genetic belirteçler uzun yıllardır araştırılmaktadır. *CAST* geni, sığırlarda et gevrekliğini etkileyen bir aday gendir. Bu meta analizi, sığırlarda *CAST* geninin c.2832A>G polimorfizmi ile et gevrekliği arasındaki ilişkiyi incelemeyi amaçlamıştır. Belirlenen kriterlere göre, 17 çalışma meta analize dahil edilmiş ve allel ve genotip frekansların birleştirilmiş oranları hesaplanmıştır. Bunun yanında, etin gevreklik özelliği için Warner-Braztler kesme kuvveti değerlerini genotiplere göre veren üç çalışmanın sonucu için birleştirilmiş kesme kuvveti değeri hesaplanmıştır. Çalışmalar arası heterojenlik ve yayın yanlılığı da test edilmiştir. Meta-analizi sonucunda AA, AG ve GG genotiplerinin birleştirilmiş oranları 0.578, 0.342 ve 0.080; A ve G alellerinin birleştirilmiş oranları 0.777 ve 0.223 olarak elde edilmiştir. En yüksek birleştirilmiş oranlar AA genotipi ve A alleli için elde edilmiştir. Birleştirilmiş Warner-Braztler kesme kuvveti değerleri AA, AG ve GG genotipleri için sırasıyla 3.707, 3.893 ve 5.137 kilogram kuvvet olarak hesaplanmıştır. En yüksek ortalama kesme kuvveti değeri GG genotipi için elde edilmiştir. Sonuç olarak, *CAST* geninin c.2832A>G polimorfizmi ile et gevrekliği arasındaki ilişkinin incelendiği meta-analiz sonuçları populasyon parametresine yakın bulunmuştur. Bu çalışma sığırlarda genetik seçimi geliştirmek ve et kalitesini arttırmak için yeni stratejiler sağlayabilir.

Anahtar sözcükler: *CAST geni, Et gevrekliği, Meta-analiz, Polimorfizm, Sığır*

Introduction

Meat tenderness is one of the characteristics that determine meat quality, and it is an essential factor affecting the satisfaction in beef consumption. Two enzymes are responsible for the meat tenderness property used to improve genetic selection: *calpain (CAPN1)* and its inhibitor, calpastatin (*CAST*) genes [1]. The *CAPN1* gene, which degrades postmortem myofibrillar proteins, is located on bovine chromosome 29. The *CAST* gene is

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located on BTA 7 [2,3]. Several markers have been developed in the *CAST* gene and three in the *CAPN1* gene [4,5]. These markers were independently examined in previous studies and have been suggested as being associated with meat tenderness in beef cattle [1,4,5].

Especially several variants of the *CAST* gene have been reported in studies conducted on different breeds of cattle [6-8]. Some of these studies examined the association between c.2832A>G polymorphism of the *CAST* gene and meat tenderness. Different results were obtained due to sample sizes [7,9,10]. To address this problem about differences, large sample sizes or meta-analysis are required to determine the genetic effects of the c.2832A>G polymorphism on meat tenderness in cattle.

Systematic reviews and meta-analysis studies combine the findings from different studies on the same subject and evaluate them using statistical methods. Accordingly, the statistical power and precision of estimating an SNP effect on a trait in individual studies with small sample sizes can be increased with meta-analysis studies that have also become widespread in genetics. Even though the previous meta-analyses had discussed the association between different gene variants and milk yield in cattle [11-13], this study examines the relationship between the related gene and meat tenderness.

This study aimed to evaluate the genotype and allele frequencies of c.2832A>G polymorphism of the bovine *CAST* gene with meta-analysis. In addition, it was aimed to examine the relationship of this SNP with meat tenderness in cattle.

Material and Methods

Ethical Statement

This study was performed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) checklist criteria [14]. The data for this research were collected from online databases, so this study does not require any ethical permission.

Literature Search Strategy

A comprehensive literature search was conducted through PubMed and Web of Science databases in January 2022 using the keywords "*calpastatin* gene" and "cattle" to detect relevant studies.

Inclusion and Exclusion Criteria

The inclusion criteria were as follows: (a) studies providing genotype and/or allele frequencies and sample sizes of *CAST* gene c.2832A>G polymorphism in cattle; (b) the full text can be obtained; (c) published in English or Turkish language. All relevant reports were screened first by title and then full-text to avoid duplication. Exclusion criteria are (a) about other *CAST* gene polymorphisms; (b) duplicated data; (c) abstract, review, and case reports; (d) provided insufficient data; (e) other species experiments. In addition, for subgroup analysis to examine the relationship between the *CAST* gene c.2832A>G polymorphism and meat tenderness, studies reporting the Warner-Braztler Shear Force (WBSF) values according to genotypes (AA, AG, and GG) were included.

Data Extraction

All authors independently screened all selected studies in full text to determine whether they met the inclusion and exclusion criteria. A standard data extraction form was used to extract the following data: first author's name, publication year, region, cattle breed, sample size, genotype (AA, AG, and GG), and allele (A and G) gene frequencies. In some studies, the frequencies of the genotypes were not given separately. In this case, only allele frequencies were included in those studies. In addition, in some studies, the frequencies were obtained by calculating ratios, while in some studies, the genotype frequencies were calculated manually. Since some studies used more than one breed and frequencies were specified for each breed in detail, each breed was taken as a separate study. Any disagreement was resolved by discussion between the authors. For subgroup analysis to examine the relationship between the *CAST* gene c.2832A>G polymorphism and meat tenderness, least-square means (with standard error) of WBSF values according to genotypes were extracted. When the standard error of the mean was reported, it was converted to standard deviation.

Statistical Analysis

Before meta-analysis, publication bias was evaluated quantitatively with Begg's adjusted rank correlation test and graphically with funnel plots. The heterogeneity of effect sizes between studies was assessed with the Cochran Q test. Q statistics and I^2 statistics were calculated. I^2 values higher than 50% were considered as high heterogeneity. Based on the heterogeneity test results, the fixed-effect model was used when heterogeneity was low; otherwise, a random effect model was employed to compute pooled effect sizes. In this study, pooled ratios were calculated with 95% confidence intervals concerning all the genotypes (AA, AG, and GG) and allele (A and G) frequencies. In evaluating genotype and allele frequencies, studies were classified according to cattle breeds as *Bos taurus*, *Bos indicus*, and cross breed. Analyzes were performed separately for both breed type and total. In addition, point estimates of WBSF values were calculated for each genotype separately (Hedges method). In the evaluation of heterogeneity and publication bias, α=0.10 was taken. R software (version 4.1.2) was utilized to perform a meta-analysis in this study.

Results

Study Characteristics

The study selection process is shown in *Fig. 1*. A total of 228 articles were retrieved. According to the inclusion and exclusion criteria, the final selected articles were 17 and were published from 2006 to 2021. The characteristics of the selected studies and frequency distribution of genotype and allele gene were presented in *Table 1*. Since frequency values of more than one cattle breed were reported in some studies, each result was considered as a separate trial result. For this reason, the relevant table includes 26 trial results from 17 publications. The findings of 21 trials reporting genotype frequencies (AA, AG, and GG) and 26 trials containing allele frequencies (A and G) were given in this table. Among the identified studies, the relationship between meat tenderness and c.2832A>G polymorphism of bovine *CAST* gene was examined, and Warner-Braztler Shear Force (WBSF) values were entirely reported in three studies. WBSF values of these studies according to genotypes were given in *Table 2*.

Meta-analysis Results

All meta-analysis results applied to combine the genotype and allele frequencies are represented in *Table 3*. Cochran's Q and I^2 test statistics were reported with p values in the relevant table.

Considering all the studies, according to calculated I^2(%) and P values, statistically significant (P<0.001) and high heterogeneity was detected in all genotypes and alleles.

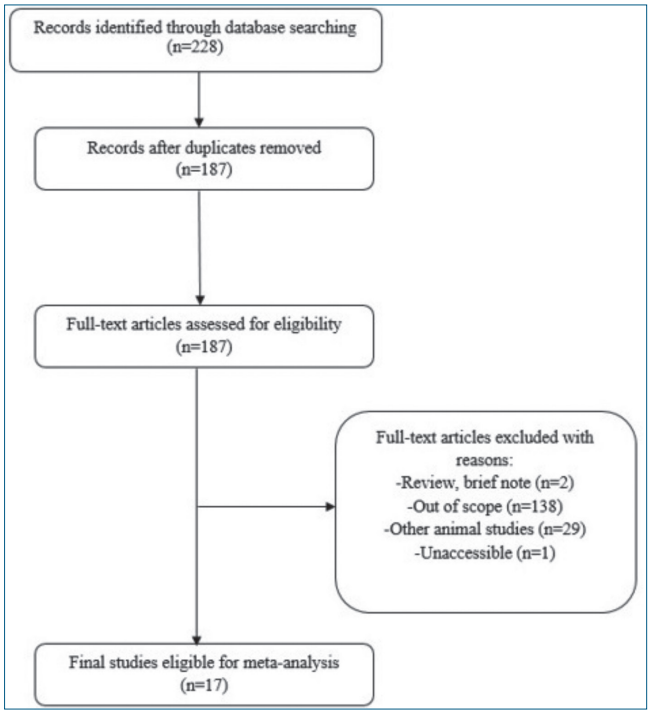

Fig 1. Flowchart of study selection for meta-analysis

Therefore, the pooled ratios were obtained with the random effect model. As a result of the meta-analysis, the AA genotype pooled ratio of 0.578 (0.485-0.666) was calculated higher than AG and GG genotypes, and the A allele pooled ratio of 0.777 (0.725-0.822) was calculated higher than the G allele. Forest plots related to the meta-analysis of genotypes and alleles for all studies were represented in *Fig. 2*. As a result of Begg's test, it was determined that there was no publication bias for all genotypes and alleles. Funnel plots represented in *Fig. 3* also showed no evidence of publication bias.

According to meta-analysis results for *Bos taurus* breed, calculated I^2(%) and p values show that there is statistically significant (P<0.001) and high heterogeneity in all genotypes and alleles. Therefore, the pooled ratios were obtained with the random effect model. The AA genotype pooled ratio of 0.626 (0.512-0.727) was calculated higher than AG and GG genotypes, and the A allele pooled ratio of 0.798 (0.742-0.845) was calculated higher than the G allele. Also, it was determined that there was no publication bias for all genotypes and alleles with Begg's test.

The meta-analysis results of the *Bos indicus* breed for AA genotype (Q=23.41, P<0.001, I^2=87.2%) and AG genotype (Q=13.12, P=0.004, I^2=77.1%) show that heterogeneity is statistically significant and high. For the GG genotype, the heterogeneity between studies is not significant (P=0.542), and a fixed-effect model was used to calculate the pooled ratio. For A and G alleles, statistically significant and high heterogeneity (Q= 9.36, I^2=67.9%, P=0.025) was detected. For studies including *Bos indicus* breed cattle, the AG genotype pooled ratio was calculated higher than AA and GG genotypes. The A allele pooled ratio was higher than the G allele.

According to meta-analysis results for the cross breed of *Bos indicus* and *Bos taurus*, calculated I^2(%) and p values show statistically significant and high heterogeneity in only the AA genotype (Q=13.02, P=0.023, I^2=61.6%). The random-effects model was used to calculate the effect size for the AA genotype, and the fixed effect model was used for other genotypes and alleles. For studies including cross breeds, the AA genotype pooled ratio was calculated higher than AG and GG genotypes, and the A allele pooled ratio was higher than the G allele. The Begg's test could not be performed because less than ten studies were included in the meta-analysis for *Bos indicus* and cross breed groups.

To examine the association between c.2832A>G polymorphism of the *CAST* gene and meat tenderness, three studies with sufficient data were combined with a meta-analysis. All results are represented in *Table 4*. High heterogeneity was determined (I^2=71.40%, P=0.030) for the AA genotype, and pooled mean of WBSF value in

Table 1. *Basic characteristics, genotype, and allele frequency distribution of studies included in the meta-analysis*

No	Study	Year	Country	Breed	n	Genotype Frequency (*n*)			Allele Frequency (n)	
						AA	AG	GG	A	G
1	Allais et al.[6]	2011	France	Blonde d'Aquitaine	971	568	358	45	747	224
2	Allais et al. [6]	2011	France	Charolais	1094	738	321	35	899	195
3	Allais et al.[6]	2011	France	Limousin	1245	838	384	23	1030	215
4	Barendse et al.[9]	2007	Australia	Brahman, Angus, Belmont Red	4936	-	-	-	3899	1037
5	Cafe et al. [7]	2010	Australia	Brahman	143	51	51	41	77	66
6	Casas et al.[1]	2006	USA	MARCIII population⁺	539	349	166	24	432	107
7	Casas et al.[15]	2013	USA	Hereford, Angus, Red Poll crosses	248	-	-	-	206	42
8	Castro et al.[16]	2016	Colombia	Braunvieh, Limousin, Normande	85	-	-	-	60	25
9	Curi et al.[10]	2009	Brazil	Nelore	114	39	49	26	64	50
10	Curi et al.[10]	2009	Brazil	Angus x Nelore	67	53	14	0	60	7
11	Curi et al.[10]	2009	Brazil	Rubia Gallega x Nelore	44	29	15	0	37	7
12	Curi et al.[10]	2009	Brazil	Canchim	41	20	17	4	29	12
13	Curi et al.[10]	2009	Brazil	Brangus three-way crosses⁺⁺	19	13	6	0	16	3
14	Curi et al.[10]	2009	Brazil	Braunvieh three-way crosses⁺⁺⁺	15	7	8	0	11	4
15	Cushman et al.[17]	2021	USA	MARCIII population	187	58	97	32	107	80
16	Frylinck et al.[18]	2009	South Africa	Brahman	19	11	8	0	15	4
17	Frylinck et al.[18]	2009	South Africa	Simmental	20	16	4	0	18	2
18	Frylinck et al.[18]	2009	South Africa	Nguni	19	14	5	0	17	2
19	Gruber et al.[19]	2011	USA	Charolais-Angus crosses	343	261	82	0	302	41
20	Johnston and Graser [20]	2010	Australia	Angus, Hereford, Murray Grey, Shorthorn	3136	-	-	-	2741	395
21	Li et al.[21]	2010	South Korea	Chinese cattle breeds	212	112	73	27	149	63
22	Morris et al.[22]	2016	New Zealand	Jersey-Limousin, Angus and Hereford-crosses	746	664	75	7	702	44
23	Pintos and Corva [8]	2011	Argentina	Argentinian Angus	268	-	-	-	211	57
24	Tait et al.[23]	2014a	USA	MARCIII population⁺	199	95	82	22	136	63
25	Tait et al.[24]	2014b	USA	MARCIII population⁺	254	101	128	25	165	89
26	Tizioto et al.[25]	2014	Brazil	Nelore	178	30	99	49	80	98
Total					15142	4067	2042	360	12210	2932

⁺ *MARCIII: (composite of ¼ Hereford, ¼ Angus, ¼ Pinzgauer, and ¼ Red Poll),* ⁺⁺ *(9/16 B. Taurus + 7/16 B. indicus),* ⁺⁺⁺ *(3/4 B. Taurus + 1/4 B. indicus)*

Table 2. *The Warner-Braztler Shear Force (WBSF) values according to the CAST - c.2832A >G genotypes*

No	Study	Year	AA		AG		GG	
			n	LSM±SE	n	LSM±SE	n	LSM±SE
1	Cafe et al.[7]	2010	51	4.74±1.93	51	4.98±1.93	41	5.54±1.93
2	Curi et al.[10]	2009	39	3.46±0.07	49	3.88±0.07	26	-
3	Li et al.[21]	2010	112	3.98±0.19	73	4.01±0.22	27	5.13±0.28

* *Values of shear force presented as least square mean ± standard error (LSM±SE) (in kgf unit);* ***LSM:*** *Least square mean*

the studies was calculated with a random effect model as 3.707 (3.210-4.203) kgf. Heterogeneity was not significant for AG and GG genotypes (I^2=0.00%). Therefore, the fixed-effect model was chosen, and pooled mean of WBSF values was calculated as 3.893 (3.762-4.024) kgf and 5.137 (4.594-5.679) kgf for AG and GG genotypes, respectively. The highest mean shear force value was obtained for the GG genotype. Begg's test could not be applied because the combined studies were less than 10.

Discussion

In this study, 17 out of 228 publications retrieved from Pubmed and Web of Science were suitable for meta-analysis. Firstly, genotype and allele frequencies obtained from studies examining the c.2832A>G polymorphism of the bovine CAST gene were combined with a meta-analysis. This step combined 21 frequency values for AA, AG and GG genotypes and 26 frequency values for

Table 3. *Evaluation results of genotype and allele frequencies of CAST gene with meta-analysis method*

Cattle Breed	Genotype/Allele		Number of Trials	Pooled Ratio	95% CI	Heterogeneity			Publication Bias
						Cochran Q	P Value	I^2 (%)	P Value*
Bos taurus	Genotype	AA	11	0.626	0.512-0.727	384.08	<0.001	97.4	0.139
		AG	11	0.316	0.243-0.399	243.83	<0.001	95.9	0.585
		GG	11	0.058	0.029-0.094	146.71	<0.001	94.5	0.697
	Allel	A	15	0.798	0.742-0.845	321.28	<0.001	95.6	0.804
		G	15	0.202	0.155-0.258	321.28	<0.001	95.6	0.804
Bos indicus	Genotype	AA	4	0.327	0.209-0.472	23.41	<0.001	87.2	-
		AG	4	0.433	0.361-0.534	13.12	0.004	77.1	-
		GG	4	0.240	0.227-0.310	1.23	0.542	0.0	-
	Allel	A	4	0.538	0.453-0.620	9.36	0.025	67.9	-
		G	4	0.462	0.380-0.547	9.36	0.025	67.9	-
Cross breed	Genotype	AA	6	0.653	0.544-0.749	13.02	0.023	61.6	-
		AG	6	0.317	0.257-0.384	8.65	0.124	42.2	-
		GG	6	0.030	0.007-0.050	13.20	1.000	0.0	-
	Allel	A	7	0.791	0.780-0.802	8.36	0.213	28.2	-
		G	7	0.209	0.198-0.220	8.36	0.213	28.2	-
Total	Genotype	AA	21	0.578	0.485-0.666	587.96	<0.001	96.6	0.717
		AG	21	0.342	0.288-0.401	300.70	<0.001	93.3	0.952
		GG	21	0.080	0.046-0.141	340.50	<0.001	96.5	0.393
	Allel	A	26	0.777	0.725-0.822	549.11	<0.001	95.4	0.774
		G	26	0.223	0.178-0.275	549.11	<0.001	95.4	0.774

** Begg and Mazumdar Rank Correlation Test*

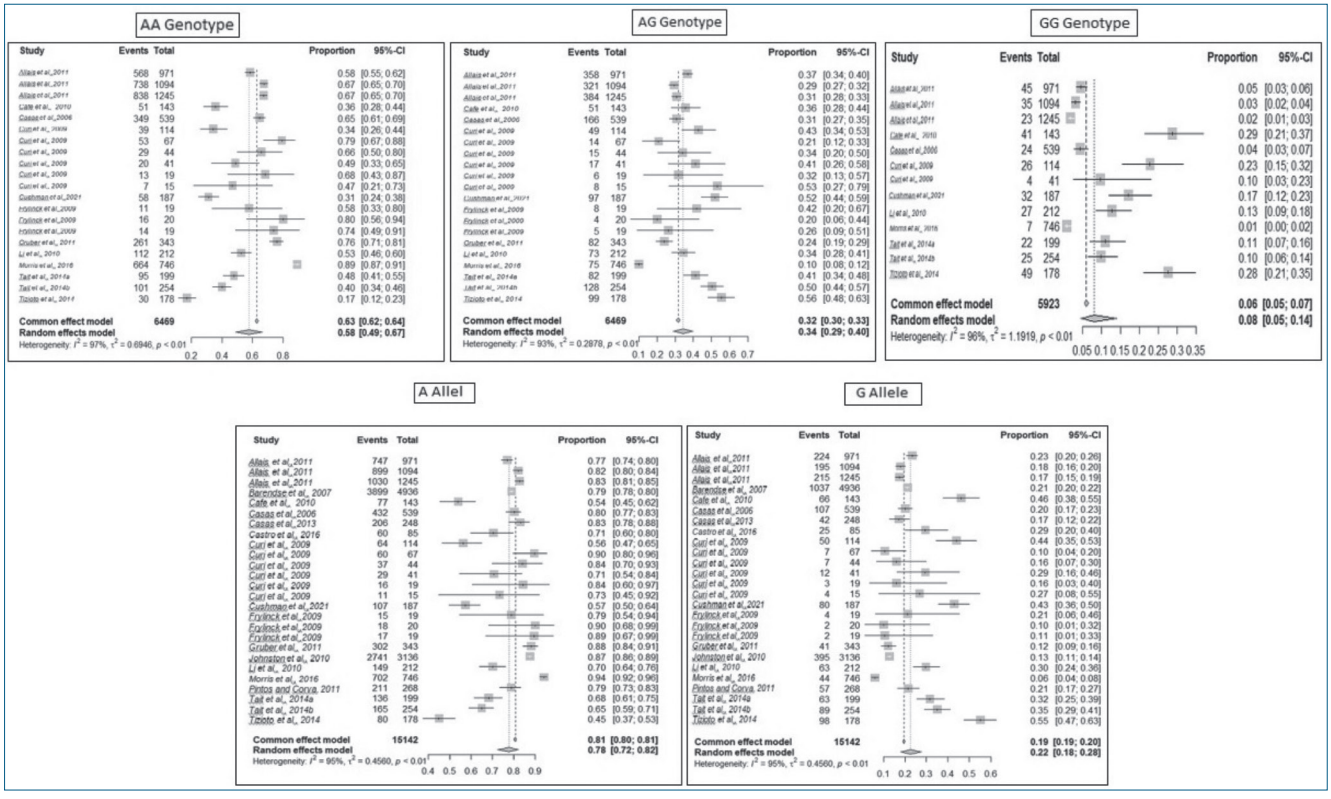

Fig 2. Forest plots of AA, AG, and GG genotypes and A and G alleles of the *CAST* gene

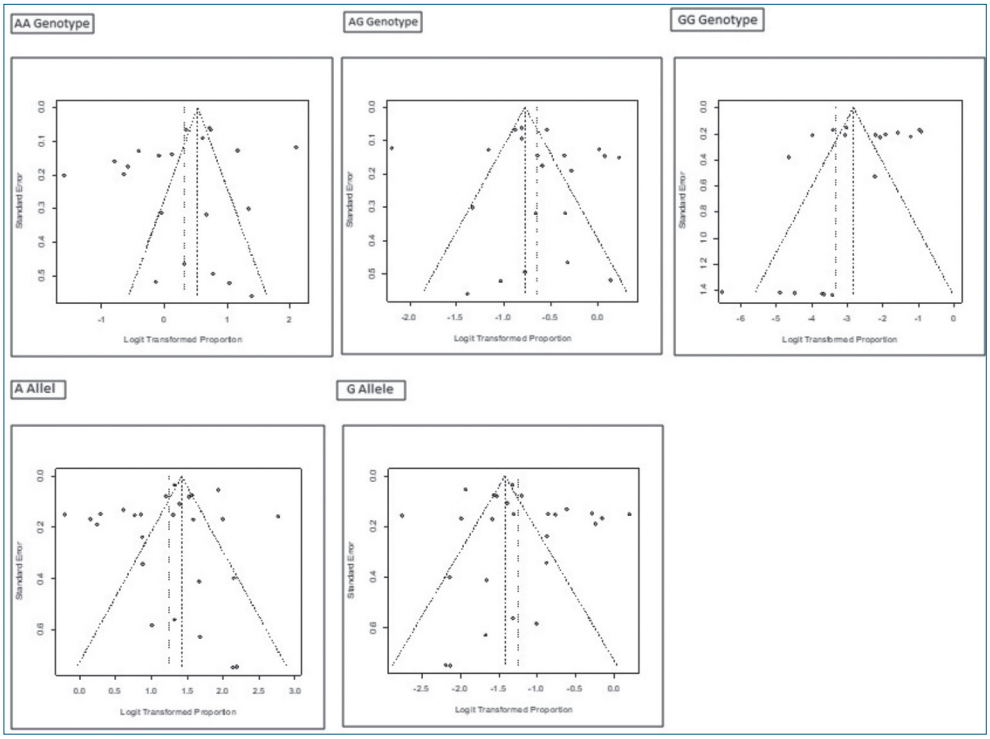

Fig 3. Funnel plots of AA, AG, and GG genotypes and A and G alleles of the *CAST* gene

Table 4. *Meta-analysis results of WBSF values by genotypes of the CAST gene*

Genotype	Number of Study	WBSF	95% CI	Heterogeneity		
				Cochran Q	P Value	I^2 (%)
AA	3	3.707	3.210-4.203	6.99	0.030	71.40
AG	3	3.893	3.762-4.024	0.63	0.728	0.00
GG	2	5.137	4.594-5.679	0.04	0.850	0.00

A and G alleles. Then, as a subgroup analysis, the results of studies giving WBSF values showing the relationship between c.2832A>G polymorphism of the *CAST* gene and meat tenderness were combined.

According to the results of this study, the pooled ratio of AA was 57.8%; however, the AA genotype proportions of individual studies ranged between 17% to 89%. The pooled ratio of AG genotype was 34.2%; however, the AG genotype proportions of individual studies ranged between 10% to 56%. For the GG genotype, the pooled ratio was 8%; however, the GG genotype proportions of individual studies ranged between 1% to 29%. In addition, the pooled ratio was 78%; however, the A allele proportions of individual studies ranged between 45% to 94%. The pooled ratio was 22%; however, the G allele proportions of individual studies ranged between 6% to 55% *(Fig. 2)*. This result shows that different individual studies vary significantly in genotype and allele gene frequencies. Therefore, the need for a meta-analysis of genotype and allele frequencies of *CAST* gene *c.2832A>G* polymorphism in cattle was justified.

Due to the genetic heterogeneity of the cattle breeds used in the studies included in meta-analysis, studies were classified in three groups in order to create more homogeneous subgroups. When the results obtained with this method were examined, it was determined that there was homogeneity between the studies in the *Bos indicus* and cross breed groups. In addition, in contrast to *Bos taurus*, cross breed and total evaluation, the pooled ratio of AG genotype was higher in the *Bos indicus* group than AA and GG genotypes.

According to meta-analysis results, the combined WBSF value for the AA genotype was higher than that observed in Li et al.[21] and lower than in other studies. WBSF value calculated by meta-analysis was very close to the value found by Curi et al.[10]. The results of the two studies for the GG genotype were combined, and the pooled WBSF value was the same as for Li et al.[21]. There was no WBSF value for the GG genotype in the study of Curi et al.[10].

Considering the studies that examined the relationship between the *c.2832A > G* polymorphism of the *CAST*

gene and meat tenderness, it was stated that this marker was significantly associated with WBSF. In the study of Li et al.[21], it was noted that the WBSF value did not differ between AA and AG genotypes, and the highest value belonged to the GG genotype, while Curi et al.[10] found that the WBSF value was significantly different between the AA and AG genotypes. The differences in the results show the necessity of the meta-analysis method.

The applications of the meta-analysis method in genetic studies in veterinary medicine are increasing rapidly. While bringing together results of studies on the same subject in genetics, it is necessary to identify studies that examine the same polymorphism of the same gene, and this is quite difficult. There are meta-analysis studies examining the effects of different gene polymorphisms on growth and yield characteristics for different animal species [26,27]. In meta-analysis studies on cattle, growth hormone gene *allul* polymorphism [11], *CSN3* gene polymorphism [12], *Beta-lactoglobulin* gene polymorphism [28], *UASMS2* polymorphism in the *leptin* gene [29], MspI derived variants of growth hormone gene [30] were examined. Especially in studies conducted on cattle, the effects of related genes on milk yield were investigated. Only one of them examined meat quality in cattle [29].

Unlike the others, the *CAST* gene in cattle was considered in this study. Among the many polymorphisms of the *CAST* gene, which were determined to affect meat tenderness, studies in which the *c.2832A>G* polymorphism in cattle were studied were included. Many criteria are examined in studies evaluating meat quality. However, only studies examining the meat tenderness feature were discussed in this study. Also, the association between the *CAST* gene's c.2832A>G polymorphism and meat tenderness has been investigated. Different results were obtained due to sample sizes [7,9,10]. Meta-analysis synthesized the results of the studies examining the relationship between *CAST* gene c.2832A>G polymorphism and meat tenderness in cattle in this research.

This study has some limitations. Because only the articles published in English and Turkish were selected, the results of the studies written in other languages could not be included. In addition, shear force values for very few studies could be obtained due to insufficient information in the analyses for meat tenderness.

In conclusion, with this meta-analysis, the genotype and allele frequencies of a polymorphism of the *CAST* gene and its association with meat tenderness were examined in detail. With the pooled ratio of genotype and allele frequencies and combined mean WBSF values for the AA, AG, and GG genotypes, closer results to the population parameter could be obtained. This study may provide valuable information on improving meat quality in cattle production. This SNP can be used as a reliable genetic marker for meat tenderness as a meat quality trait in cattle breeding.

Availability of Data and Materials

The dataset generated during the current study is available from the corresponding author (E. Uzabaci) on reasonable request.

Ethical Statement

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

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Conflict of Interests

The authors declared that there is no conflict of interest.

Author Contributions

EU, DD: Conception and design; DD: Acquisition of data; EU: Analysis and interpretation of data, writing the article; EU, DD: Final approval of the article.

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RESEARCH ARTICLE

A Comparison of the Efficacy of Selamectin and Fluralaner in the Treatment of the Canaries Infected with *Dermanyssus gallinae*

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Abstract: *Dermanyssus gallinae* is a hematophagous ectoparasite responsible for anaemia, weight loss, itching, dermatitis, and also decreased egg production in many domestic and wild bird species. The use of various synthetic acaricides such as avermectin and isoxazoline group is common in its treatment. This study aimed to compare the efficacy of Selamectin and Fluralaner on canaries during *D. gallinae* infestations. In the study, all canaries before treatment constituted the control group. Two groups of canaries were included in the study (n:40): Selamectin was applied to the first group (n:20) and Fluralaner to the second group (n:20). Selamectin was applied topically and infrascapularly at a 20 mg/kg dose, while Fluralaner was administered orally by a dose of 0.5 mg/kg. Parasite load was evaluated before and after treatment. With regard to the reduction in the mean number of red mites, the treatment successes of Selamectin and Fluralaner were evaluated as percentages of the efficacy. The percentage of efficacy of Selamectin and Fluralaner treatments were 80.0%, and 90.90%, respectively. We conclude that both drugs provide effective treatment in the treatment of *D. gallinae* infestation in canaries, nevertheless short-term clinical efficacy of Fluralaner was superior to Selamectin.

Keywords: *Dermanyssus gallinae, Canaries, Selamectin, Fluralaner*

Dermanyssus gallinae İle Enfekte Kanaryaların Tedavisinde Selamektin ve Fluralaner Etkinliğinin Karşılaştırılması

Öz: *Dermanyssus gallinae* evcil kuş türlerinde ve yabani kuş türlerinde görülen anemi, kilo kaybı, kaşıntı ve dermatite yol açan ayrıca yumurta üretiminin azalmasına neden olan hematofagöz bir ektoparazit türdür. *D. gallinae* tedavisinde avermektin ve izoksazolin grubu gibi çeşitli sentetik akarisitlerin kullanımı oldukça yaygındır. Bu çalışma, kanaryalarda *D. gallinae* enfestasyonlarının tedavisi amacıyla kullanılan Selamektin ve Fluralaner'in etkinliğini karşılaştırmayı amaçladı. Çalışmada tedavi öncesi tüm kanaryalar kontrol grubunu oluşturdu. Çalışmada yer alan kanaryalar (n:40) iki farklı gruba ayrıldı: birinci gruptaki kanaryalara (n:20) Selamektin tedavisi ve ikinci gruptaki kanaryalara (n:20) ise Fluralaner tedavisi uygulandı. Selamektin tedavisi lokal olarak, infraskapular bölgeye 20 mg/kg dozda uygulanırken, Fluralaner tedavisi ise 0.5 mg/kg dozda oral olarak uygulandı. Parazit yükü tedavi öncesinde ve tedavi sonrasında değerlendirildi. Ortalama kırmızı akar sayısındaki azalmaya bağlı olarak Selamektin ve Fluralaner'in tedavi başarıları etkinlik yüzdesi hesaplanarak değerlendirildi. Selamektin tedavisindeki etkinlik yüzdesi %80.0 iken Fluralaner tedavisindeki etkinlik yüzdesi ise %90.90 olarak tespit edildi. Çalışma sonucunda kanaryalarda *D. gallinae* enfestasyonlarının tedavisinde uygulanan hem Selamaktin hem de Fluralaner'in etkili tedavi sağladığı tespit edildi. Bu çalışmada tedavi edilen kanaryaların kısa süreli klinik etkinliği değerlendirildiğinde ise Fluralaner ile tedavinin etkinlik yüzdesinin Selamektin ile tedavinin etkinlik yüzdesine göre daha yüksek olduğu gözlemlendi.

Anahtar sözcükler: *Dermanyssus gallinae, Kanarya, Selamektin, Fluralaner*

INTRODUCTION

Dermanyssus gallinae is a nocturnal, hematophagous mite that is very common in poultry farms around the world. It causes weight loss, anemia, and dermatological lesions, and adversely affects reproductive functions [1]. There are some reports regarding human transmission [2]. These mites live in clusters in cracked and recessed environments [3]. They seem white, but turn red while sucking. Females are 0.7-1 mm and males are smaller, 0.3-0.6 mm in size. The optimum living ambient temperature is 20-25°C, however they cannot survive below -20°C and above 45°C [4]. The major struggle with these mites is that they can survive 5-9 months without sucking blood and they proliferate

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rapidly [5]. They are not species-specific as they can also hold on other domestic animals apart from humans and poultry [6]. They are vectors of many zoonotic diseases such as *Chlamydia psittaci*, *Coxiella burnetti*, *Salmonella* spp., *Listeria monocytogenes, E. coli* [1,7]. Clinical symptoms associated with this infestation include itching, insomnia, self-pecking, cannibalism, dermatitis, weakening and anemia, reduced laying rate, increased percentage of downgraded eggs, and death [8,9].

Several acaricides are widely used against *D. gallinae*. Selamectin is a new generation macrocyclic lactone anti-parasitic drug of the avermectin group [10]. Like other avermectins, it binds to gamma-amino-butyric acid (GABA) receptors in the nervous system and activates glutamate chloride ion channels in muscle synapses, thus disrupting the neuromuscular transmission of nematodes and arthropods [11]. Selamectin is widely used in small animals and exotic companion practices due to its broad spectrum of coverage, high margin of safety, and straightforward administration and packaging [10]. Fluralaner is a strong ligand-gated chloride channel inhibitor targeting insect nervous systems [12]. It works by blocking GABARs specifically and is a good acaricide for ticks and mites. Fluralaner has significant antagonistic actions for these GABARs and its high pest control activities against parasitic agents [13]. In comparison to other classic GABAergic chemicals, fluralaner demonstrates no cross-resistance against numerous insect species in both *in vivo* and *in vitro* investigations [14].

The aim of this study was to compare the clinical efficacy of Selamectin and Fluralaner against red mites, including the rate of killing, and their possible effects on mite reproduction after administration to canaries.

Material and Methods

Ethical Statement

This study was conducted in clinical cases naturally infested with *Dermanyssus gallinae* in Amasya and Samsun regions. For this reason, Ethics Committee approval was not required in our study, and the animal ethical rules in force during the study were followed. In addition, an Informed Consent Form (for each patient) was obtained from the owners.

Animal and Groups

A total of 40 owned gloster canaries (25 females and 15 males) *(Serinus canaria)* were enrolled in the study, in Amasya and Samsun, Türkiye. The age range of the canaries was 8-36 months, and body weights were 18-20 g.

Clinical Application Procedure

In the anamnesis, the canaries had itching, insomnia, moult, skin lesions, and decreased spawning rate and reproductive performance reported. In the clinical examination, the mites in the cage and on the canaries were observed with the naked eye. Traps constructed of folded cardboard papers were made and used before treatment and after treatment to measure the infestation rate. The mites were collected by punching holes in the surfaces of cardboard papers to create a gap. All traps were placed at a safe distance from each other on the floor. For 72 h, traps were placed inside perches. The parasitic population was counted as an estimated before the treatment and then 7 and 15 days after the initial treatment. Samples collected for ectoparasitic examination were examined under a light microscope (x10) and *D. gallinae* mites were detected. The canaries were housed in 4 cages with 10 canaries in each. They were randomly assigned to 2 study groups of 20 canaries (Group 1 was the Selamectin treatment group and group 2 was the Fluralaner treatment group) in 2 cages each, based on their health status, age (8-18 months), and body weight (18-21 g). The canaries were not exposed to any ectoparasite control product prior to initiation of treatment.

All canaries in both groups represented the control group before treatment. As group 1 Selamectin treatment; A drop solution containing 0.25 mL/15 mg Selamectin was used. With the help of a 0 size fine brush, 1 drop of solution (20 mg/kg) was applied topically and infrascapularly from the gap between the wing and neck. During the application, the hairs in the area were separated and the skin was exposed. The efficiency of the solution was augmented by applying to the hairless area at the neck-wing junction, where subcutaneous vascularization is intense. The Selamectin treatment was performed as a single dose. As for group 2 Fluralaner treatment; A stock solution was diluted with tap water to achieve the intended dosing concentration of 0.03 mg fluralaner/0.1 mL and administered orally to the canaries. The Fluralaner treatment was performed 2 times with an interval of 7 days.

Statistical Analysis

Changes in parasite load were assessed by examining mites in cage perches and canaries before and after treatments to confirm the presence of natural infestations. For each post-treatment, the antiparasitic efficacy (E) was calculated using the formula [15]:

$$E\ \% = \frac{A\text{-}B}{A} \ x100$$

where A is the number of mites before treatment and B is the number of mites after treatment.

Results

No adverse effects were reported in a total of 40 canaries in four cages following treatment. The mite population before treatment was approximately 450 in group 1 and

400 in group 2. At day 7, in Selamectin group (S1), the mite population declined significantly to 61.11% and in Fluralaner group (F1), the mite population declined significantly to 72.50% *(Table 1)*.

After 15 days of both treatments, in Selamectin group (S2), the mite population dropped significantly to 80.0% and in Fluralaner group (F2), the mite population dropped significantly to 90.90% *(Table 2)*. After the both treatments, the number of mites was significantly reduced.

When the short-term clinical efficacy of the two treatments was compared, Fluralaner showed a higher percentage of efficacy than Selamectin *(Fig. 1)*.

Discussion

Dermanyssus gallinae infestation is observed in many bird species, but also studies reports that many other species are affected, nearby being a zoonotic infection [16]. *D. gallinae* infestations result in death in progressive cases [17]. Studies show that skin lesions similar to those in birds are also seen in humans [18]. More than 35 compounds have been mentioned for the control of *D. gallinae* in birds (organophosphates, organochlorines, pyrethroids, carbamates amitraz, and endectocytes). Although some of these are theoretically efficient, they are insufficient in practice [19]. Selamectin is an efficient antiparasitic drug in veterinary medicine. Its long duration of action and broad therapeutic activity facilitate its use against a wide variety of internal and external parasites. In addition, it is preferred in domestic and wild bird species as it is suitable for widespread and safe use [10]. Selamectin can also be used successfully in canary species, and it has been reported in various publications that the appropriate dose should be investigated [10,19].

Selamectin was used in the treatment of *D. gallinae* in canaries and was found to be quite successful in a study comparing different preparations. However, the dose rate was not mentioned in the study [20]. In another study, Selamectin applied topically at a dose of 20 mg/kg reached its maximum level in 3 days, and it was reported that effective plasma concentrations were reached for 19 days [21]. Nevertheless, Selamectin can be used as an efficient anti-parasitic drug in bird species [19,21].

Oral administration of Fluralaner is routinely used in dogs to provide a safe and fast acting antiparasitic treatment that also disrupts flea reproduction [22,23]. Fluralaner's proven acaricidal effects after oral treatment suggested that a similar approach would be effective for the treatment of *D. gallinae*. Fluralaner has no cross-resistance against a variety of insect species in both *in vivo* and *in vitro* studies, unlike other conventional GABAergic compounds [24]. There has been no research on the use of oral Fluralaner to control *D. gallinae* in canaries. It has been reported that

Table 1. *First application efficiancy of Selamectin treatment (ES1) and Fluralaner treatment (EF1)*

Groups	Number of Mites Before Treatment	Number of Mites After Treatment	Antiparasitic Efficacy
ES1	450	175	61.11%
EF1	400	110	72.50%

Table 2. *Efficiancy of Selamectin treatment (ES2) and Fluralaner treatment (EF2).*

Groups	Number of mites Before Treatment	Number of Mites After Treatment	Antiparasitic Efficacy
ES2	175	35	80.0%
EF2	110	10	90.90%

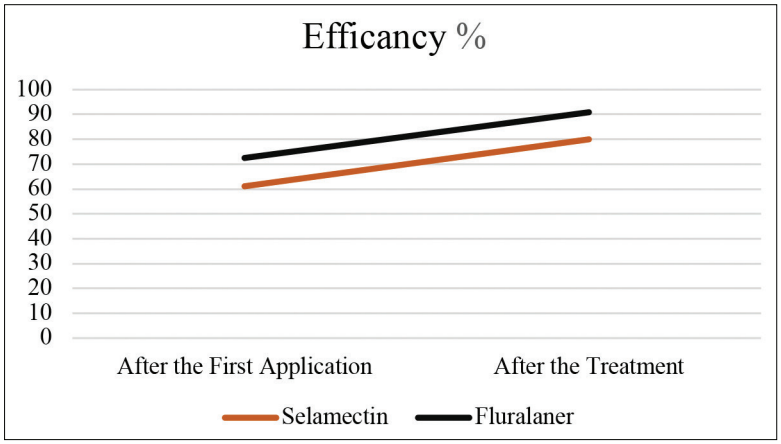

Fig 1. Efficacy of Selamectin and Fluralaner treatment

the application of 0.5 mg Fluralaner/kg body weight twice to hens with 7-day intervals is effective in controlling mites [25].

Brauneis et al.[26], added Fluralaner to the drinking water of laying hens twice with 7-day interval and, reported a reduction of up to 99% of *D. gallinae* mites within 3 days from the first application. It has been reported that up to 100% success was achieved within 2 days after the second application. Two oral applications of Fluralaner, 7 days apart, to canaries provide a maximum efficacy from the end of the second week, against bird red mite. The rapid mite killing effect is achieved within 4-8 h after application, it prevents the mite spawning and disrupts the life cycle of the mite. Therefore, oral administration of Fluralaner can effectively treat existing poultry red mite infestation leading to eradication of mites in canaries as well as poultry [26]. In another study, it is suggested that Fluralaner should be tried on eagles in the treatment of bird mites such as *D. gallinae* [27].

Antiparasitic treatment protocols should be held with caution especially in birds as they are very frail. Many agents are found to be toxic and most have many potential side effects. For example, Gozalo et al.[28] reported mortality associated with Fenbendazole, a very widespread used antiparasitic agent, with hemorrhagic enteritis, diffuse lymphoplasmacytic enteritis, small intestinal crypt necrosis, bile duct hyperplasia etc. In the present study, the absence of any side effects related with medications, contributes to the promising potential of Selamectin and Fluralaner in canary *D. gallinae* enfestations.

This study concluded that subcutaneous administration of a macrocyclic lactone antiparasitic drug, Selamectin, and oral administration of Fluralaner, a systemic acaricide, would offer an alternative treatment option for the control and treatment of *D. gallinae* in canaries. Under the light of the present data, both of the antiparasitic medications may be considered in *D. gallinae* infestations of canaries.

Ethical Statement

There is no connection between the authors of this study and the commercial companies of the products used in the study.

Availability of Data and Materials

The datasets and analysed during the current study available from the corresponding author (Ç. Esin) on reasonable request.

Funding Support

There is no funding source.

Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Contributions of Authors

ÇE, UA and SR are listed according to the determination of the subject, experimental design and writing stages, their contribution rates to laboratory studies and literature review, and corrections.

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Research Article

A Meta-Analysis of the Associations Between Prolactin (PRL) Gene Polymorphism and Milk Production Traits in Cattle

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Abstract: The meta-analysis method is used to combine results from many independent studies conducted in a different region or area on a specific subject qualitatively and quantitatively and achieve a consensus. The current meta-analysis was conducted to investigate the association between prolactin gene polymorphism and milk production traits in dairy cattle. The data were collected between 2002 and 2021, and a total of 26 published studies were included in this study. The standardized mean difference (SMD) between genotypes was obtained for statistical evaluation, and to this end, the genetic models (dominant; AA + AB and BB, recessive; AB + BB with AA, co-dominant; AA, BB and AB genotypes) were compared with each other. In the analysis results, cattle with the AB versus BB genotype (SMD=0.289, 95% CI 0.005, 0.573) were found to have a statistically significant higher protein yield in comparison with the AA versus AB and AA versus BB genotypes. The relationships between prolactin gene polymorphism and milk yield, fat yield, fat content, and protein content traits were not found to be statistically significant.

Keywords: *PRL gene, Polymorphism, Co-dominant, Meta-analysis, Standardized mean difference*

Sığırlarda Prolaktin (PRL) Geni Polimorfizmi İle Süt Verim Özellikleri Arasındaki İlişkilerin Meta Analizi

Öz: Meta Analizi, belirli bir konu üzerinde farklı bölge veya alanlarda yapılmış birçok bağımsız çalışmanın sonuçlarını niteliksel ve niceliksel olarak birleştirmek ve fikir birliğine varmak amacıyla kullanılan bir yöntemdir. Meta-analizi, süt sığırlarında prolaktin geni polimorfizmi ile süt verim özellikleri arasındaki ilişkiyi araştırmak için uygulanmıştır. Veriler 2002-2021 yılları arasında yayınlanmış 26 çalışma analizlere dahil edildi. İstatistiksel değerlendirme için genotipler arasında standartlaştırılmış ortalama farkı (SMD) elde edildi ve bu amaçla genetik model: baskın; AA + AB ile BB, çekinik; AA ile AB + BB, eş baskın; AA, BB ve AB genotipleri birbirleriyle karşılaştırıldı. Analiz sonuçları, AB ile BB genotipine sahip sığırların (SMD=0.289, %95 CI 0.005, 0.573), AA ile AB ve AA ile BB genotipli sığırlara kıyasla istatistiksel olarak anlamlı bir yüksek protein verimine sahip olduklarını göstermiştir. Prolaktin geni polimorfizmi ile incelenen diğer süt verimi, yağ verimi, yağ içeriği ve protein içeriği özellikleri arasındaki ilişkiler istatistiksel olarak anlamlı değildi.

Anahtar sözcükler: *PRL geni, Polimorfizm, Ko-dominant, Meta-analizi, Standartlaştırılmış ortalama farkı*

Introduction

Meta-analysis is a method used to combine and re-evaluate results from at least two or more studies for the purpose of estimating or evaluating the joint effect size. It is applied to the results of further research in a particular field and ensures a strong analysis and sample effect [1]. It is necessary to conduct a meta-analysis in case of the repeating sample number [2]. The research on genetics in farm animals mainly focuses on identifying genes with an effect on economically important traits. Most studies on dairy cows have identified genes determining or specifying variations in milk traits. In livestock, milk genes and hormones are researched as perfect candidate genes because of their biological significance and linkage with important quantitative traits.

The prolactin gene is a quantitative trait locus and is considered a potential genetic marker that can be utilized in enhancing production traits in dairy cattle. The prolactin (PRL) gene polymorphisms are associated with milk yield traits [3]. The process of milk production is very

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complicated with numerous interrelated factors (genetic and hormonal factors), and prolactin is one of the most essential hormones in this process [4,5].

Prolactin is one of the polypeptide hormones produced by the anterior pituitary gland and plays a very important role in lactogenesis, synthesis, regulating the growth of the udder, and secretion of milk proteins in dairy cows [6-10]. The prolactin (PRL) gene is located on chromosome 23, is approximately 10 kb in size, includes 5 exons and 4 introns, and encodes a 199 amino-acid polypeptide [6,11,12]. This site plays an essential role as a genetic marker for the genetic characterization of cattle populations [13]. There are significant associations between the PRL-*Rsa*I locus and milk and fat yield in dairy cows. The RFLP technique can detect a small change naturally occurring through deletions or insertions of one or more pairs of nucleotides in the genome [5,11]. A shift of adenine to guanine (A103G) in the PRL gene causes point mutations, resulting in two alleles (A and B). The adenine at position 103 is referred to as the A allele. The restriction site for the *Rsa*I restriction enzyme was caused by a mutation from adenine (A) to guanine (G), and the allele was referred to as the B allele. Three genotypic patterns were revealed from the digestion of a 156 bp fragment of exon 3 region in the prolactin gene by RsaI. The first uncut pattern fragment of 156 bp was referred to as the AA genotype, the second pattern fragments (82 and 74 bp) were denoted as the BB genotype (the presence of a restriction site), and the third one with three fragments (156, 82, and 74 bp) was referred to as the AB genotype [12,14]. Several studies have demonstrated that polymorphisms in the genomic sequence of the prolactin (PRL) gene have significant associations with different economic traits in dairy cattle [6]. The present meta-analysis was conducted to combine results from many independent studies on prolactin gene polymorphism and its association with milk production traits in dairy cattle.

Material and Methods

Scientific journals were searched for meta-analysis on prolactin (PRL) gene polymorphism and its association with milk production traits in dairy cattle. To obtain the standardized mean difference, the random effect model and the fixed effect model were used. The model selection was performed based on whether the effects of studies were homogenous or heterogeneous: when the study effects were homogeneous, the fixed model was employed, and when the study effects were heterogeneous, the random model was utilized. Q statistic was employed with the objective of determining heterogeneity between studies, and the index was used for its further quantification. Cohen's method [15] was used for the standardized mean difference.

Data Extraction

The authors extracted data independently, and the Microsoft Excel format was used for data collection. The researchers carried out discussions to avoid mistakes or errors with regard to studies and data collection. At first, 51 studies were searched, and a total of 26 studies on the same genotypes of the prolactin gene were reached and selected for this study. The important information, involving the author's name, the year of publication, country, sample size, genotypes, breed, lactation milk yield (LMY), fat yield (kg), and protein yield, was collected from the related studies.

The Search Strategy of Sources

To carry out the meta-analysis, essential criteria for systematic reviews and meta-analyses were used for study selection. A comprehensive search was done among studies published in different languages, journals, and databases on the associations between prolactin gene polymorphisms and milk production traits between 2002 and 2021. Google Scholar, ResearchGate, Wiley, Springer, Taylor & Francis, PubMed, and Elsevier were searched for studies, and a number of keywords (meta-analysis, milk production traits, polymorphism, PRL, association, cattle) were used to find the required studies.

Selection and Exclusion of Studies

During study selection, certain criteria were applied, including; (I) the number of animals of each genotype, (II) association between the gene polymorphism and milk production traits, (III) the reported least square means for milk production traits, and (IV) standard deviation/errors and average means of the involved trait for every genotype (if it is a standard error, it is converted to a standard deviation with the presence of the animal number).

Studies were rejected or excluded if they had the following characteristics;

- Publications as a summary,
- No indication of the number of animals of each genotype,
- Studies did not have standard deviation/errors and average means per genotype, and
- Duplicate studies.

From a total of 51 studies, 25 studies were excluded since they did not have the above-mentioned criteria, and accordingly, 26 studies were selected for analysis.

Statistical Analysis

The meta-analysis was carried out using the Stata 11.2 software (StataCorp 2001; Stata Statistical Software), and $P<0.05$ was accepted as a significance level. For the gene locus, the database was arranged individually, and the four methods below were followed;

- Concerning differences between means, two effect models (random and fixed) were used in the metanalysis. According to the I^2 statistics, the fixed model was employed in case of the homogenous study results, while the random model was employed in case of the heterogeneous study results (the levels of significance for I^2 were defined as 0.10 in the heterogeneity analysis) [2,16].

- In the study, the following four genetic models were used: dominant; AA + AB versus BB, recessive; AA versus AB + BB, co-dominant; AA versus BB versus AB.

- The standardized mean differences (SMDs) and standard deviation were computed at a 95% CI (Confidence Interval) to estimate the capabilities between the three genetic models for every trait.

- For standardized mean differences, Cohen's method [15] was used to assess the effect size.

Estimation of Heterogeneity

In this study, the I^2 test was conducted with the objective of estimating heterogeneity between the studies. The random effect model was applied due to the high heterogeneity [2,16].

Results

In this study, three performance traits (lactation milk yield, fat yield, and protein yield) were analyzed in a total of (26) studies by the meta-analysis technique, using the random and fixed effect models. Each trait was analyzed separately. *Table 1* and *Table 2* contain the results for heterogeneity, standardized mean difference (SMD) with a 95% CI, and significances.

The following alleles were considered and used in this study: dominant (AA+AB versus BB), recessive (AA versus AB+BB), complete over-dominant (AA+BB versus AB), and co-dominant (AA + versus AB, AA versus BB, and AB versus BB). The statuses of the heritage pattern of alleles are shown in *Table 1*.

Upon analyzing 26 studies for lactation milk yield (LMY), all genetic models showed no significant (P>0.05) association between the genotypes of the prolactin gene and milk yield (P>0.05).

When 14 studies were analyzed in terms of fat yield, the results showed no significant (P>0.05) association between prolactin gene polymorphisms and fat content. Concerning fat content from the analysis of 21 studies, no significant association (P>0.05) was detected between prolactin gene polymorphisms and all genetic models.

With regard to protein yield (n=14), a significant association was found between prolactin gene polymorphisms and the AB versus BB genotype (P<0.05). No significant association (P>0.05) was identified between protein yield and the other genetic models. Concerning protein content (n=18), no significant association was determined between prolactin gene polymorphisms and all genetic models (P>0.05).

Table 1. *Genetic model analysis of the associations between PRL polymorphism and milk production traits*

Traits	n	AA+AB versus BB Dominant Model						AA versus AB+BB Recessive Model						AA+BB versus AB Complete over Domn. Model					
		I^2	Model	SMD	95%	CI	P	I^2	Model	SMD	95%	CI	P	I^2	Model	SMD	95%	CI	P
Lactation Milk Yield	26	14.6	F	-0.009	-0.102	0.084	0.850	46.3**	R	-0.01	-0.067	0.095	0.727	27.9*	R	-0.003	-0.068	0.063	0.937
Fat Yield	14	2.5	F	-0.040	-0.186	0.105	0.587	0.0	F	-0.03	-0.099	0.033	0.329	0.0	F	-0.025	-0.090	0.040	0.451
Fat Content	21	74.0**	R	-0.226	-0.466	0.014	0.065	47.8**	R	0.03	-0.061	0.120	0.522	54.0**	R	0.093	0.000	0.186	0.050
Protein Yield	14	6.8	F	0.025	-0.121	0.170	0.739	3.1	F	-0.05	-0.116	0.016	0.140	0.0	F	-0.054	-0.119	0.011	0.106
Protein Content	18	0.0	F	0.013	-0.101	0.126	0.823	0.0	F	0.009	-0.048	0.067	0.753	0.0	F	0.001	-0.057	0.059	0.974

** P<0.10; ** P<0.01;* ***n:*** *number of publication;* ***I²:*** *variation in SMD attributable to heterogeneity;* ***F:*** *Fixed;* ***R:*** *Random*

Table 2. *Results of the meta-analysis on the associations between PRL polymorphism and milk production traits*

Traits	AA Versus AB							AA Versus BB						AB Versus BB					
	n	I^2	Model	SMD	95%	CI	P	I^2	Model	SMD	95%	CI	P	I^2	Model	SMD	95%	CI	P
Lactation Milk Yield	26	47.1**	R	0.011	-0.074	0.096	0.796	32.7*	R	0.1	-0.010	0.022	0.470	39.9*	R	0.087	-0.063	0.238	0.254
Fat Yield	14	0.0	F	-0.034	-0.102	0.034	0.328	15.8	F	-0.09	-0.269	0.090	0.327	8.6	F	0.004	-0.147	0.156	0.956
Fat Content	21	45.1*	R	0.041	-0.052	0.133	0.388	76.8**	R	-0.3	-0.610	0.001	0.051	74.3**	R	-0.218	-0.477	0.041	0.099
Protein Yield	14	4.3	F	-0.057	-0.125	0.011	0.101	15.8	F	-0.03	-0.213	0.146	0.717	62.2**	R	0.289	0.005	0.573	**0.046**
Protein Content	18	0.0	F	0.006	-0.054	0.066	0.851	0.0	F	-0.01	-0.145	0.118	0.846	0.0	F	0.030	-0.096	0.156	0.642

** P<0.10; ** P<0.01;* ***n:*** *number of publication;* ***I²:*** *variation in SMD attributable to heterogeneity;* ***F:*** *Fixed;* ***R:*** *Random*

Discussion

The meta-analysis method is used to combine results from numerous independent studies conducted in a different region or area on a specific subject qualitatively and quantitatively and assist in achieving a consensus [17]. Meta-analysis represents a test method using the results of numerous studies conducted in the area, offering a more powerful analysis. There is a need for meta-analysis if a lot of similar studies have been performed on a particular subject, and there has been a difference of opinion in some of these studies [2]. Therefore, the current research was conducted for a meta-analysis on PRL gene polymorphism and its association with milk traits in dairy cattle. The meta-analysis showed that the relationships of the prolactin gene with other factors must be studied by means of the co-dominant genetic model in general.

In the analysis of 26 studies for lactation milk yield (LMY), all genotypes showed no significant (P>0.05) association between PRL gene polymorphism and milk yield. This result is similar to the studies carried out by many researchers [12,18-25]. They suggested that the polymorphisms of the prolactin gene did not affect milk yield. However, the obtained results contradict the findings obtained by some researchers [7,8,11,26,27], indicating that the polymorphisms of the prolactin gene had an impact on milk yield.

Upon analyzing 14 studies for fat yield, no significant association was identified between prolactin gene polymorphisms and fat content (P>0.05). Similar results have previously been reported by various authors [8,18,21,28] for different dairy cattle breeds. Moreover, these results are in disagreement with the findings of other authors [7,19,26]. When 21 studies were analyzed with regard to fat content, no significant (P>0.05) association was detected between prolactin gene polymorphisms and all genetic models. While similar results were reported by some authors [8,11,21,25-27], contradictory results were indicated by the others [7,28].

Considering protein yield, there was a significant (P<0.05) association between prolactin gene polymorphisms and the AB and BB genotype. Similar results were reported by some authors [7,8,18,19,21,28]. However, this result is in disagreement with the studies by [25,26].

Considering protein content, no significant association was revealed between prolactin gene polymorphisms and all genetic models (P>0.05). Similar results were reported by a number of authors [7,8,18,19,21,22,25,28].

It is thought that the results obtained in this study will make a contribution to developing new strategies necessary for breeding dairy cattle.

In accordance with the results obtained from the current meta-analysis, a single significant association was found between prolactin gene polymorphism and protein yield in the AB and BB genotypes. No significant associations were observed between milk yield, fat yield, fat content, protein yield, and protein content and PRL genotypes. Since various conflicting results have been reported by the authors on this subject, more studies should be conducted to determine the PRL gene polymorphism and investigate its impact on milk yield traits. Moreover, to obtain more reliable results, the genotype x environment interaction should be taken into account, group comparisons should be made to minimize outcome variability with more articles, and the usability of the results obtained by applying the correct statistical model for breeding purposes should be discussed.

Availability of Data and Materials

Data sets are not deposited in different repositories, and data from a third party were not used. The data are original, and users can get it from corresponding author (M. Özdemir).

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Competing Interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Author's Contributions

MÖ and ZM designed the study, ZM, KE, ES, and EB collected all the data and arranged them for analysis. MÖ conducted the statistical analysis, and MÖ and ZM wrote the paper.

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Research Article

Evaluation of Microplastic Presence in Yogurt Production Process

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Abstract: Plastics, which have made our lives easier since their invention and have found a wide range of applications because they offer numerous solution alternatives, are currently being investigated as a potential food safety risk. Microplastics (MPs) are defined as plastic waste particles smaller than 5 mm in size. Microplastics are commonly consumed orally, and their presence in various foods has been reported. The purpose of this study was to investigate the presence of MPs in yogurt production steps. The study's samples were drawn from a medium-sized national yogurt producing facility in İstanbul. Initially, samples were subjected to artificial digestion. They were subsequently filtered by a vacuum pump. Suspicious MP in the filters were examined with a binocular microscope and classified based on their size, color, and shape. Finally, SEM and ATR-FTIR techniques were utilized to characterize MPs. According to the results, the filters of twelve process steps/sampling locations contained a total of 171 microplastic particles. MPs were prevalent within the range of 20 to 580 particles L^{-1}. The concentration of MPs in raw milk and yogurt containers were found extremely high. To evaluate the level of risk associated with MP and to reduce MP contamination at plants of varying sizes, interdisciplinary research is required.

Keywords: *Food pollution, Microparticles, Microplastics, Polymer particles, Yogurt*

Yoğurt Üretim Sürecinde Mikroplastik Varlığının Değerlendirilmesi

Öz: Buluşlarından bu yana hayatımızı kolaylaştıran ve çok sayıda çözüm alternatifi sunduğu için geniş bir uygulama alanı bulan plastikler, günümüzde potansiyel bir gıda güvenliği riski olarak araştırılmaktadır. Mikroplastikler (MP'ler), boyutu 5 mm'den küçük plastik atık parçacıkları olarak tanımlanmaktadır. MP'ler genellikle ağız yoluyla organizmaya alınırlar ve çeşitli gıdalarda da bulundukları rapor edilmiştir. Bu çalışmanın amacı, yoğurt üretim basamaklarında mikroplastik partiküllerin varlığını araştırmaktır. Araştırmanın örnekleri İstanbul'da faaliyet gösteren orta ölçekli bir ulusal yoğurt üretim tesisinden alınmıştır. Toplanan örnekler ilk önce yapay sindirime tabi tutulmuştur. Daha sonra bir vakum pompası ile filtrasyon işlemi uygulanmıştır. Filtrelerdeki şüpheli MP'ler, binoküler mikroskopla incelenmiş, boyutlarına, renklerine ve şekillerine göre sınıflandırılmıştır. Son olarak, SEM ve ATR-FITR teknikleri kullanılarak MP'ler karakterize edilmiştir. Sonuçlara göre, on iki işlem aşamasının/örnekleme lokasyonunun filtrelerinin toplam 171 mikroplastik parçacık içerdiği tespit edilmiştir. Mikroplastikler, 20 ila 580 partikül L^{-1} aralığında bulunmuştur. Çiğ süt ve yoğurt kaplarındaki mikroplastik konsantrasyonunun son derece yüksek olduğu görülmüştür. Farklı ölçeklerde yoğurt üretimi yapan tesislerde MP risk seviyesini ortaya koyacak, MP kontaminasyonunu önleyecek veya asgari seviyeye indirebilecek tedbirlerle ilgili interdisipliner çalışmalar yapılması gereklidir.

Anahtar sözcükler: *Gıda kirliliği, Mikropartiküller, Mikroplastik, Polimer parçacıkları, Yoğurt*

Introduction

Plastics, which have made our lives easier since the day they were invented and have found a wide variety of applications because they offer abundant solution alternatives, are now being investigated as a potential food safety risk. Plastics have become a major source of problems for the environment, animal health, and human health, with their waste and residues spreading uncontrollably into the environment during their production, use, and disposal after use [1,2]. Microplastics (MPs) are defined as plastic waste particles smaller than 5 mm in size [2,3]. Based on their origin, MPs are classified as primary or secondary material. The secondary groups of MPs are microparticles that are the result of environmental degradation and whose base material is not MPs and that

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are subsequently formed by mechanical tearing, abrasion, and the fragmentation of larger plastic objects or related debris [4].

In scientific studies, the harmful effects of MPs on the human body have been reported. In addition to being a physical hazard, MPs can serve as carriers for other chemicals with known toxicity, such as environmental pollutants and plastic additives [5-7]. MPs have been shown to cross the placental barrier, which is known to be permeable to numerous toxic substances [6]. These dangers fall into four major categories: gastrointestinal toxicity, liver toxicity, neurotoxicity, and reproductive toxicity [2]. MPs also prepare the environment for biofilm formation and allow pathogenic microorganisms to enter the body, which is a significant potential threat [8].

Microplastics are commonly consumed orally, and their presence in various foods has been reported. Research has focused on foods of marine origin, including invertebrates, crustaceans and fish [9,10], microplastics have also been found in table salt [11,12]; sugar [13]; beer [12]; water [12,14-16], soft drinks [17], honey [13,18], and broilers [19,20]. There are two major microplastic sources. The granular raw material used to mold new plastic products and polymer microparticles added to cosmetics as exfoliants and abrasives are the primary sources. Microparticles formed by abrasion access environmental water systems, inevitably causing secondary pollution and increasing the dangers for humans [21]. The secondary source reflects the deterioration of larger plastics [22]. Numerous MPs, which are environmental contaminants of public concern, can accumulate in the food chain. Yogurt plays a significant role in global nutrition strategies and is recommended for all age groups. Moreover, yogurt is essential as the first recommended complementary food to be added to breast milk in the complementary feeding of infants after the sixth month [23].

The potential presence of MP contamination in yogurt production may pose a significant public health risk. The purpose of this study was to investigate the presence and the source of MP particles in yogurt production steps.

Material and Methods

Sampling

The study's samples were drawn from a medium-sized national yogurt producing facility in İstanbul. In this facility, which routinely continues to produce "set type yogurt", process steps have been determined and critical control points and hazard types have been marked on the workflow chart according to the food safety management system applied in the enterprise. On a randomly selected production day, parallel sampling was performed at enterprise locations and equipment where MP contamination was expected during the process. Before accepting milk, the bulk tank's wall, which is the first point of contact with raw milk on the CIP (Clean-in-place) process band, was rinsed with MP-free ultrapure water, and 100 mL of this water was obtained as a sample. 100 mL-g samples were taken from the phases of raw milk acceptance, filtration, clarification, separated cream, pasteurization, starter culture addition, filtration before filling, and ready-to-eat last product. Furthermore, samples were taken from the starter culture and the empty yogurt buckets, which were added to the process line later *(Fig. 1)*. A 100 mL sample of ready-to-use starter culture was taken for starter culture. The empty yogurt buckets were filled with 100 mL of MP-free water and brought to the laboratory with all of the sealed samples for analysis.

Analyse Safety

Plastic-free materials have been selected for all sampling and analysis consumables and equipment. The solutions used in the analysis, including ultrapure water, multi-

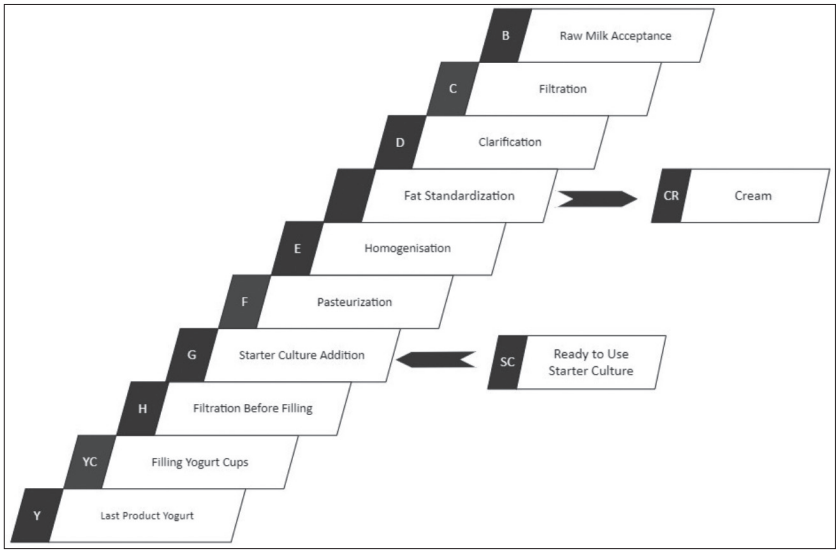


Fig 1. Process steps/sampling locations of yogurt production

enzymatic detergent, ethylenediaminetetraacetic acid disodium salt (EDTA), and tetramethylammonium hydrate, were analyzed for the presence of MP. Before use, empty sample bottles were rinsed with MP-free water and subjected to microscopic examination. All filtration processes were performed in a laminar flow cabinet to prevent MP contamination by airborne particles.

Positive control samples were prepared by adding Polypropylene (PP), Polystyrene (PS), polyethylene (PE), Polyvinyl Chloride (PVC), Linear low-density polyethylene (LLDPE), Thermoplastic elastomers (TPE) to ultrapure water were included in the study to control the analysis. Air and ultrapure water blank samples were examined under a microscope, and it was determined that there was no contamination caused by the analysis method.

Artificial Digestion

In our study, the methodology proposed by Costa Filho et al.[4], Kutralam-Muniasamy et al.[24], and Diaz-Basantes et al.[22] was implemented. Prior to analysis, each milk, cream, and yogurt sample were transferred to an erlenmeyer flask that had been cleaned with MP-free ultrapure water. Due to their density, yogurt and cream were difficult to filter through. In a glass bottle, 25 mL of a yogurt/cream sample was combined with 40 mL of MP-free ultrapure water by vigorously shaking. In contrast, milk samples did not require this step. The samples were then mixed for two minutes at 40°C after 2 mL of a multi-enzymatic detergent was added. Then, 10 mL of sodium ethylenediamine tetra acetate was added and stirred for an additional three minutes at a temperature of 40°C. Finally, 30 mL of tetramethylammonium hydrate was added, and the mixture was incubated at 40°C for 24 hours. After removing yogurt samples from the incubator, they were filtered immediately.

Sample Filtration

Every piece of filtration equipment was washed with MP-free ultrapure water before and after each sample filtration. Filtration was performed at a pressure of approximately 0.5 bar using glass microfiber filters (Whatman, Grade GF/B circles, 47 mm) with a pore size of 1 µm via a vacuum pump [12,13,15].

Microscopic Analysis and Visual Imagination

Filters were carefully transferred to glass petri dishes by using metal tweezers. They were air-dried at room temperature and subsequently stored for analysis. Filter papers were examined using a binocular biological microscope (Olympos CX31) with a camera (Canon A640) attached at a magnification of 4x. Filter papers were examined under the binocular biological microscope (Olympos CX31) with a camera (Canon A640) attached at magnification level 4x. Particles believed to be MPs were photographed using Kameram Software 1.3.0.8 (Mikrosistem, Turkey). The particles were measured and sorted by color, shape, and size using IC Measure (The Imaging Source®, 2.0.0.286, Germany).

SEM and FTIR Analysis

The morphological characterization and elemental composition of the particles that give a polymer image detected and marked by the light microscope were performed in high vacuum pressure mode and at different magnifications operating at an acceleration voltage of 10.00 kV in the secondary electron and backscattering modes using scanning electron microscopy (SEM, JSM-7001F, Jeol). The material chemical composition of microplastics was identified using FTIR spectroscopy (Agilent Cary 630). The results were evaluated using Agilent Polymer Handheld ATR Library, Agilent Elastomer Oring and Seal Handheld ATR Library and Agilent ATR General Library.

Calculation of Microplastic Ingestion by Humans

The recommended daily amount of yogurt (dairy product) for adults is three servings (1 serving = 240 mL)., while children, adolescents, pregnant-breastfeeding women, and postmenopausal women should consume two to four servings. These groups consume 720 mL and 480-960 mL, respectively, per day [23]. The total number of microplastic particles consumed orally is the basis for our risk assessment. This evaluation does not contain any toxicokinetic components.

$$EDI = \frac{W \times C}{100}$$

EDI = Estimated daily intake of MPs with yogurt

W = Recommended amount of yogurt (mL/day)

C = Microplastic concentration (particle number/100 mL)

Statistical Evaluation

Descriptive statistics were used to summarize the characteristics (number of MPs, length of MPs) and distribution of the dataset by SPSS 21.0.

Results

In our research, glass microfiber filters were examined through a microscope. To prevent erroneous assessments, the optical properties of the glass fiber filters have been thoroughly examined. After this procedure, the evaluation of the filters commenced. The properties of microplastic particles, including their color, shape, and number, are detailed in *Table 1*. Except for the ready-to-use starter culture filtration, MP was detected in all of the observed filters. Examples of microscopic images of typical micro-

Table 1. *MPs descriptive statistical data at all steps*

Process Step/ Sampling Location	*Total MP Number/100 mL	MPs' Size Dispersion in Samples (µm)	
		Range (mean±sd)	Median
A	2	179-1256 (717.50±761.55)	717.50
B	10	32-3697 (1248.60±1357.80)	913.50
C	8	68-4933 (1685.50±1950.76)	765.50
D	9	96-2533 (1036.33±734.70)	915.00
E	9	114-4994 (973.78±1563.28)	373
CR	9	103-956 (313.56±253.79)	239
F	13	36-3747 (1280.23±1263.22)	605
SC	0	-	-
G	16	9-2675 (380.38±653.42)	155
H	9	30-1688 (284.13±407.37)	152.50
YC	58	10-2913 (345.21±554.82)	128.50
Y	28	31-4946 (702.71±1200.11)	109

**MPs greater than 5000 µm are not evaluated.*
***A:** Bulk tank, **B:** Raw Milk Acceptance, **C:** Filtration, **D:** Clarification, **E:** Homogenisation, **CR:** Cream, **F:** Pasteurization, **SC:** Ready to Use Starter Culture, **G:** Starter Culture Addition, **H:** Filtration Before Filling, **YC:** Filling Yogurt Cups, **Y:** Last Product Yogurt*

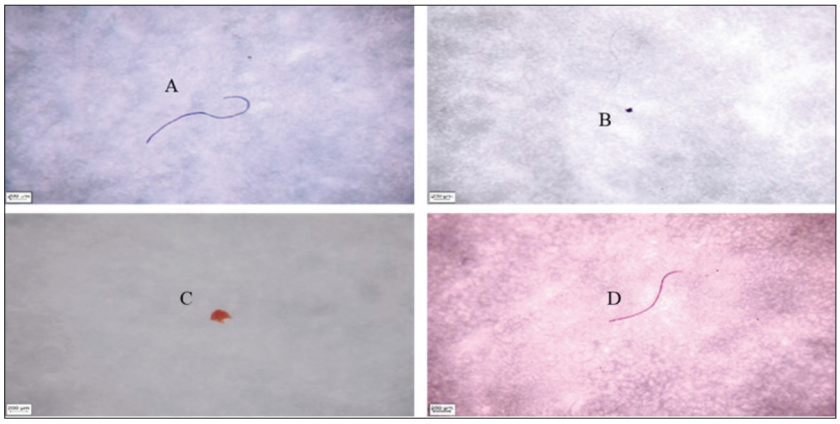


Fig 2. Image of microplastics in binocular biological microscope

A: Ethylene propylene fibre (1974 µm), **B:** Neoprene fragment (96 µm), **C:** Polytetrafluoroethylene fragment (275 µm), **D:** Polyacrilamide fibre (1394 µm)

plastics collected from process steps and sampling locations are shown in *Fig. 2*. The results of microplastic ratios based on colors, shapes and sizes are shown in *Table 1, Table 2, Fig. 3* and *Fig. 4*.

The filters of twelve process steps/sampling locations contained a total of 171 microplastic particles. In the range of 20 to 580 particles L^{-1}, microplastics were abundant. Example process step YC had the most microplastics in its filter (580 particles L^{-1}) and process step A contained 20 particles L^{-1} of microplastics at the lowest concentration.

Black, blue, brown, gray, green, orange, pink, red, purple, reddish brown, and transparent white were among the many hues exhibited by the microplastics described.

IC Measure software was used to determine the size distribution of microplastics, with fibres measured along their true length and parts measured along their longest dimensions. Of the total microplastics detected, 1-150 µm (43.27%) were dominated by microplastics *(Table 2)*. Using SEM, the surface morphologies of representative microplastics were observed, and the outcomes are depicted in *Fig. 5*.

Table 2. MPs size dispersion at all process step/sampling location

Process Step/ Sampling Location	MPs' Size Categorisation (µm)					
	1 - 10 n (%)	10.1 - 50 n (%)	50.1 - 150 n (%)	150.1 - 500 n (%)	500.1 - 1000 n (%)	1000.1 - 5000 n (%)
A	0	0	0	1 (50.00%)	0	1 (50.00%)
B	0	1 (10.00%)	3 (30.00%)	1 (10.00%)	0	5 (50.00%)
C	0	0	4 (50.00%)	0	0	4 (50.00%)
D	0	0	1 (11.11%)	1 (11.11%)	4 (44.44%)	3 (33.33%)
E	0	0	2 (22.22%)	4 (44.44%)	1 (11.11%)	2 (22.22%)
CR	0	0	1 (11.11%)	7 (77.77%)	1 (11.11%)	0
F	0	1 (7.69%)	2 (15.38%)	2 (15.38%)	2 (15.38%)	6 (46.15%)
SC	0	0	0	0	0	0
G	1 (6.25%)	2 (12.50%)	5 (31.25%)	5 (31.25%)	2 (12.50%)	1 (6.25%)
H	0	1 (11.11%)	3 (33.33%)	4 (44.44%)	0	1 (11.11%)
YC	1 (1.72%)	6 (10.34%)	24 (41.38%)	16 (27.59%)	6 (10.34%)	5 (8.62%)
Y	0	4 (14.29%)	12 (42.86%)	4 (14.29%)	3 (10.71%)	5 (17.86%)

***A:** Bulk tank, **B:** Raw Milk Acceptance, **C:** Filtration, **D:** Clarification, **E:** Homogenisation, **CR:** Cream, **F:** Pasteurization, **SC:** Ready to Use Starter Culture, **G:** Starter Culture Addition, **H:** Filtration Before Filling, **YC:** Filling Yogurt Cups, **Y:** Last Product Yogurt*

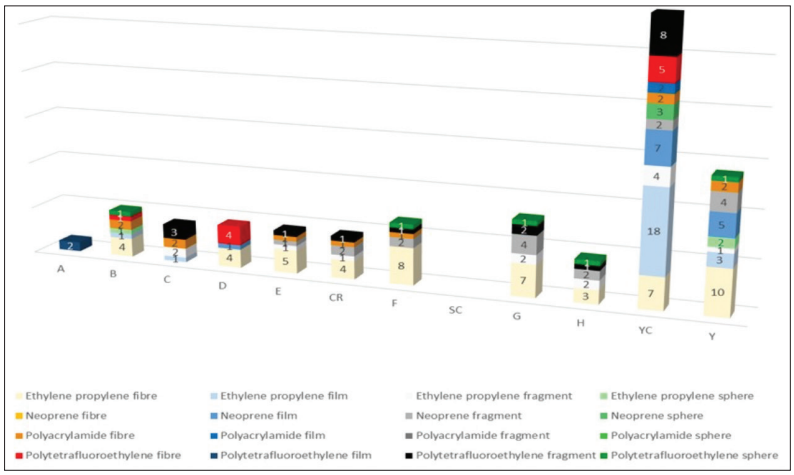


Fig 3. MPs shape and polymer type dispersion at all process step/sampling location (n)

The results of the ATR-FTIR analysis of the microplastic particles' chemical composition are depicted in *Fig. 6*. In the samples, four different types of microplastics were identified: ethylene propylene, neoprene, polyacrylamide, and polytetrafluoroethylene. Ethylene propylene was the most common type of microplastic found in the specimens.

Microplastic risk assessment in yogurt was calculated according to the consumption amounts recommended in Turkish Nutrition Guide [23]. Considering the age groups and important life stages, the consumption amounts given in the Turkish Nutrition Guide were evaluated as 201.60±14.40 MP ingestion/day in adult individuals, and children, adolescents, pregnant-breastfeeding women, and post-menopausal women 134.40±9.6 - 268.8±19.2 MP ingestion/day.

Discussion

In this study, the number, type, size, color, and shape of microplastics found in the yogurt manufacturing process were evaluated. Microplastic contamination may pose a

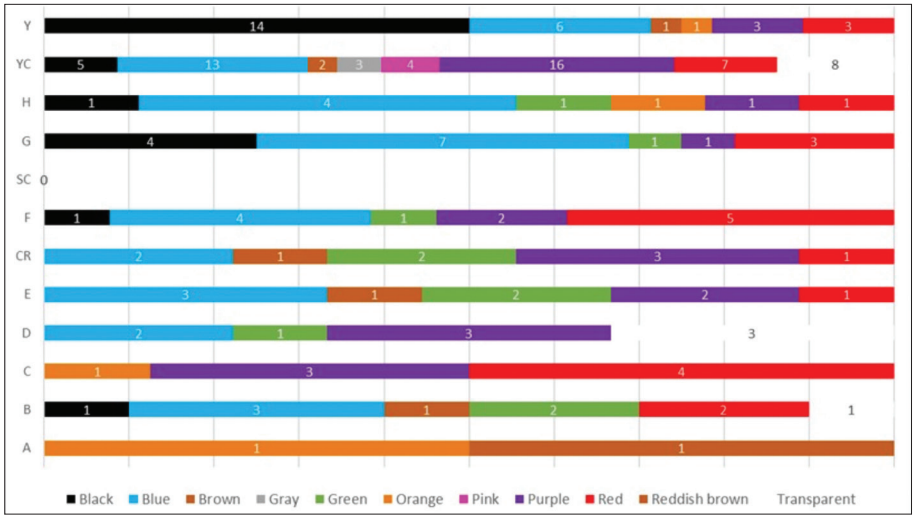
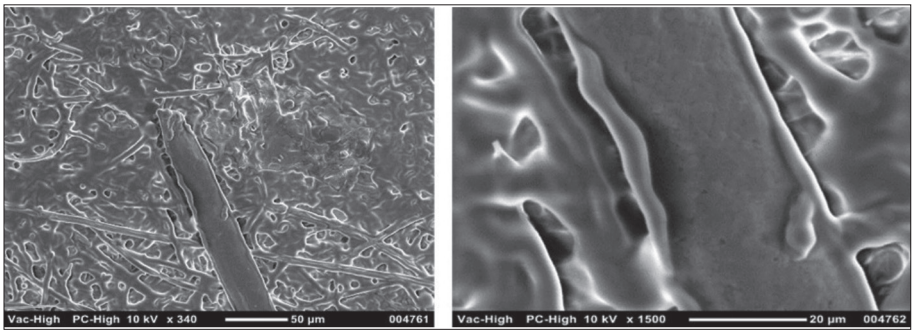


Fig 4. MPs color dispersion at all process step/sampling location (n)

Fig 5. SEM image of microplastics



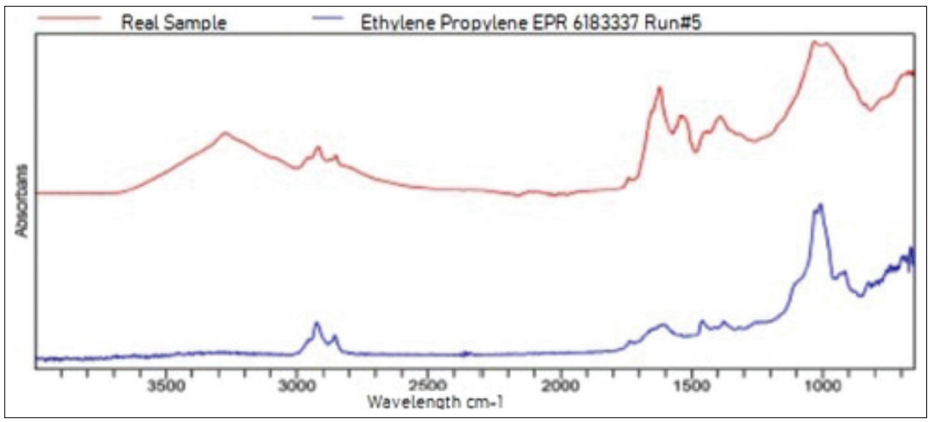


Fig 6. Identification of microplastic in ATR-FTIR

risk to human health, as demonstrated by the findings of our study indicating that microplastics are prevalent in the production process steps of yogurt. Despite the presence of microplastics at each process step and sampling location, the amounts of microplastics varied considerably between process steps *(Table 1)*.

The majority of analyzed samples (83%) contained an abundance of 80 particles L^{-1} microplastics; 20 particles of L^{-1} microplastic were found in one sample (8.33%); and microplastic could not be detected in one sample (8.33%). According to our research results, the concentration of microplastics in raw milk and yogurt containers was extremely high.

There were no comprehensive studies on the presence of microplastics in raw milk. However, when dairy cattle operations and milk logistics operations in Turkey are analyzed, numerous potential microplastic contamination risk points are anticipated. There are processes that must be regulated when a step-by-step backward flow chart is drawn from the moment milk is accepted by the enterprise. Plastic pipes and valves used to transport milk from tankers can be a significant source of contamination for MP. Tankers transporting milk from milk collection points to yogurt manufacturing facilities can be considered a risk factor. Water and detergent residues used to clean tankers, valves, and pipes may pose a risk to the MP load.

Moreover, rubber pipes themselves can be a source of contamination.

Refrigerated collection tanks at milk collection points, churns used to transport milk to milk collection points, milking buckets or automatic milking units with vibrating vacuum rubber-coated teat cups attached around the udder are additional potential contamination points. Cloths, gloves, plastic equipment, as well as detergents, disinfectants, and water used at every stage of cleaning, can contaminate plastic [25,26].

Another possibility is that MPs could be present in the udder and contaminate the milk. No study was found on this subject. Nevertheless, in mammals, plastics smaller than 0.1 μm can cross the blood-brain barrier and the placenta [27]; similarly, in a study with inhalation and translocation of MPs, 10 μm particles were reported to be absorbed from the alveolar epithelium [28,29].

The gaskets and filtration units located between the pipes through which the milk circulates in the enterprise are also the points that need to be checked. The increased permeability of filtration units over time could facilitate the migration of microplastics into dairy products. The milk accepted by the enterprise is ultrafiltered to reduce its microbial load and physical contamination and to prevent its transportation into yogurt [30,31]. Keeping organic materials, mineral substances, and colloids on the surface and/or pores of the filter can increase its pore size and its MP permeability if the milk filters are not cleaned and/or replaced on a regularly [32].

The microplastics' composition provides hints about their origin. Due to their hydrolytic stability, low and high pH stability, and excellent flow rates, sulfone family polymers are generally used as ultrafiltration and microfiltration membranes in the food and dairy industry [33-35]. Ethylene propylene, detected in our study, is used for air and water tightness in closed circuit systems, whereas neoprene is used as an oil tightness gasket and polyacrylamide is a polymer that is utilized in filtration processes [36]. Politetrafloroetilen (PTFE) are the most ubiquitous in the environment, and their presence in milk samples may be due to environmental contamination, the milking process (a series of macro, micro, and ultrafiltration using polymeric membranes), and packaging conditioning from farms to dairy processing facilities [22,24].

MP size determines the efficiency of uptake through the gastrointestinal, alveolar, and dermal epithelium. It has been reported that >90% of ingested MPs, especially those larger than 150 μm, are eliminated in the faeces. However, particles with a size range of 0.1-10 μm can cross the blood-brain barrier and placenta. Particles 150 μm can cross the gastrointestinal epithelium. Endocytosis allows particles 2.5 μm in size to enter the systemic circulation [37]. The risk assessment of MPs in food products should focus not only on the effects of MPs themselves, but also on the effects of the chemical pollutants that MPs absorb. MPs, which can adsorb the majority of pollutants such as bisphenol A, phthalates, and some brominated flame retardants, which are endocrine disruptors and can cause serious health problems, are able to absorb these contaminants [38].

In our study, we observed MPs of various hues, including black, blue, brown, gray, green, orange, pink, red, purple, reddish brown, and transparent white. While fibres are blue, green, red, pink, purple, brown, black, and transparent; fragments are blue, green, red, pink, orange, purple, brown, gray, black, and transparent white; films are black, blue, brown, gray, orange, pink, purple, red, and reddish brown; and spheres were found to be red, green, blue, black, and orange. Color is an essential feature for visually distinguishing the chemical composition of MPs [39]. In our study, FTIR analysis revealed that black and blue MPs were ethylene propylene and neoprene; brown, red, and reddish-brown MPs were PTFE and polyacrylamide; gray MPs were neoprene; green and purple MPs were ethylene propylene; and orange, pink, and clear white MPs were PTFE. It should not be forgotten that different color pigments can be added to polymer mixtures during the production of polymers. It should also be considered that the colors detected with a microscope may be the result of the color pigments and additives used in the manufacturing of plastic packaging [40]. Consequently, FTIR verification is necessary for a conclusive diagnosis.

A risk assessment was carried out by considering the data on ready-to-eat yogurt, which is the final product of the process. The risk assessment for microplastics provides the total number of microplastics consumed. It does not indicate how much of the microplastics ingested are excreted in feaces. It excludes the rate of translocation from the intestinal epithelium [41]. It may be subject to MP absorption of particles smaller than 1.5 μm; it has been reported that larger particles can be taken into the organism via endocytosis and phagocytosis [37,41-43]. This study evaluated the physical destruction of microplastics within the body. It excludes the microbial and toxicological risks adsorbing to the plastic's content or surface. Regarding the potential toxic effects of MPs on humans, little is known. MPs 20 μm in size can reportedly penetrate biological membranes, accumulate in tissues, cause cytotoxicity, and elicit immune responses when inhaled or ingested [37].

In our study, according to Turkey Nutrition Guide [23], considering the yogurt consumption of adults and children, adolescents, pregnant-breastfeeding women, post-menopausal women, the number of microplastic particles that could be included in the evaluation groups was found to be approximately 73.500-98.000, respectively.

Considering these results, it is thought that the risk should be evaluated carefully. Cox et al.[44] estimated the total intake of MPs utilizing 402 data sets from 26 studies. According to the authors, the annual microplastics consumption ranges from 74.000 to 121.000 particles, depending on the person's age and size. The annual MP intake per capita is estimated to range from 39.000 to 52.000 items, including 37-1.000 from sea salt, 4.000 from tap water, and 11.000 from shellfish.

The average daily consumption of PP MPs by infants is estimated to be 1.580.000 particles per capita in the range of 14.600-4.550.000 particles, depending on the region. The average value corresponds to approximately 3000 times the total adult consumption of MPs from water, food and air (up to 600 particles per day for adults) [45].

In conclusion, analyses were conducted on raw materials, semi-finished materials, finished products, intermediate products, and starter cultures gathered from the production line of a national medium-sized yogurt manufacturer. The results indicated the microplastic particle source of the collected samples. This study's findings may provide a clear indication of the contamination risk associated with this product. When investigating microplastic concentrations in yogurt and other dairy products, it is necessary to collect additional information on the contamination of raw milk with plastic residues. Microplastic contamination in yogurt buckets is quite severe. Before filling, precautions must be taken to prevent contamination. Considering the potential health risks posed by microplastics, food research must be intensified.

Availability of Data and Materials

The datasets and analyzed during the current study available from the corresponding author (K. Muratoglu) on reasonable request.

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Competing Interests

The authors declared that there is no competing interests.

Ethical Approval

N/A

Authors Contributions

SRZ, KM, and SKB planned and designed the experiment. SRZ collected the samples and performed the experiment. KM and SKB analyzed the data, wrote and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Research Article

Comparison of Histopathological, Immunohistochemical and Real-Time PCR Methods for Diagnosis of Listeriosis in Ruminants with Encephalitis [1][2]

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Abstract: Encephalitic listeriosis is the most significant purulent encephalitis in ruminants and is a very common endemic problem in sheep, cattle, and goats. In this study, it was aimed to compare the presence of *Listeria (L.) monocytogenes* revealed by immunohistochemical (IHC) and Real-Time PCR methods with histopathological findings obtained from the archive materials. The study material consisted of pons and medulla oblongata paraffin tissue of 100 ruminants (9 cattle, 4 calves, 44 sheep, 38 lambs, and 5 goats). Positivity was obtained by the IHC method in 46 (46%) and by the Real-Time PCR method in 21 (21%) of 100 cases. In the *L. monocytogenesis* antigen IHC scoring, more severe staining was observed in sheep and goats (P>0.05). In the IHC positive cases, microabscess was more severe in sheep and goats than in cattle and lambs (P<0.05). In addition, 19 patients had *Coenurus cerebralis* cysts, and 3 of them were found to be positive for the IHC agent of *Listeria*. It was concluded that IHC and PCR methods can be used to detect *L. monocytogenes* from paraffin blocks, but the IHC method is a more effective method than PCR in revealing the presence of antigen from paraffin blocks stored for many years.

Keywords: *Histopathology, Immunohistochemistry, Listeriosis, Real-Time PCR, Ruminants*

Ensefalitisli Ruminantlarda Listeriyozisin Tanısı İçin Histopatolojik, İmmünohistokimyasal ve Real-Time PCR Yöntemlerinin Karşılaştırılması

Öz: Ensefalitik listeriozis, ruminantlardaki purulent ensefalitisler arasında en önemlisidir ve tüm dünyada koyun, sığır ve keçilerde oldukça yaygın görülen endemik bir problemdir. Bu çalışmada, immünohistokimyasal (IHC) ve Real-Time PCR yöntemleri ile ortaya konan *Listeria (L.) –monocytogenes*'in varlığının arşiv materyallerinden elde edilen histopatolojik bulgularla karşılaştırılması amaçlandı. Çalışma materyalini 100 ruminantın (9 sığır, 4 buzağı, 44 koyun, 38 kuzu ve 5 keçi) pons ve medulla oblongata parafin dokuları oluşturdu. İncelenen 100 olgunun 46'sında (%46) IHC yöntemi ile, 21'inde ise (%21) Real-Time PCR yöntemi ile pozitiflik elde edildi. IHC skorlamasında *L. monocytogenes* antijeni koyun ve keçilerde daha belirgin boyandığı gözlendi (P>0.05). IHC pozitif vakalarda, koyun ve keçilerde, sığır ve kuzulara göre mikroapse daha şiddetliydi (P<0.05). Ayrıca 19 hastada *Coenurus cerebralis* kisti vardı ve bunlardan 3'ünde IHC yöntemiyle *Listeria* etkeni pozitif bulundu. IHC ve PCR yöntemlerinin parafin bloklarından *L. monocytogenes* tespitinde kullanılabileceği, ancak IHC yönteminin uzun yıllar saklanan parafin bloklarından antijen varlığını ortaya çıkarmada PCR'den daha etkili bir yöntem olduğu sonucuna varıldı.

Anahtar sözcükler: *Histopatoloji, İmmünohistokimya, Listeriyozis, Real-Time PCR, Ruminant*

Introduction

Listeriosis caused by *Listeria (L.) monocytogenes* is a zoonotic disease progressing with three basic forms as meningo-encephalitis, abortion, and septicemia. Although rare, it also causes mastitis, purulent conjunctivitis, keratitis, and endocarditis [1,2]. Encephalitic listeriosis, which is seen as the most important of the purulent encephalitis in ruminants, is an endemic problem that is common all over the world. While encephalitic listeriosis is observed at a

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rate of 7.5 - 29.4% in ruminants in Europe, it is emphasized that it is the main cause of encephalitis observed in goats and sheep in Switzerland [2,3].

Patient or asymptomatic animals scatter the agent with feces, urine, runny nose, milk, and placenta [4,5]. Contaminated straw, grass, pulp, water, and especially poorly fermented silages cause the disease to occur in herds as epidemics [6-8]. Encephalitic listeriosis is mostly seen in late winter and early spring, in indoor silage fattening [6,9]. Disease agent enters the body through abrasions in the buccal mucosa, teething wounds, and portantres in the intestinal mucosa [7]. Macroscopic lesions are often unremarkable in encephalitic listeriosis. However, sometimes thickening of the membranes covering the medulla oblongata due to greenish gelatinous edema, and hemorrhage with gray-colored melting foci with a diameter of a few millimeters on the cross-sectional surface of the medulla oblongata may be observed. Besides, turbidity can be determined in the cerebrospinal fluid (CSF). Lesions begin in the brain parenchyma and meningitis forms as secondary. The typical histopathological finding of the disease is microabscesses formed in the parenchyma of the pons and medulla oblongata. Microabscesses can be formed due to too many neutrophils and macrophage infiltrations, as well as due to microglial reaction [10-12]. Lymphocyte, histiocyte, plasma cells, and less commonly neutrophil and eosinophil granulocytes are seen around the vessels (perivascular cuffing) near the microabscesses [13-15]. In leptomeningitis, exudate accumulation consisting of macrophages, lymphocytes, plasma cells, and very few neutrophil granulocytes is seen and is often severe [11,16].

In our previous studies [17,18], we have revealed that brainstem cytology and immunocytological methods are significant and can be used in the rapid diagnosis of listeriosis. In the present study, it was aimed to compare the histopathological findings with the positivity of encephalitic listeriosis determined by IHC and RT-PCR methods from the archive materials (pons and medulla oblongata in paraffin blocks) of the cases, which were necropsied with the suspicion of listeriosis between 2000 and 2015 and encephalitis was detected.

Material and Methods

Ethical Statement

This study was approved by Ethics Committee of Selcuk University, Faculty of Veterinary Medicine, Experimental Animal Production and Research Center (Approval no: 2015/49).

Cases and Samples

In the study, the brainstems of 100 ruminants (9 cattle, 4 calves, 44 sheep, 38 lambs, and 5 goats) brought to Selcuk University, Faculty of Veterinary Medicine, Department of Pathology between 2000-2015 for necropsy were used. The archive material of pons and medulla oblongata of these animals, which were reported to have neurological symptoms in anamnesis and encephalitis was found in necropsy, were evaluated.

Histopathology

Five micron-thick sections were taken from paraffin blocks of the pons and medulla oblongata and stained with hematoxylin&eosin (H&E) and examined under a light microscope (Olympus BX51, Tokyo, Japan). Changes observed in histopathological examination of sections were evaluated as per scoring criteria of Oevermann et al.[19]. Accordingly; none (0); no microabscess, mild (+1); 1 small microabscess, moderate (+2); several small to medium-sized microabscesses, severe (+3); diffuse microabscesses of medium size, some of which coalesce, very severe (+4); multiple and extensive microabscesses in the parenchyma. Perivascular cell infiltration (perivascular cuffing) histopathological scoring; none (0); no lesion, mild (+1); 1-2 layers, moderate (+2); 3-4 layers, severe (+3); 5 to 6 layers, very severe (+4); more than 6 layers.

Immunohistochemistry

After the samples were cut into the size of 5 micron-thick and taken to polylysine slides, were deparaffinized, rehydrated, and stained as per the NovoLinkTM Max Polymer Detection System (RE7280-K) kit procedure. Antigen retrieval was performed with Proteinase K for 15 minutes at room temperature. Then, 3% hydrogen peroxide solution was dripped to remove endogenous peroxidase activity. The Protein Block was dripped and then incubated with the primary antibody (Rabbit polyclonal Anti-*L. monocytogenes* antibody - ab35132) for 1 hour at room temperature. Then, NovoLink Post Primer Block and NovoLink Polymer were incubated for 30 min at room temperature, respectively. Finally, the DAB solution was dripped onto the sections and incubated for 3-5 min at room temperature, then counterstained with Hematoxylin and closed with entellan. All stained sections were examined under a light microscope (Olympus BX 51) and scored as per the number of positively stained cells at x400 magnification. IHC staining scores: none (0); no staining, mild (+1); IHC positive staining in 1-10 cells, moderate (+2); IHC positive staining in 11-20 cells, severe (+3); IHC positive staining in more than 20 cells.

Real-Time PCR

Deparaffinization of Samples: Paraffin-blocked medulla oblongata, pons, and cerebellum tissues were cut in microtome of 5 µm-thick and taken into 1.5 mL Eppendorf tubes, and 1000 µL of xylene was added to remove the paraffin and shaken slowly in a vortex device (Drogan Lab). Then, eppendorf tubes were kept in the heat block (Dry

Bath) brought to 56°C. Eppendorf tubes were centrifuged at 13200 rpm and the supernatant was discarded. This process was repeated two more times. Then, 500 µL of xylene was added to the tubes and vortexed and kept in a 56°C heat block. By vortexing again, spinning was done and 500 µL of ethanol was added. The tubes were vortexed and kept in a 56°C heat block centrifuged at 13200 rpm and the supernatant was discarded. The tubes were centrifuged at 13200 rpm by adding 1000 µL of ethanol, and the same process was repeated once more by discarding the supernatant. The deparaffinization process was completed by allowing the ethanol to evaporate for 10 min in the 56°C heat block with the lids of the eppendorf tubes open.

DNA Isolation: After deparaffinization, 180 µL of tissue lysis buffer and 70 µL of Proteinase K were added to each tube. The tubes were vortexed and incubated at 56°C and 90°C for 1 h in a dry heat block. After the tubes were brought to room temperature, 200 µL of DNA Binding Buffer was added and kept at 15-20°C for 10 min. Spin (Scilogex) was performed by adding 100 µL of isopropanol to each tube. The lysate (average 550 µL) in the tubes was taken into Spin Filter tubes and centrifuged at 8000 rpm for 1 min, and the collective tubes at the bottom were changed after each procedure. 500 µL Wash Buffer I and II were added to each tube, respectively, and centrifuged (8000 rpm x 1 min.). Filtered tubes were taken into new Eppendorf tubes, 35 µL of DNA Elution buffer was added, kept at 15-20°C for 5 min, and centrifuged (8000 rpm X 1 min.) DNA was obtained. 1 µL Probe, 1 µL Primer Forward, 1 µL Primer Reverse, 10 µL Master, 2 µL H_2O; 5 µL of the sample (total volume 20 µL) was added into each well using DNA Master Hydrolysis Probes. Base sequences (LM1: CCTAAGACGCCAATCGAA, LM2: AAGCGCTTGCAACTGCTC) determined by Border et al.[20] were used as primers. In the Roche 96 Cycle device, the plates were set for pre-incubation at 95°C 600 sec-1 cycle, for amplification at 95°C 15 sec and 64°C 45 sec-45 cycles, and cooling at 37°C 30 sec-1 cycle.

Statistical Analysis

IBM SPSS Statistics 25.0 software was used for the comparison of histopathology, immunohistochemical, and Real-Time PCR results. Histopathological scores of immunohistochemically positive cases and immunohistochemical scores among ruminates were analyzed using the Kruskal-Wallis test using non-parametric statistics, followed by the Mann-Whitney U test as a post-hoc analysis between the two groups. Chi-square test was applied to IHC and PCR scores of *L. monocytogenes* among ruminant groups between 2000 and 2015. The value of $P<0.05$ is considered statistically significant. Pearson correlation analysis was applied to determine the significant and positive relationship between microabscess, perivascular cuffing, and IHC ($P<0.01$).

Results

Macroscopic Results

In the macroscopic examination, hyperemia and edema were found in the meninges, and diffuse hyperemia and melting areas of substantia alba on the cross-sectional surface of the brainstem were seen in 4 lambs and 1 calf. Besides some melting areas with a 1x2 mm size and yellowish-green consistency exudate in the brainstem of 2 lambs and 1 sheep were noticed. Also, a *Coenurus cerebralis* cyst was detected in 8 lambs and 11 sheep.

Histopathology Results

The number and scores of cases for which microabscess and perivascular cuffing were observed in the study are given in *Table 1* by the ruminant species. When microscopic findings were evaluated, microabscess was observed in a total of 57 cases. In 13 of these 57 cases, 1 small microabscess (+1) with diffuse neutrophil infiltration and glia cells was found in the brainstem. In 11 cases, microabscess (+2 and +3) *(Fig. 1-A)* was observed, from small to medium-sized, and some of them even united.

Table 1. *Histopathological, IHC and Real-Time PCR results according to animal species*

Parameters	Histopathological Changes										IHC and Real-Time PCR Results					
	Microapse					Perivascular Cuffing					IHC				Real-Time PCR	
Lesion score	0	+1	+2	+3	+4	0	+1	+2	+3	+4	0	+1	+2	+3	Positive	Negative
Cattle	4	2	1	1	1	2	0	3	0	4	4	3	0	2	2	7
Calf	1	2	0	1	0	2	1	1	0	0	4	0	0	0	0	4
Sheep	17	3	3	3	18	8	4	2	2	28	19	6	4	15	13	31
Lamb	20	6	2	0	10	10	2	4	3	19	25	4	1	8	6	32
Goat	1	0	0	0	4	1	0	0	0	4	2	0	0	3	0	5
Total	43	13	6	5	33	23	7	10	5	55	54	13	5	28	21	79
		57					77					46				

Histopathological and immunohistochemical score; 0; none lesion, +1; middle, +2; moderate, +3; severe, +4; very severe

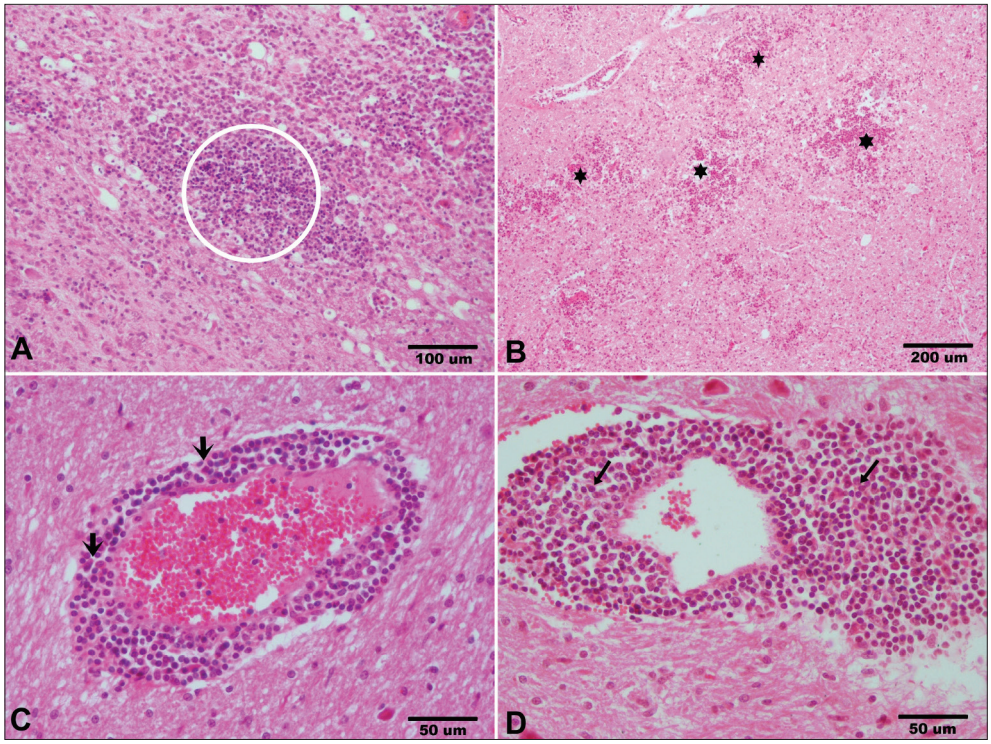

Fig 1. A-D. Brainstem, H&E. **A.** Microabscess *(circle)* (moderate), Bar, 100 µm, **B.** Numerous and widespread microabscesses *(stars)* (very severe), Bar, 200 µm, **C.** Perivascular mononuclear cell and neutrophil infiltrations *(arrows)* (moderate) and hyperemia, Bar, 50 µm, **D.** Perivascular mononuclear cell and neutrophil infiltration (very severe) *(arrows)*, Bar, 50 µm

Large microabscesses (+4) and *(Fig. 1-B)* were detected in the parenchyma in 33 cases. Besides, perivascular cuffing consisting of lymphocytes, histiocytes, and plasma cells and neutrophils in the Virchow-Robin spaces around the vessels in the brainstem parenchyma was observed in a total of 77 cases (+1 in 7 cases, +2 in 10 cases *(Fig. 1-C)*, +3 in 5 cases, and +4 in 55 cases *(Fig. 1-D)*. No microabscess was found in 20 of these 77 cases in which perivascular cell infiltration was observed in the study. In cases with *L. monocytogenes* antigen IHC positive, microabscess was observed histopathologically in sheep and goats (P<0.05), and more severe in cattle and lambs *(Fig 3-A)*. Perivascular cuffing was found at a similar rate (P>0.05) in cattle, sheep, goat, and lambs, while it was most severe in goats *(Fig. 3-B)*. In the cases with *Coenurus cerebralis* cyst, it was determined that the cyst walls and foreign body giant cells formed against them and an inflammatory zone in which eosinophil granulocytes were also found.

Immunohistochemical Results

Listeria monocytogenes antigen in the cytoplasm of neutrophils found in microabscesses in the brainstem was stained by IHC method and its severity was scored. IHC staining results, lesion scores, and general distribution by the ruminant species are given in *Table 1*. When the IHC findings were evaluated collectively, 46 of 100 cases (5 cattle, 25 sheep, 13 lambs, and 3 goats) were found to be positive for *L. monocytogenes* antigen *(Fig. 2-A,D)*. *L. monocytogenes* antigen was detected mild in 13 cases *(Fig. 2-A)*, moderate in 5 cases *(Fig. 2-B)* and severe staining in 28 cases *(Fig. 2-C,D)* by IHC method. *Listeria* antigens were determined by IHC in 46 (80.7%) of 57 cases with microabscess in histopathological examinations.

While there was no statistical difference between ruminant species in *L. monocytogenes* antigen IHC scoring (P>0.05) *(Fig. 3-C)*, more intense staining was observed in sheep and goats. A significant difference (P<0.05) was determined between the positivity and negativity of *L. monocytogenes* in sheep and no significant difference (P>0.05) was seen in cattle, goats and lambs *(Table 2)*. According to pearson correlation analysis, a strong, positive and significant relationship was found between microabscess, perivascular cuffing, and IHC *(Table 3)*. In addition, the incidence of *L. monocytogenes* was determined to be highest in spring and winter months, respectively *(Fig. 4)*. When the findings were evaluated according to years, the highest *L. monocytogenes* positivity was detected in 2012 with 8 cases, while IHC positivity was not detected in 2002 and 2006 *(Fig. 5)*.

Real-Time PCR Results

The distribution of Real-Time PCR results by animal species is given in *Table 1*. Accordingly, *L. monocytogenes* positivity was found in 21 of 100 cases *(Fig. 6)* (2 cattle,

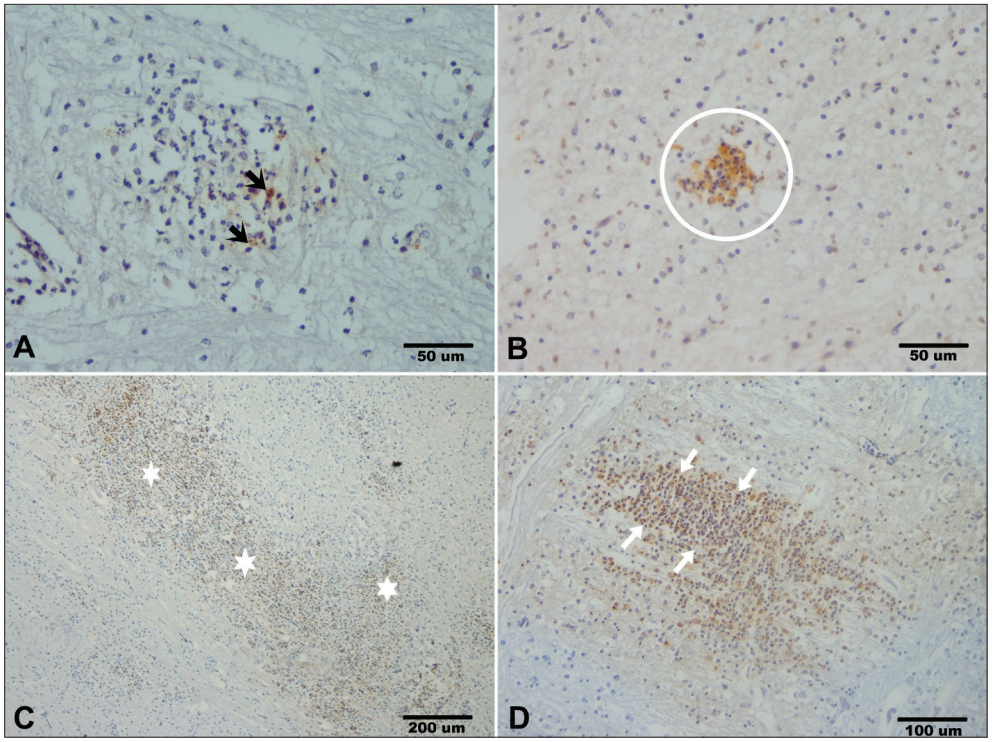

Fig 2. A-D. Microabscess in the brainstem and *L. monocytogenes* antigen in the cytoplasm of neutrophils, IHC positive staining. DAB. **A.** Mild *(arrows),* Bar, 50 µm, **B**. Moderate *(circle),* Bar, 50 µm, **C.** Severe *(stars),* Bar, 200 µm, **D.** Severe *(arrows),* Bar, 100 µm

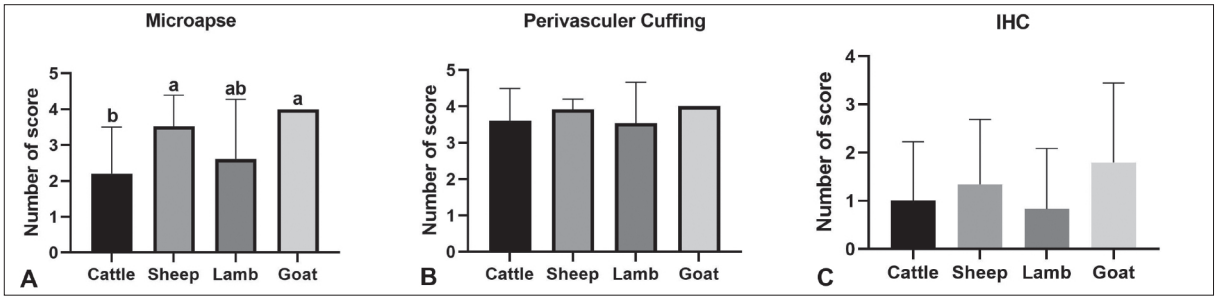

Fig 3. A. Statistical expression of microabscess severity among *L. monocytogenes* positive cattle, sheep, lamb and goat, (Data presented mean value ± standard deviation, significant differences (p<0.05) marked with different superscripts), **B.** Statistical expression of perivasculer cuffing severity among *L. monocytogenes* positive cattle, sheep, lamb and goat, (Data presented mean value ± standard deviation (P>0.05), **C.** Statistical expression of IHC scores of *L. monocytogenes* antigen in ruminants. Data presented mean value ± standard deviation (P>0.05)

Table 2. *IHC and Real-Time PCR results and ratios of L. monocytogenes in ruminant species*

Animals	IHC			PCR		
	Number (%) Negative Cases	Number (%) Positive Cases	x^2 (P Value)	Number (%) Negative Cases	Number (%) Positive Cases	x^2 (P Value)
Cattle	4 (44.4%)	5 (55.6%)	6.496 (0.167)	7 (77.8%)	2 (22.2%)	5.774 (0.417)
Sheep	19 (43.2%)	25 (56.8%)	23.183 (0.004*)	31 (70.5%)	13 (29.5%)	20.104 (0.013*)
Lamb	25 (65.8%)	13 (34.2%)	6.902 (0.920)	32 (84.2%)	6 (15.8%)	8.490 (0.782)
Goat	2 (40.0%)	3 (60.0%)	5.000 (0.172)	5 (100%)	0 (0.00%)	-

** Statistically significant test (Chi square, P<0.05)*

13 sheep, 6 lambs). In sheep, a significant difference was found between positive and negative aspects with the *L. monocytogenes* PCR method, and no statistically significant difference was found in cattle, goats and lambs *(Table 2)* between 2000 and 2015. When PCR findings were evaluated by years, PCR positivity could not be detected in

Table 3. *Degree of correlation between histopathological results and IHC method. Pearson Correlation Test*			
Findings	**Microabscess**	**IHC**	**Perivascular Cuffing**
Perivascular cuffing	$r^2 = +0.74$ P<0.01*		
Microabscess		$r^2 = +0.92$ P<0.01*	
IHC			$r^2 = 0.66$ P<0.01*
* *P<0.05*			

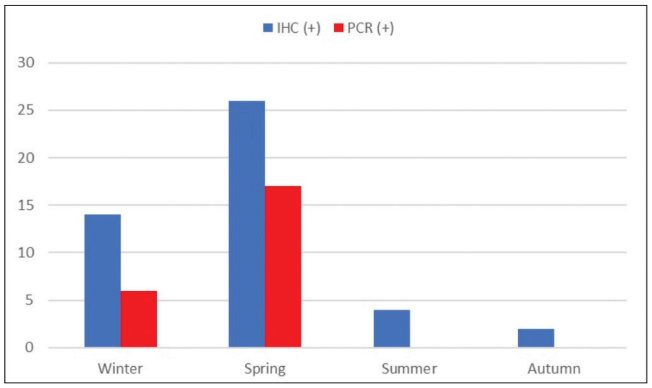

Fig 4. Distribution of *L. monocytogenes* IHC and Real-Time PCR positive cases by season

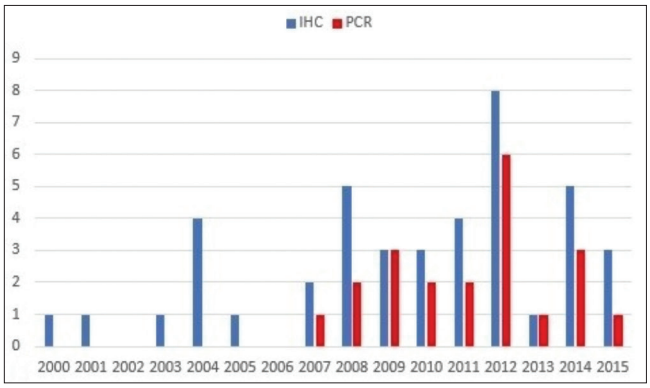

Fig 5. IHC and Real-Time PCR results of listeriosis-positive cases in ruminant between 2000 and 2015

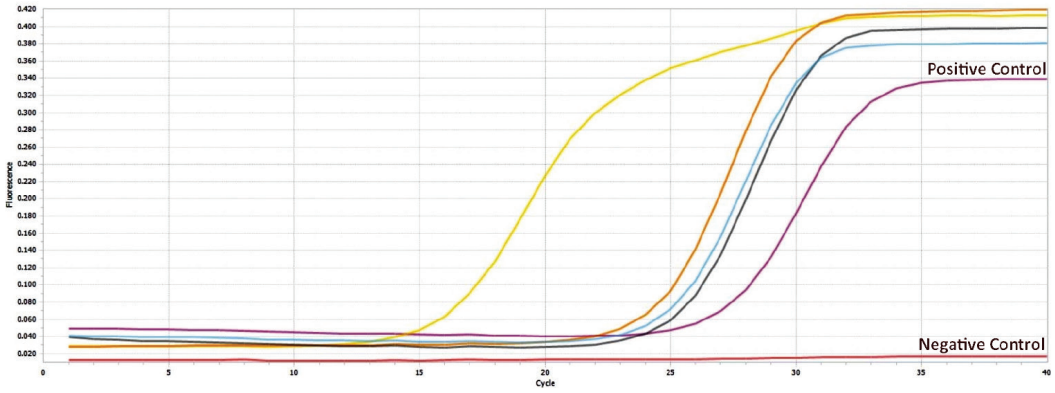

Fig 6. Real-Time PCR reactions: positive control, negative control and positive cases

cases between 2000 and 2006, *L. monocytogenes* was found positive between 2007 and 2015, the most positivity was detected in 2012 with 6 cases *(Fig. 4)*. It was determined that the incidence of *L. monocytogenes* increased in the in spring and winter months by PCR method, respectively, similar to IHC findings *(Fig. 3)*.

Discussion

Encephalitic listeriosis causes loss of productivity and sometimes death in farm animals, causing economic losses in the world. Also, listeriosis is very important for human health, since it is a foodborne infection in humans. While encephalitic listeriosis is sporadic in cattle, it occurs as epidemics in sheep and goat herds [6]. Encephalitic listeriosis is usually seen as a result of feeding with grass, straw, which are contaminated with *L. monocytogenes*, especially silage that are not well fermented, juicy beet, and malt pulps [21]. Nightingale et al.[5] and Wesley et al.[22] reported that they encountered listeriosis in the winter and spring months (December-May). In the study presented, it was determined that 14 of 46 cases diagnosed with encephalitic listeriosis came in the winter months and 26 at the beginning of spring. The conclusions were considered that the disease caused epidemics in the herds in Konya and the surrounding provinces. The reason for this could be in these region, sheep and goat breeding are common, during the periods

when the animals are not taken to the pasture, due not pay attention to the cleaning of the barns and water, and the incorrect storage of wet feeds such as silage and pulp, and/or the feeding with spoiled silage.

Disease agents enter the body through abrasions in the buccal mucosa, teething wounds, and portantres in the intestinal mucosa and come to the trigeminal ganglia and from there to the brain (medulla) by motor branches and cause purulent encephalitis. When moved the animals are seen circular movements clinically, and tongue and facial paralysis due to paralysis of the 7th nerve [6-8]. Since similar findings can also be seen in parasitic infestations such as *Coenurus cerebralis*, it makes it difficult to distinguish clinically encephalitic listeriosis and coenuriasis. However, macroscopically, the appearance of *Coenurus cerebralis* cysts in the cerebral hemispheres or the cerebellum provides a macroscopic diagnosis of coenuriasis. In the study presented, macroscopically *Coenurus cerebralis* cysts were determined in the brain hemispheres of 8 lambs and 11 sheep, and histopathologically, it was determined that there is an inflammatory zone with eosinophil granulocytes and foreign body giant cells formed against the *Coenurus cerebralis* cyst walls. In 3 of these 19 cases, in which a *Coenurus cerebralis* cyst was detected, *Listeria* was also found to be positive by IHC. The results obtained in the study presented showed that ruminants found to have *Coenurus cerebralis* cyst should also be investigated for encephalitic listeriosis and the coexistence of both diseases should not be ignored.

In encephalitic listeriosis, it was stated that microabscesses formed due to a large number of neutrophil and macrophage infiltrates and a small number of microglial reactions are the characteristic findings, histopathologically [11,23]. Oevermann et al.[19] scored histopathologically microabscess and perivascular cuffing formation in 220 ruminants (59 goats, 89 sheep, and 72 cattle) to determine encephalitic listeriosis and reported that they found microabscess in 59 goats (100%), 89 sheep (100%), and 69 cattle (95.8%). In the presented study, microabscess formation to varying degrees in the brainstem was detected in the histopathological examinations of 57 out of 100 ruminants with neurological findings. The presence of varying degrees of microabscess in all 46 cases (80.7%) confirmed to have listeriosis by IHC reveals that although microabscess finding is not pathognomonic in terms of listeriosis, it is a very important histopathological finding. In cases with intense microabscess (moderate, severe, and very severe), this rate increases to 90.9% (40/44). In sheep and goats, microabscess formation was found to be statistically more severe (P>0.05) in *L. monocytogenes* positive cases by IHC method. These findings were evaluated as acute according to the scoring made by Oevermann et al.[19], and the fact that acute cases were more common in small ruminants in the study supports the view that encephalitic listeriosis progresses more severely.

Lymphocyte, histiocyte, plasma cells, and less commonly neutrophil and eosinophil granulocytes are seen around the vessels (perivascular cuffing) near the microabscesses [11]. Histopathologically, perivascular cell infiltration was detected in 17 cases with mild and moderate scores *(Fig. 1-C)*, and in 60 cases with severe and very severe scores *(Fig. 1-D)* in our study. In the study, perivascular cuffing scores were found to be similar in cattle, sheep, goats, and lambs in the *L. monocytogenes* positive cases, while it was most severe in goats (P>0.05). In addition, microabscess was not detected in 20 cases with perivascular cuffing in the brain parenchyma. Since perivascular cell infiltration can also be seen in parasitic, viral, and other bacterial infections in histopathological examinations [24,25], it was concluded that it is more appropriate to evaluate the cases in which microabscess is observed with perivascular cell infiltration in terms of encephalitic listeriosis. Although microabscess and perivasculer cuffing is not pathognomonic for listeriosis, the presence of a strong, positive, and significant correlation between microabscess, perivascular cuffing, and IHC reveals that it is a very important histopathological finding.

Although clinical, macroscopic, and histopathological findings are important in the diagnosis of encephalitic listeriosis, it is also necessary to demonstrate the agent with methods such as bacteriological culture, IHC, and PCR in the definitive diagnosis of the disease [26,27]. On the other hand, although culture is shown as the gold standard in the diagnosis of the disease, there are disadvantages such as inability to produce bacteria, especially in animals using antibiotics, long duration, and high cost [28,29]. In addition to this method, molecular methods such as IHC and PCR have been developed, which are more specific, less costly, and give results in a shorter time. In the presented study, *L. monocytogenes* antigen was detected as positive in 46 cases (46%) by the IHC method from paraffin blocks *(Fig. 2A-D)*. This result supports the studies.

Immunohistochemistry is an effective method for the detection of bacteria and their antigens, since the morphological features of tissues and organs are preserved [30]. Allen et al.[31] and Loeb [32] reporting that IHC can be used as an alternative to the culture method in the *L. monocytogenes* diagnosis. In the current study, a strong and positive correlation was determined between the histopathological and immunohistochemical findings of *L. monocytogenes* in archival paraffin block tissue of small ruminates. Besides, it showed parallelism with the studies [33,34] showing that it is possible to determine the causative agent from archive materials by IHC method, and supported the view that it can be used

safely in the diagnosis of diseases from previous years' cases.

We determined the presence of *L. monocytogenes* in cases with encephalitic listeriosis findings by using immunohistochemical and Real-Time PCR methods in archival paraffin block tissues. In recent years PCR technology has been instrumental in identifying infectious agents and therefore in some cases complements or even surpasses conventional methods in terms of sensitivity [35]. Mygind et al.[36] found a good correlation between IHC and PCR methods in their study for the detection of *Chlamydia pneumoniae* in mouse lung paraffin block tissues. In the study, the IHC method is a safe method in archival paraffin block tissues and it is thought to affect the sensitivity of the Real-Time PCR method depending on the age of the paraffin block tissue.

Tissue samples that are fixed with formalin or embedded in paraffin are considered a suitable source for DNA analysis as their structures and proteins are preserved. Besides, the high sensitivity and specificity provided by the PCR method are important in terms of its applicability to a wide variety of samples. By PCR method in animals, *Listeria* agents were determined in brain tissue [37], in cerebrospinal fluid [38] and colonies formed as a result of the culture of brain samples taken. In the literature that could be examined, PCR studies to determine *Listeria* agents from paraffin block tissues were not encountered. In the present study, *Listeria* agent was detected as positive by Real Time-PCR method in 21 (21%) of archive brain tissues in paraffin blocks *(Fig. 6)* belonging to 100 ruminants who showed neural findings and were diagnosed with encephalitis between 2000-2015. Since positive results were found in cases after 2007 in the study, it was concluded that positive results could not be obtained using the PCR method from paraffin block tissues, belonging to 8-10 years ago or older, in the determination of *Listeria* agent, and therefore paraffin blocks older than 10 years were not suitable for PCR method.

When IHC and PCR results were evaluated together, PCR was found to be positive in only 1 of 13 IHC positive cases with lesion score of mild, but PCR was positive in 19 of 33 cases with IHC scores moderate and severe. On the other hand, in only 1 lamb, while IHC was found to be negative, it was positive by the PCR method. From the results obtained, it was concluded that IHC and PCR methods can be used in archive paraffin block tissues, but the IHC method is more sensitive and specific for old tissues in the diagnosis of encephalitic listeriosis. It was noted that the majority of PCR positive cases were IHC positive cases with moderate and severe, and this situation made us think that for the safe use of the PCR method, the presence of more intense agents in the paraffin-embedded tissue is needed. On the other Szafranska et al.[39] showed the inability to completely remove inhibitory substances such as paraffin, alcohol, or xylene as the reason for the inability to obtain pure DNA from archive tissues in the paraffin block by PCR method. The fact that positivity could not be determined by PCR in cases where the number or density of agents was low in the study supports the opinion of the researchers, considering that the inhibitory substances in question may have suppressed the detection of a small number of agents. Based on this idea, it was predicted that such inhibitory substances should be removed more carefully and adequately in similar studies to be carried out in the future.

It was concluded that IHC and PCR methods can be used to detect *L. monocytogenes* from paraffin blocks, but in the detection of thick-walled bacteria such as *Listeria* sp. in paraffin-embedded archival materials, due to the difficulties in the disintegration of the bacterial wall, the low amount of the agent, and/or the residue of inhibitory substances. It was evaluated that IHC method is a more effective method than PCR in revealing the presence of antigen from paraffin blocks stored for many years. The fact that IHC was found to be positive in 3 of 19 cases with a macroscopically and microscopically seen *Coenurus cerebralis* cyst showed that encephalitic listeriosis may occur together with coenuriasis and it was concluded that the cases in which the *Coenurus cerebralis* cysts were determined should also be examined in terms of *Listeria*. It was also noted that both diseases that cause herd problems can progress together, and it is recommended to take the necessary precautions for both diseases in the treatment of such cases. Also, it was considered that prospective studies on live animals or fresh materials could be useful to more clearly determine the effectiveness of the PCR method and to compare it with IHC.

Availability of Data and Materials

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

Ethical Statement

This study was approved by Ethics Committee of Selcuk University (Approval no: 2015/49).

Author Contributions

All authors contributed to the study conception and design. FH: Conceptualization, Methodology, Writing - Original Draft. FT: Formal analysis, Resources. ÖÖ: Investigation, Resources. MO: Writing - Review & Editing, Validation. MKÇ: Writing - Review & Editing. MBA: Resources, Investigation.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Research Article

Morphometrical Analysis of the Egyptian Mongoose *(Herpestes ichneumon)* Hind Limb Bones (Pelvis, Femur and Crus) Using Three-Dimensional Reconstructed Images

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Abstract: The Egyptian mongoose *(Herpestes ichneumon)* is on the International Union for Conservation of Nature's Red List of Threatened Species, and very little information is available on its morphometric characteristics. This study aimed to create the three-dimensional (3D) reconstruction of the pelvis, femur and crus bones in the hind limb of the Egyptian mongoose using two-dimensional (2D) multidetector computed tomography (MDCT) images, perform morphometric measurements on these models, and compare these measurements to other Carnivora members described in the literature. For this purpose, MDCT was performed on the hind limb bones of two adult mongooses (one male, one female) who died as a result of traffic accidents at different times and were brought to the anatomy laboratory, and the data were stored in the DICOM format. These images were transferred to a computer with 3D reconstruction software Mimics 14.1 loaded, and the reconstruction of the hind limb bones was undertaken. Some morphometric measurements were performed from the 3D models of the pelvis, femur and crus bones. Also, cortical thickness, endosteal and periosteal diameter values of the femur and tibia were measured using MDCT images. According to the results, the measurements of the male mongoose were larger than those of the female mongoose, except for the angle of arcus ischiadicus. The Egyptian mongoose was found to have a minor form among the members of the order Carnivora. It is expected that the morphometric measurements revealed as a result of this study will contribute to the knowledge concerning wild animals and guide future clinical studies.

Keywords: *Carnivora, Mongoose, Morphometry, Three-dimensional reconstruction, Wild animal*

Kuyruk Süren *(Herpestes ichneumon)* Arka Bacak Kemiklerinin (Pelvis, Femur ve Crus) Üç Boyutlu Rekonstrüksiyon Görüntüleri Kullanılarak Morfometrik Analizi

Öz: Kuyruk süren *(Herpestes ichneumon),* Uluslararası Doğayı Koruma Birliği Tehdit Altındaki Türlerin Kırmızı Listesi'nde yer almaktadır ve morfometrik özellikleri hakkında çok az bilgi mevcuttur. Bu çalışmanın amacı; arka ekstremite'nin yapısında bulunan pelvis, femur ve crus kemiklerinin iki-boyutlu (2B) multidedektor bilgisayarlı tomografi (MDBT) görüntülerini kullanarak, üç-boyutlu (3B) rekonstrüksiyonlarını oluşturmak ve bu modeller üzerinden alınan morfometrik ölçümleri ortaya koyarak, literatürde bulunan Carnivor ile karşılaştırmaktır. Bu amaçla farklı zamanlarda trafik kazası sonucu ölen ve Anatomi laboratuvarına getirilen erişkin 2 adet (1 erkek, 1 dişi) kuyruk süren'in arka ekstremite kemiklerinin MDBT görüntüleri alındıktan sonra, veriler DICOM formatında stoklandı. Bu görüntüler 3B rekonstrüksiyon programı olan Mimics 14.1'in yüklü olduğu bir bilgisayara aktarılarak, arka ekstremite kemiklerinin rekonstrüksiyonları gerçekleştirildi. Pelvis, femur ve crus kemiklerinin 3B modellerinden bazı morfometrik ölçümler alındı. Ayrıca femur ve tibia'nın kortikal kalınlığı, endosteal ve periosteal çap değerleri MDBT görüntüleri kullanılarak ölçüldü. Ölçüm sonuçlarına göre, erkeğe ait değerlerin arcus ischiadicus açısı dışında dişiye ait olanlardan daha büyüktü. Kuyruk süren'in Carnivora takımının üyeleri arasında küçük bir yapıya sahip olduğu tespit edildi. Bu çalışma sonucunda ortaya konulan morfometrik ölçüm değerlerinin yaban hayvanlarının bilgi birikimine katkı sağlaması ve klinik uygulamalarda yardımcı olması beklenmektedir.

Anahtar sözcükler: *Karnivor, Kuyruk süren, Morfometri, Üç-boyutlu rekonstrüksiyon, Yabani hayvan*

Introduction

The Egyptian mongoose *(Herpestes ichneumon)* is a member of the family *Viverridae* from the order Carnivora [1]. It is found across the African continent, from the Sinai Peninsula in the Levant to the south of Turkey, as well as in the southwest of the Iberian Peninsula in Europe [2]. In Turkey, it has a limited habitat in Hatay, Mersin, Adana,

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and Aydın [3,4]. It generally lives in villages and rural areas, and does not hesitate to approach residential areas in search of food. Therefore, some mongooses can be found dead on roadsides as a result of traffic accidents [4]. Since the mongoose legs are short, their movement is like crawling on the ground [1]. While it has the ability to dig into the ground to change its nest, it lacks climbing skills [5].

Among the members of the Carnivora, the hind limbs only carry the animal forward [6]. This has a stronger effect on the shaping of the movement than the front extremities [7]. The morphology of these bones is very important, since the hind limb bones provide most of the driving force [8]. Animal bone morphometry studies provide important data for many scientific fields [9].

Three-dimensional (3D) models are created by the 3D reconstruction of data obtained from two-dimensional (2D) imaging modalities, such as computed tomography (CT), magnetic resonance imaging (MRI), and X-ray [10]. Since measurements performed on 3D models are very sensitive, they are used for measurement and assessment. In addition, models can be customized to cut, flip, and rotate images [11]. 3D models are also very useful in planning surgery and creating prostheses [12].

A review of the literature on the mongoose shows that there are studies performed on their body morphology and metric measurements [3], investigating their karyological and some morphological features [4], and examining their skull morphology [13], as well as comparing the craniometric measurements of mongooses living in Asia and Africa [14]. In addition, research has been undertaken on the extremities of many members of the order Carnivora, including morphometric studies on the pelvis of the retriever and German shepherd dog breeds, and the red fox [15-17], microanatomic investigation of the hind bones of the lynx *(Lynx lynx)* [18], comparison of the long bones of the modern red fox *(Vulpes vulpes)* and arctic fox *(Alopex lagopus)* [19], measurements of the canine fore and hind bones [20], 3D reconstruction and morphometric analysis of the long bones of the hind limb and hip bones (ossa coxae) of the Van cat [21,22], and 3D model measurements performed from the ossa cruris of the brown bear *(Ursus arctos)* [23].

This study was conducted to create the 3D reconstruction of the pelvis, femur and crus bones in the hind limb using 2D MCDT, perform morphometric measurements using these models, and compare these measurements to other Carnivora members described in the literature.

Material and Methods

Ethical Approval

This study was performed with permission from the Turkish Ministry of Forestry and Water Management (permission number: 92554751-445.04-148699). Ethical approval was obtained from the Research Ethics Committee of Cukurova University on December 7, 2021 (decision number: 14/05).

Animals

In this study, two adult mongooses *(Herpestes ichneumon)* [(one male (4.5 kg), one female (4.2 kg)] were used. Both mongooses died at different times by hitting their heads in traffic accidents and were brought to the anatomy laboratory. The cadavers were in good condition with intact hind limb bones.

Study Design

The animals were placed in the prone position to obtain MDCT images. The parameters of the MDCT instrument (Somatom Sensation 64; Siemens Medical Solutions, Germany) were adjusted as follows: physical detector collimation, 32 x 0.6 mm; final section collimation, 64 x 0.6 mm; section thickness, 1 mm; gantry rotation time; 330 msec; kVp; 120; mA, 300; resolution, 512 x 512 pixel; and resolution range, 0.92 x 0.92. The dosage parameters and scans were in agreement with standard protocols described in the literature [24,25]. Radiometric resolution (MONOCHROME2; 16 bits) was obtained at the lowest radiation level with optimum image quality. The images were stored in the DICOM format and transferred to a personal computer containing the 3D modelling software Mimics 14.1. (Materialise Group, Belgium). The boundaries of the pelvic bones (os coxae, sacrum), femur, and crus bones (tibia, fibula) were determined using the same software. The demarcated images were overlapped, and reconstruction was performed with this translator program. Mimics 14.1 program was used for measurements on 3D models.

Measurements

In this study, the measurements of the pelvic cavity and os coxae (1. dorsal transverse diameter, 2. intermediary transverse diameter, 3. ventral transverse diameter, 4. right sacrocotyloid diameter, 5. left sacrocotyloid diameter, 6. angle of arcus ischiadicus, 7. length of ischium, 8. total length of the symphysis pelvis, 9. mid-pubis width 10. cranial transverse diameter, 11. medial transverse diameter, 12. caudal transverse diameter, 13. total length of pelvis, 14. length of ilium, 15. length of pubis, 16. greatest length of the foramen obturatorium, 17. greatest width of the foramen obturatorium, 18. conjugata vera, 19. conjugata diagonalis, 20. vertical diameter, and 21. inclinatio pelvis), the femur (1. total length of femur, 2. distal width of femur, 3. femoral head diameter, 4. narrowest neck width, 5. proximal width, 6. angle between center of head of femur and center point of fossa trochanterica), the fibula and tibia (1. total length of the fibula, 2. width of fibula at

mid-shaft, 3. mid-shaft transverse diameter of the corpus tibia, 4. greatest breadth of the proximal end of the tibia, 5. greatest breadth of the distal end of the tibia, 6. total length of the tibia, 7. angle between medial and lateral malleolus) were performed using the 3D computer model of each bone according to the literature [8,16,19,21,26,27] *(Fig. 1, Fig. 2, Fig. 3, Fig. 4, Fig. 5)*. The cortical thickness (medial and lateral aspects) of the femur and tibia in the proximal, mid-shaft and distal were measured using tomography sections [28] *(Fig. 6)*. Also endosteal diameter and periosteal

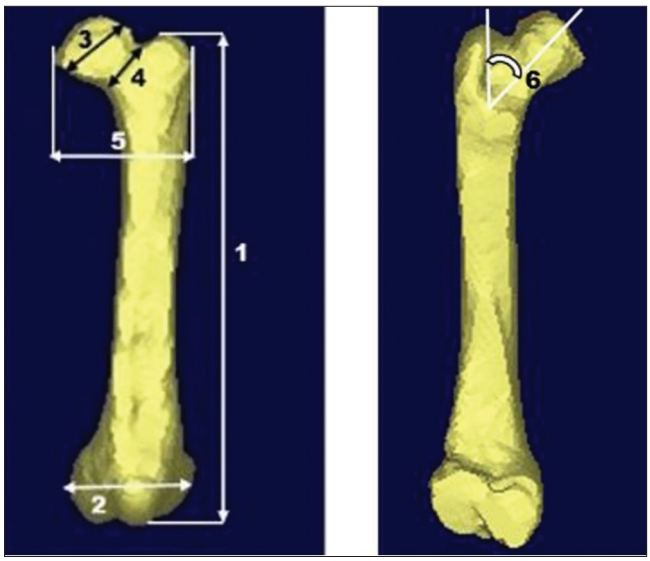

Fig 1. Cranial and caudal view of the femur: 1. total length of the femur, 2. distal width of the femur, 3. femoral head diameter, 4. narrowest neck width, 5. proximal width of the femur, 6. angle between center of head of femur and center point of fossa trochanterica

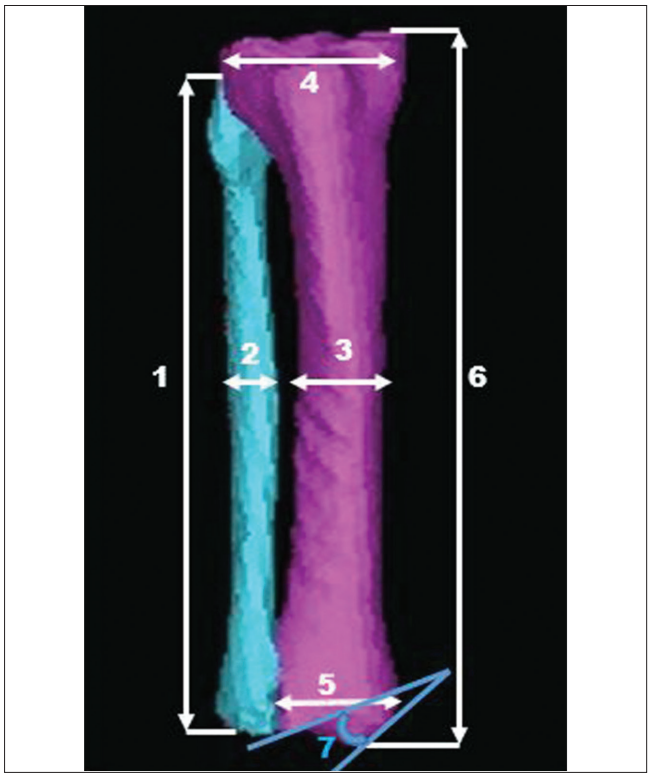

Fig 2. Cranial view of the fibula and tibia: 1. total length of the fibula, 2. width of the fibula at mid-shaft, 3. mid-shaft transverse diameter of the corpus tibia, 4. proximal width of the tibia, 5. distal width of the tibia, 6. total length of the tibia, 7. angle between medial and lateral malleolus

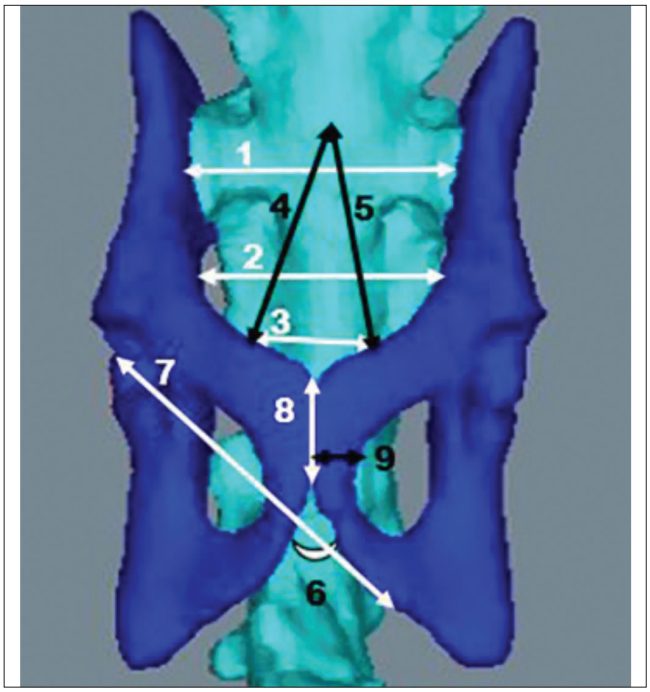

Fig 3. Ventral view of the pelvic cavity: 1. dorsal transverse diameter, 2. Intermediary transverse diameter, 3. ventral transverse diameter, 4. right sacrocotyloid diameter, 5. left sacrocotyloid diameter, 6. angle of arcus ischiadicus, 7. length of ischium, 8. total length of the symphysis pelvis, 9. mid-pubis width

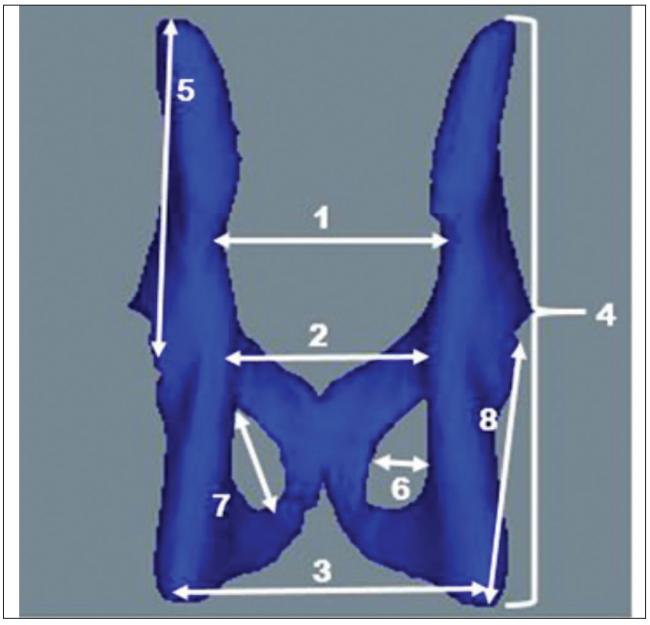

Fig 4. Dorsal view of the pelvic cavity: 1. cranial transverse diameter, 2. medial transverse diameter, 3. caudal transverse diameter, 4. total length of pelvis, 5. length of ilium, 6. greatest width of the foramen obturatorium, 7. greatest length of the foramen obturatorium, 8. length of pubis

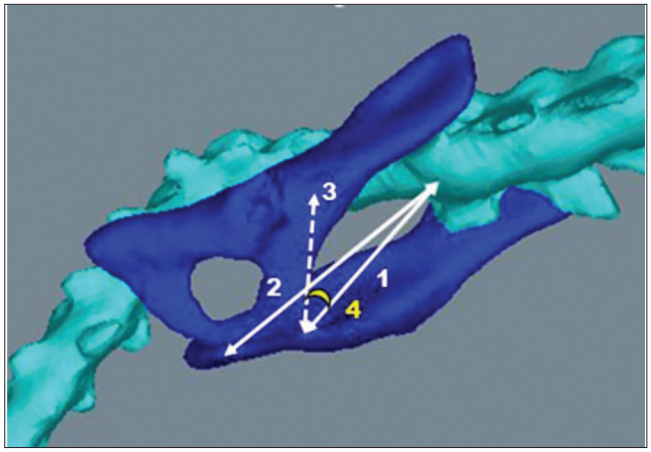

Fig 5. Lateral view of the pelvic cavity: 1. conjugata vera, 2. conjugata diagonalis, 3. vertical diameter, 4. inclinatio pelvis

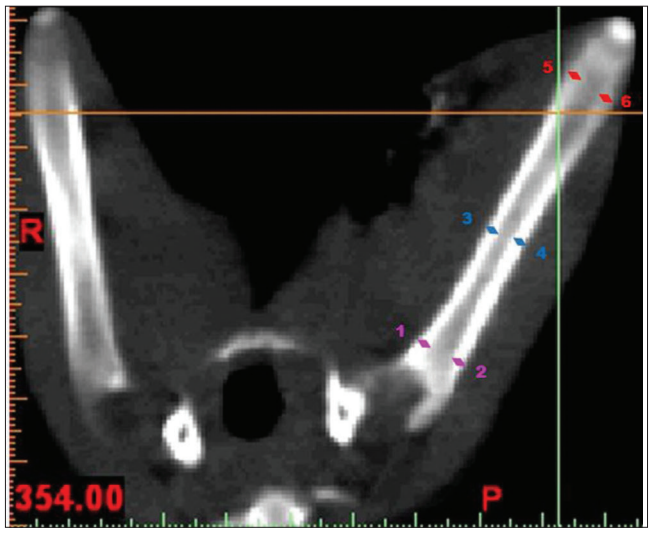

Fig 6. Measurements taken at the proximal, mid-shaft and distal level on MDCT images: 1. medial cortical thickness at proximal, 2. lateral cortical thickness at proximal, 3. medial cortical thickness at mid-shaft, 4. lateral cortical thickness at mid-shaft, 5. medial cortical thickness at distal, 6. lateral cortical thickness at distal

diameter in X-axis and Y-axis direction of the femur and tibia were taken from MDCT images [28] *(Fig. 7)*.

Results

The first of the bone forming the os coxae, the os ilium, was in sagittal position; the second bone, os pubis, was in cranio-caudal position and the third bone, os ishcii, was in horizontal position. The shape of the pelvis was narrow and small. Aperture pelvis cranialis was highly oblique *(Fig. 3, Fig. 4. Fig. 5)*.

The data of the morphometric values obtained from the 3D model of the mongoose are showed in *Table 1* and *Table 2*. All the measurements, except the angle of arcus ischiadicus, which is one of the measurement values of the pelvis, indicated greater values in the male mongoose than

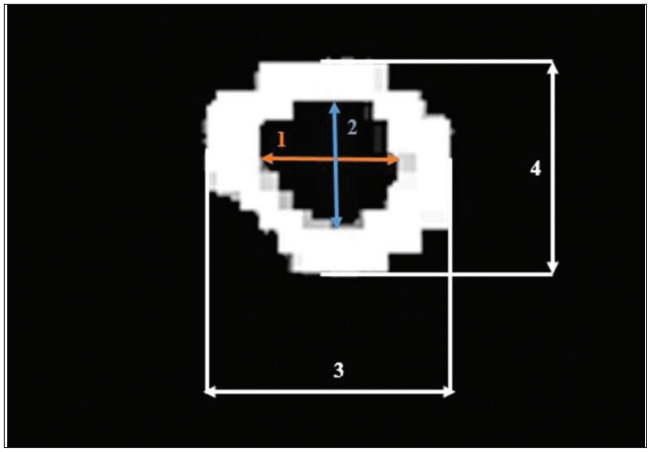

Fig 7. Measurement of endosteal and periosteal diameters at the mid-shaft of the long bone on MDCT: 1. endosteal diameter in X-axis direction, 2. endosteal diameter in Y-axis direction, 3. periosteal diameter in X -axis direction, 4. periosteal diameter in Y-axis direction

Table 1. *Results of the morphometric measurements of the pelvic cavity obtained from 3D reconstruction*

Measurements	Female	Male
Dorsal transverse diameter (mm)	24.26	25.24
Intermediary transverse diameter (mm)	23.07	24.94
Ventral transverse diameter (mm)	15.64	16.93
Right sacrocotyloid diameter (mm)	26.14	31.72
Left sacrocotyloid diameter (mm)	26.03	31.72
Angle of arcus ischiadicus (°)	50.43	45.30
Length of ischium (mm)	35.78	40.77
Total length of the symphysis pelvis (mm)	9.98	14.72
Mid-pubis width (mm)	1.87	2.89
Cranial transverse diameter (mm)	23.40	27.98
Medial transverse diameter (mm)	22.72	26.69
Caudal transverse diameter (mm)	28.04	35.16
Total length of pelvis (mm)	66.55	71.04
Length of ilium (mm)	37.54	40.53
Length of pubis(mm)	25.06	33.39
Greatest length of the foramen obturatorium (mm)	16.21	17.93
Greatest width of the foramen obturatorium (mm)	12.79	14.64
Conjugata vera (mm)	32.67	37.05
Conjugata diagonalis (mm)	40.81	49.25
Vertical diameter (mm)	23.28	31.11
Inclinatio pelvis (°)	26.54	27.22

in the female mongoose. It was determined that the dorsal transverse diameter of the cranial opening of pelvis was larger than the other transverse diameters, but close to intermediary transverse diameter. Among the transverse diameters of the cavum pelvis, the caudal transverse diameter had the greatest value. Aperture pelvis caudalis were wide. The sacrocotyloid diameter was similar on both sides *(Table 1)*.

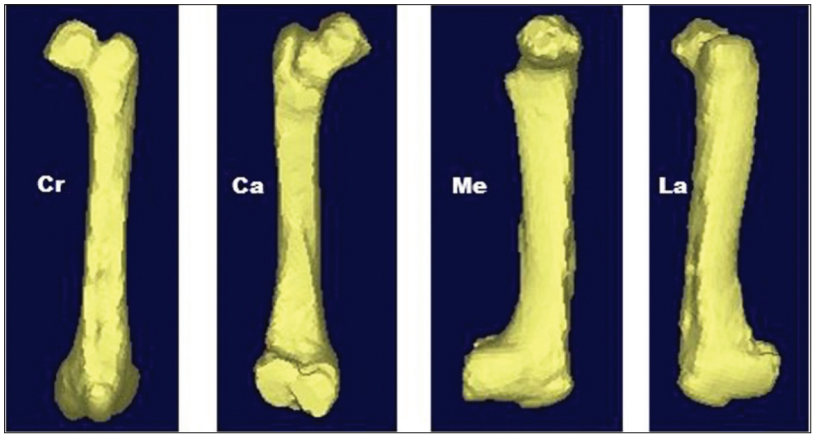

Fig 8. 3D model of the femur obtained from MDCT images. Cr. cranial view, Ca. caudal view, Me. medial view, La. lateral view

Fig 9. 3D model of crus bones obtained from MDCT images. Cr. cranial view, Ca. caudal view, Me. medial view, La. lateral view

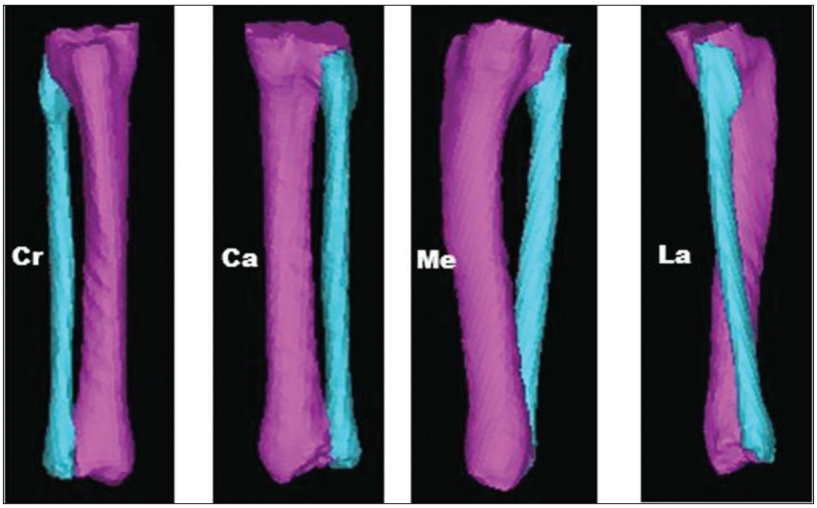

Table 2. Results of the morphometric measurements of the femur, tibia and fibula obtained from 3D reconstruction

Measurements	Female		Male	
	Right	Left	Right	Left
Total length of the femur (mm)	75.53	74.84	78.11	78. 56
Distal width of the femur (mm)	15.43	15.12	16.22	16.38
Femoral head diameter (mm)	8.92	8.67	9.43	9.78
Narrowest neck width (mm)	6.72	6.72	7.00	7.12
Proximal width of the femur (mm)	16.27	16.73	19.26	19.43
Angle between center of head of femur and center point of fossa trochanterica (°)	30.73	30.73	31.30	31.28
Total length of the fibula (mm)	61.16	61.50	63.87	63.39
Width of fibula at mid-shaft (mm)	3.68	3.98	4.25	4.36
Total length of the tibia (mm)	68.14	67.32	69.25	69.74
Mid-shaft transverse diameter of the corpus tibia (mm)	6.94	6.83	7.38	7.21
Proximal width of the tibia (mm)	15.46	15.95	18.38	18.23
Distal width of the tibia (mm)	11.26	11.35	12.58	12.36
Angle between medial and lateral malleolus (°)	41.27	41.35	41.54	41.60

The femur was proximally inclined towards its long axis in the cranio-ventral direction. The fibula extended to the distal end of the tibia. A wide interosseous space was formed between tibia and fibula *(Fig. 1, Fig. 2, Fig. 8, Fig. 9)*.

The measurement values of the femur, tibia and fibula of the female mongoose were smaller than those of the male mongoose *(Table 2)*. In addition, the data on the right and left sides were very similar to each other in both

Table 3. *Results of the morphometric measurements of the femur and tibia obtained from MDCT images*

Measurements		Female		Male	
		Right	Left	Right	Left
Femur	Medial cortical thickness at proximal (mm)	1.87	1.92	2.03	2.10
	Lateral cortical thickness at proximal (mm)	2.11	2.06	2.19	2.21
	Medial cortical thickness at mid-shaft (mm)	1.85	1.79	1.91	1.96
	Lateral cortical thickness at mid-shaft (mm)	2.65	2.27	2.86	2.75
	Medial cortical thickness at distal (mm)	2.95	2.85	3.07	3.10
	Lateral cortical thickness at distal (mm)	3.70	3.58	3.85	3.82
	Endosteal diameter in X-axis direction at mid-shaft (mm)	3.63	3.52	3.73	3.67
	Endosteal diameter in Y-axis direction at mid-shaft (mm)	3.12	3.17	3.22	3.19
	Periosteal diameter in X-axis direction at mid-shaft (mm)	6.82	6.89	7.08	7.01
	Periosteal diameter in Y-axis direction at mid-shaft (mm)	6.21	6.15	6.28	6.39
Tibia	Medial cortical thickness at proximal (mm)	1.21	1.55	1.36	1.48
	Lateral cortical thickness at proximal (mm)	1.55	1.74	1.62	1.69
	Medial cortical thickness at mid-shaft (mm)	1.74	1.80	1.90	1.93
	Lateral cortical thickness at mid-shaft (mm)	1.48	1.51	1.59	1.62
	Medial cortical thickness at distal (mm)	1.56	1.64	1.68	1.74
	Lateral cortical thickness at distal (mm)	1.42	1.48	1.56	1.59
	Endosteal diameter in X-axis direction at mid-shaft (mm)	1.73	1.73	2.64	2.52
	Endosteal diameter in Y-axis direction at mid-shaft (mm)	2.25	2.08	2.52	2.39
	Periosteal diameter in X-axis direction at mid-shaft (mm)	4.66	4.83	5.53	4.91
	Periosteal diameter in Y-axis direction at mid-shaft (mm)	5.88	5.54	6.42	6.17

sexes. Among the long bones, the femur had the greatest values, followed by the tibia and fibula in that order. In addition, the femur had a thicker structure than the tibia. The proximal ends of both the femur and tibia had larger values than their distal ends. Among the long bones in the hind limb, the fibula had the least values.

According to the medial and lateral cortical thickness measurements obtained from the tomography images of the femur, it was seen that lateral cortical thickness had higher values than medial cortical thickness at proximal, mid-shaft and distal. Medial and lateral cortical thickness was measured the most in the distal part of the femur. It was observed that the lateral cortical thickness was greater than the medial cortical thickness in the proximal, and on the contrary, the medial cortical thickness was greater than the lateral cortical thickness in the mid-shaft and distal part of the tibia. It was found that medial cortical thickness was the most in the mid-shaft and lateral cortical thickness was the most at proximal part of tibia. Endosteal and periosteal diameter values in the femur were greater on the X-axis direction than on the Y-axis direction. In the tibia, on the contrary, endosteal and periosteal diameter values on the Y-axis direction were higher than on the X-axis direction *(Table 3)*.

Discussion

It has been reported that the pelvis of the retriever dog and red fox statistically significantly differed according to sex [16,17], while there is no sexual dimorphism in the German shepherd dog [15]. Except for the angle of arcus ischiadicus, all the measurement values have been reported to be greater in the male retriever, German shepherd and red fox compared to their female counterparts [15-17]. In the current study, the measurement values of the pelvis were smaller in the female mongoose, except for the angle of arcus ischiadicus, similar to the previous studies on dogs and red foxes.

It has been stated that the size of the pelvis and the thickness of the leg bones determine the differences that distinguish dog breeds from each other. Dogs with a large pelvis have thin limb bones and dogs with a small pelvis have thicker limb bones. For example, greyhounds have relatively thinner legs than pit bulls. Fast running dogs have a wider pelvis and thinner leg bones [29]. In this study, it was observed that the mongoose had a small and narrow pelvis, thick and short leg bones. This anatomical structure caused the mongoose not run fast and move in a crawling manner on the ground [1].

In the red fox, it was reported that the aperture pelvis cranialis was widest in the middle and caudal transverse diameter had greatest value of the cavum pelvis [17]. In this study, unlike the fox, the dorsal transverse diameter of the aperture pelvis cranialis was the largest, but the values were close to the intermediary transverse diameter. The caudal transverse diameter of the mongoose was also the largest transverse diameter of the pelvic cavity, just like in the fox.

For *Metailurus parvulus* belonging to the family *Felidae* of the order Carnivora, the total length of the femur was reported as 216.0 mm, transverse diameter at the midshaft of the femur as 19.7 mm, greatest anteroposterior diameter of the distal end of the femur as 40.7 mm, anteroposterior diameter of the caput femoris as 20.7 mm, greatest anteroposterior diameter of the proximal end of the femur as 44.1 mm, total length of the tibia as 207.8 mm, transverse diameter at the mid-shaft of the tibia as 15.2 mm, greatest anteroposterior diameter of the proximal end of the tibia as 42.2 mm, greatest anteroposterior diameter of the distal end of the tibia as 19.2 mm, and total length of the fibula as 186.0 mm [30]. In the current study, the measurement values of the femur, tibia and fibula of the mongoose were approximately one-third of those reported for the same bones in *Metailurus parvulus.* This finding is consistent with the representatives of the family *Herpestidae* being characterized by a relatively small-to-medium body size [31]. Body size is the main factor determining limb bone morphology in Carnivora [5]. Among the hind limb bones of the mongoose, the femur was the largest, and the measurement between the distal end of the femur and the proximal ends of the tibia, which are involved in the formation of the articularis genus, were close to each other, which is also in agreement with the anatomy of *Metailurus parvulus.* In our study, it was observed that the distal end of the femur was narrower in the female than in the male, as described for humans [32].

As a result of the measurements performed on the long bones of the modern red fox *(Vulpes vulpes)* and arctic fox *(Alopex lagopus),* it was reported that the male animals had statistically significantly larger measurements than their female counterparts. The total length of the femur in the red fox was determined as 122.2 mm in the female and 130.4 mm in the male, the proximal breadth of the femur was 23.2 mm and 25.2 mm, respectively, and the distal breadth of the femur was 19.1 mm and 20.8 mm, respectively [19]. In the mongoose, the total length of the femur on the right side was measured as 75.53 mm in the female and 78.11 mm in the male, the proximal breadth of the femur was 16.27 mm in the female and 19.26 mm in the male, and the distal breadth of the femur was 15.43 mm in female and 16.22 mm in male. This shows that the measurement values of the male mongoose were larger than those of the female, and the proximal end of the femur was wider than the distal end, similar to the red fox. It has also been reported that there is a difference between the male and female femurs in humans, with the former being larger than the latter [32].

The total length of the tibia in the red fox was reported to be 137.5 mm in the female and 147.3 mm in the male, the proximal breadth of the tibia was 20.9 mm in the female and 22.0 mm in the male, and the distal breadth of the tibia was 14.2 mm in the female and 14.8 mm in the male [19]. In the current study, the total length of the tibia on the right side was 68.14 mm in the female mongoose and 69.25 mm in the male mongoose, the proximal breadth of the tibia was 15.46 mm and 18.38 mm, respectively, and the distal breadth of the tibia was 11.26 mm and 12.58 mm, respectively. Thus, as reported for the red fox, the male mongoose also presented with larger measurements than the female mongoose. In a human study, it was also reported that the tibia was statistically significantly larger in men than in women [32].

From the canine remains, which were found during excavations in Van-Yoncatepe, the femoral length was determined as 181.6 mm and the tibial length as 180.2 mm [20]. In another study, the mean femoral length of the Makah and Coast Salish dog was reported as 164.4 mm, and the mean tibial length as 158.5 mm [33]. While the femur of the mongoose was observed to be longer compared to the length of the tibia, as in dogs [20,33], the tibia was reported to be larger than the femur in the red fox [19].

In the literature, it is stated that the mean length of the fibula in the Makah and Coast Salish dog is 148.2 mm [33], while the total length of the fibula in the red fox is 130.2 mm in the female and 138.1 mm in the male [19]. In our study, the total length of the fibula on the right side was measured as 61.16 mm in the female mongoose and 63.87 mm in the male. Thus, the shortest bone among the hind limb bones of the mongoose was determined to be the fibula, which is similar to the reports on the red fox and dog.

For the Nigerian local dog *(Canis lupus familiaris),* no statistically significant difference was reported between the right and left sides according to the biometric measurement values of the hind limb bones [34]. Similarly, in the current study, the measurement values of the femur, tibia, and fibula were similar for the right and left sides in both the male and female mongooses.

It has been reported that the medial cortical thickness of the femur in dogs gradually decreases from proximal to distal. It was stated that the lateral cortical thickness remained more or less the same in the proximal and mid-shaft, and decreased in the distal. It has been reported

that the medial cortical thickness in the tibia increases from proximal to distal, and the lateral cortical thickness decreases from proximal to distal [35]. In our study, the mongoose unlike the dog [35], it was observed that the medial and lateral cortical thickness was the most in the distal of the femur. It was seen that medial cortical thickness was the most in the mid-shaft and lateral cortical thickness was the most at proximal part of tibia unlike the dog. In the literature [28], it was reported that the endosteal and periosteal diameter measurement values on the X-axis direction of the rabbit femur were higher than those on the Y-axis direction. In our study, it was determined that the values of endosteal and periosteal diameter measurements on the X-axis direction were greater than those on the Y-axis direction in the mongoose femur as in the rabbit femur [28].

According to the data obtained from the hind limb of the mongoose, the measurement values of the male were greater than those of the female. This is consistent with the previous knowledge that male mongooses are larger than females [14]. The male mongoose has been reported to be 20% heavier on average than the female [36]. According to the results of the morphometric study performed on the long bones of the hind limb of the Van cat, it was stated that there was sexual dimorphism, with the male having greater values than the female [21]. The measurements of the coxae, femur and tibia of the hind limb are also reported to differ according to sex in *Felis catus* [9].

The Egyptian mongoose *(Herpestes ichneumon)* is on the International Union for Conservation of Nature's Red List of Threatened Species [37] and lives in a limited area in Turkey; therefore, the number of animals in our study was limited. Due to the few number of animal used in the study, statistical results could not be expressed by performing a statistical analysis. In future studies, it is recommended to study with more animals, if possible.

In conclusion, when the hind limb bones of the Egyptian mongoose were compared between sexes, all the measurement values were higher in the male mongoose than in the female. In addition, the right and left side measurements were very close to each other in both sexes. The 3D reconstruction of the entire bone from 2D computed tomography images is very useful for morphometric studies and clinical applications. It is expected that the morphometric data presented in this paper will contribute to the knowledge of the anatomy of wild animals and guide future clinical studies.

Ethical Approval

This study was performed with permission from the Turkish Ministry of Forestry and Water Management (permission number: 92554751-445.04-148699). Ethical approval was obtained from the Research Ethics Committee of Cukurova University on December 7, 2021 (decision number: 14/05).

Availability of Data and Materials

Data sets analyzed during the current study are available from the corresponding author (S. Özkadif) on reasonable request.

Competing Interests

There was no conflict of interest in regards to authors reporting their findings.

Author Contributions

The conception of the investigation was performed by SÖ and AH. Collection of materials and obtaining MDCT images were done by two of the authors. Reconstruction of bones, taking morphometric measurements and writing of the article were done by SÖ. Revision of manuscript and preparation of the figures were performed by AH.

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