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Molecular identification using 18S ribosomal RNA of *Sarcocystis* spp. in bovine minced meat in Van Province, Turkey

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Abstract: This study aimed to determine the prevalence and molecular characterization of *Sarcocystis* spp. in bovine minced meat that is sold in various grocery stores and butcher shops in Van Province of Turkey. For this purpose a total of 150 samples were obtained from İpekyolu, Tuşba, Edremit, Erciş, and Gevaş districts of Van Province in monthly periods from May to October 2019. 28% (42/150) were found positive for *Sarcocystis* species as a result of the microscopic analyses and 96.6% (145/150) were found positive for *Sarcocystis* species as a result of the multiplex-PCR and RFLP methods. *Sarcocystis cruzi* (96.6%) was detected in all samples that were detected positive using molecular methods. *Sarcocystis hominis*-like was found in 143 (95%) samples whereas *S. hirsuta* was detected in only 4 (2.6%) samples. According to the Basic Local Alignment Search Tool (BLASTN) analysis of the 18S rRNA gene region of the *S. cruzi* (MN832695) and *S. hirsuta* (MN832697) isolates, they showed 100% similarity to the samples (MH681972; MH681973) that were submitted to GenBank from China. The BLASTN analysis of the 18S rRNA gene region of the *S. hominis*-like isolate (MN832696) revealed that it was 99.45% identical to the *S. bovini* (KT901155) isolated from a water buffalo in New Zealand. In conclusion, the molecular characterization of *Sarcocystis* spp. has been provided for the first time in Van Province, and the first unverified scientific data for *S. bovini* has been established in this study.
Keywords: 18S rRNA, bovine minced meat, PCR, *Sarcocystis* spp.

Türkiye'nin Van ilindeki sığır kıymalarında *Sarcocystis* türlerinin 18S ribosomal RNA gen bölgesi kullanılarak moleküler tanımlanması

Özet: Bu çalışmanın amacı Türkiye'nin Van ilinde çeşitli market ve kasaplarda satılan sığır kıymalarında *Sarcocystis* türlerininin yayılışını ve moleküler karakterizasyonlarını ortaya koymaktır. Bu amaçla 2019 yılının Mayıs-Ekim aylarında aylık periyotlarla Van'ın İpekyolu, Tuşba, Edremit, Erciş ve Gevaş ilçelerinden toplam 150 numune toplanmıştır. Mikroskobik analizler sonucunda %28'i (42/150) *Sarcocystis* türleri yönünden pozitif bulunmuş, multiplex-PZR ve RFLP yöntemleri sonucunda ise %96,6'sı (145/150) *Sarcocystis* türleri yönünden pozitif bulunmuştur. Moleküler yöntemlerle pozitif olarak belirlenen tüm örneklerde *S. cruzi* (%96,6) tespit edilmiştir. *Sarcocystis hominis*-like 143 (%95) örnekte bulunurken, *S. hirsuta* ise sadece 4 (%2,6) örnekte tespit edilmiştir. *Sarcocystis cruzi* (MN832695) ve *S. hirsuta* (MN832697) izolatlarının 18S rRNA gen bölgesinin Basic Local Alignment Search Tool (BLASTN) analizlerine göre, GenBank'a Çin'den kaydedilen (MH681972; MH681973) örneklerle %100 benzerlik göstermiştir. *Sarcocystis hominis*-like izolatının 18S rRNA gen bölgesinin BLASTN analizlerine göre (MN832696) Yeni Zelanda'da mandadan izole edilen *S. bovini* (KT901155) izolatı ile %99,45 identik olduğu belirlenmiştir. Sonuç olarak bu çalışma ile Van ilinde ilk kez *Sarcocsytis* türlerinin moleküler karakterizasyonları sağlanmış ve *S. bovini* için doğrulanmamış ilk bilimsel veriler oluşturulmuştur.
Anahtar sözcükler: 18S rRNA, PZR, *Sarcocystis* spp., sığır kıymaları.

Introduction

Sarcocystis species are protozoan parasites, with an obligatory two-host cycle and classified in the phylum Apicomplexa. Over 220 species were identified in the genus *Sarcocystis* and widely seen across the world (32). *Sarcocystis* spp., heteroxen and protozoan parasites, form cysts in tissues of intermediate hosts and are thrown out as sporocysts with definitive hosts. Cysts of these species are found in hearts, oesophagus, diaphragms, tongues, jaws and other skeletal muscles of herbivores, which are their intermediate hosts. Among herbivores, cattle are reported to be the intermediate hosts for seven species, i.e., *S. cruzi*, *S. bovifelis*, *S. hirsuta*, *S. bovini*, *S. hominis*, S. *rommeli* (formerly known as *S. sinensis*-like) and *S. heydorni.* The definitive hosts of these species are canids (*S. cruzi*), felids (*S. hirsuta*, *S. bovini*, *S. bovifelis* and *S. rommeli*) and

humans (*S. hominis* and *S. heydorni*). It has been found that among these species *S. hominis* and *S. heydorni* caused zoonotic infections, infected humans with infected pork and beef or contaminated food, and calves were infected with sporocysts excreted in feces as a result of infection (6, 10, 15, 18).

Sarcosporidiosis is generally considered non-pathogenic for cattle. Lymphadenopathy, petechial hemorrhages in serious membranes, degeneration, sarcocysts and hemorrhages in the heart, kidney, lung and muscles are detected in necropsy. Sarcocystosis in humans is found in the chronic form in the digestive system, and the symptoms are not significant or specific. Acute gastroenteritis may occur; abdominal pain and clinical symptoms such as nausea, vomiting, diarrhea can be seen. Some species in humans may cause muscular sarcocystosis, and progress to myalgia and arthralgia (8).

In 2010, the European Food Safety Authority (EFSA) highlighted the lack of reliable methods for detecting the presence of *Sarcocystis* species in animals and meat products (40). These species that cause sarcosporidiosis in cattle are generally microscopic and the size of the cysts, and the structure and thickness of the cyst walls vary according to the species. Previous studies (36, 39) conducted in the province, have identified these parasites under the light microscope which has several disadvantages. The thin cyst wall with the presence of hair-like villar protrusions of *S. cruzi* and *S. heydorni* enables them to be separated from the other two species. However, the cysts of *S. hirsuta*, *S. hominis*, *S. bovini*, and *S. rommeli* are thick-walled, and electron microscopic examination or molecular methods are required to be able to distinguish them from each other. The use of electron microscopes is strictly dependent on qualified microscopists, long working hours and demanding practices. Nevertheless, because these microscopes are expensive and not easily available, molecular applications have become more common in the diagnoses of *Sarcocystis* species (6, 8, 37).

In recent years, several molecular diagnostic techniques have been applied in the identification of *Sarcocystis* spp. in meat products (such as minced meat, meatball, raw hamburger, and sausage) that are most frequently offered for human consumption. These techniques include multiplex PCR, PCR-RFLP (Restriction Fragment Length Polymorphism), PCR-RAPD (Random Amplified Polymorphism DNA) and DNA Sequencing. The 18S rRNA, cytochrome oxidase 1 (cox1) and internal transcribed spacer 1 (ITS1) gene regions are used in the molecular analyses of species causing sarcosporidiosis. In particular, the 18S rRNA gene region is more widely used for the identification of *Sarcocystis* spp. than the other gene regions (4, 16, 18, 23, 24, 27, 33, 42). However; it has been reported that *S. hominis*, *S. bovifelis*, *S. bovini*, and *S. rommeli* species cannot be distinguished by the 18S rRNA gene region, and the term '*S. hominis*-like' should be used for all these species (35). *Sarcocystis sinensis* is not included in this group, as it was not considered a cattle species (35).

The rate of *Sarcocystis*-infection detected in cattle tissues is considerably high (91.6-100%) in Turkey. Therefore, it is thought that Turkish people consuming raw or undercooked beef products may have serious health problems. In addition, these studies have been mostly performed using conventional diagnostic methods (2, 30, 31, 36, 39, 41), whereas there are only a limited number of molecular diagnostic studies in Turkey (12, 20). Remarkably, the molecular characterization studies are insufficient in Turkey.

The objectives of this study were to determine the prevalence of *Sarcocystis* spp. and which species are found in bovine minced meat sold in various grocery stores and butcher shops by using the molecular techniques (multiplex-PCR and RFLP) in Van Province of Turkey.

Materials and Methods

Bovine minced meat samples: In this study, 150 bovine minced meats that were purchased from various butcher shops and markets in İpekyolu, Tuşba, Edremit, Erciş and Gevaş districts of Van Province in monthly periods from May to October 2019 were used as the sample. The meats were freshly ground, (approximately 200 gr each), taken into sterile sampling bags under aseptic conditions, and brought to the laboratory in refrigerated heat-insulated containers within 30-60 minutes at the latest. The approval of the Ethics Committee for this research was obtained from the Animal Experiments Local Ethics Committee of Van Yüzüncü Yıl University (dated 28/06/2018 and numbered 06).

***Isolation and microscopic examination of Sarcocystis* spp.:** Five grams of samples were taken into 50 cc trypsin solution (1.3 g pepsin, 3.5 ml 25% HCl, 2.5 g NaCl and 500 ml distilled water), kept at 40°C for 30 minutes by thawing and chopping method and homogenized through tissue homogenizer (14). Thereafter, it was strained through a 63-µm strainer, and the filtrate was placed into a tube. The homogenizer was cleaned with boiling water before each tissue. This filtrate was centrifuged at 3500 rpm for 10 minutes. Subsequently, a small quantity of sediment at the bottom was taken by a pipette and was morphologically examined by the light microscope (x40) for *Sarcocystis* tissue cysts (18, 28).

DNA extraction: The minced meats, on which thawing and chopping methods were applied, were taken into 1.5 ml microcentrifuge tubes in a 30 µl distilled water, and the DNA isolations were performed with commercial DNA extraction kit (NucleoSpin® Tissue, Macherey-Nagel, Düren, Germany) by applying the manufacturer's

tissue protocol. The DNA samples were stored at -20ºC until the PCR stage. After this step, two different PCR methods (multiplex-PCR and RFLP-PCR) were applied.

Multiplex-PCR: Genomic DNAs were subjected to the multiplex-PCR by using the specific primers; F: ACAGAACCAACACGCTC and R: AACCCTAATTC CCCGTTA (amplifying ~180 bp) for *S. hominis*-like; F: ATCAGATGAAAATCTACTACATGG and R: AACCCTAATTCCCCGTTA (amplifying ~284 bp) for *S. cruzi* and F: CATTTCGGTGATTATTGG and R: AACCCTAATTCCCCGTTA (amplifying ~108 bp) for *S. hirsuta* (4, 35). Five microliter of DNA sample was mixed with 12.5 µl of AmpliTaq Gold 360 Master Mix (Applied Biosystems, Life Technologies, California, USA), 0.5 µM of forward and reverse primers, and 5.5 µL of DNase- and RNase-free sterile distilled water (Biobasic, Canada), respectively, in a total volume of 25 µl. The thermal profile was set to be initial denaturation: at 95 ºC for 3 min; 40 cycles, denaturation: at 95 ºC for 60 sec, annealing at 56 ºC for 30 sec, extension: at 72 ºC for 30 sec and final extension: at 72 ºC for 5 min. The PCR products were loaded onto a 1.5% agarose gel, stained with ethidium bromide (0.5 mg/ml) (Sigma-Aldrich, St-Louis MO, USA) and subjected to electrophoresis at 100 volts for 2 hours (Biorad, USA). It was visualized with gel documentation system (Avegene, Taiwan).

RFLP-PCR: Genomic DNAs were subjected to the PCR by the specific primers; F: CGTGGTAATTC TATGGCTAATACA and R: TTTATGGTTAAGA CTACGACGGTA (amplifying ~900 bp) gene region from 18S rRNA for *Sarcocystis* spp. (43). Five microliter of DNA sample was mixed with 12.5 µl of AmpliTaq Gold 360 Master Mix (Applied Biosystems, Life Technologies, California, USA), 0.5 µM of forward and reverse primers, and 6.5 µL of DNase- and RNase-free sterile distilled water (Biobasic, Canada), respectively, in a total volume of 25 µl. The thermal profile was set to be initial denaturation: at 94 ºC for 5 min; 35 cycles, denaturation: at 94 ºC for 30 sec, annealing at 58 ºC for 1 min, extension: at 72 ºC for 1 min and final extension: at 72 ºC for 5 min. The PCR products were subjected to 1.5% agarose gel electrophoresis (90 volts for 60 min), visualized and analyzed by gel documentation system (Avegene, Taiwan).

The amplified PCR products were digested with BcII restriction endonuclease enzyme (ER0721, Thermo Fisher Scientific, USA) and different *Sarcocystis* spp. were detected. Fragments of ~358 and 595 bp lengths for *S. hirsuta* and ~782 and 140 bp lengths for *S. hominis*-like were expected whereas it was expected to be single band (i.e. uncut) for *S. cruzi* (16). For RFLP analysis; 8 µl PCR product, 5 µl distilled water, 1 µl restriction enzyme and 1 µl buffer were added into a total of 15 µl reaction mixture, and incubated at 55ºC for 3 h. After that the enzyme was inactivated at 80ºC for 20 min. The fragments (10 µl) were detected on 1.5% agarose gel electrophoresis (90 volts for 60 min) and visualized by Gel Documentation System or UV Transilluminator Equipment.

Sanger sequencing: Following the multiplex and RFLP-PCR stages, expected bands were determined, and the PCR process was applied again to form a single band for each parasite by switching the conventional PCR (Figure 1). The PCR products and primers coding the 18S rRNA gene region were properly packaged and sent to the Sentebiolab Company (Ankara) for performing DNA sequence analysis. The sequence chromatograms were controlled and arranged using the BioEdit software (13). The final consensus sequences of our isolates were subjected to the "BLAST analysis" (http://www.ncbi.nlmn.nih.gov/BLAST) in the GenBank

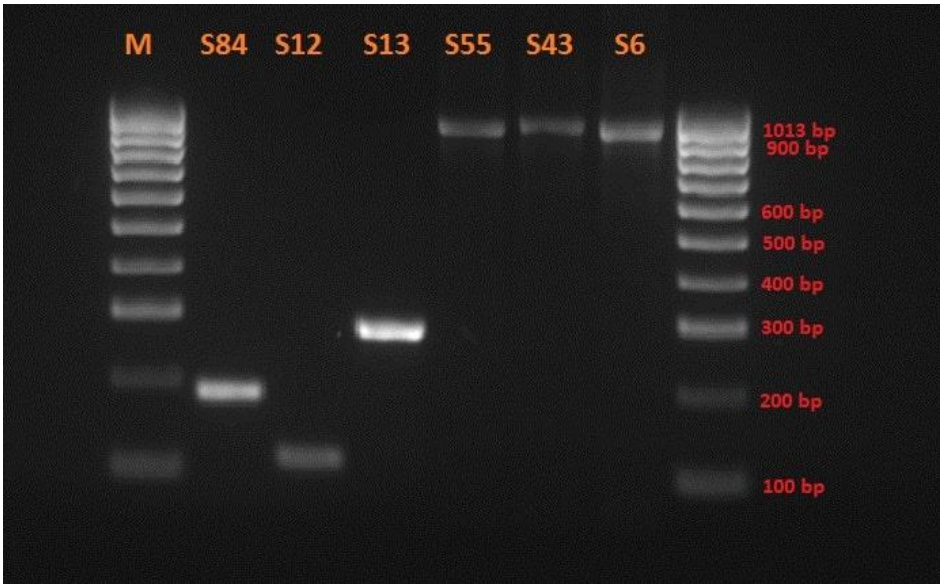

Figure 1. The single-PCR products of *Sarcocystis* species. M: 100 bp molecular size marker, S84: *S. hominis-like* (~180 bp), S12: *S. hirsuta* (~108 bp), S13: *S. cruzi* (~284 bp). S55, S43 and S6: *Sarcocystis* spp. (~900 bp).

Database, and their similarity rates were compared with the isolates reported from different countries. Genetic distances were calculated using the Kimura 2 parameter model in MEGA 7.0 (21). The 18S rRNA phylogenetic analysis data set was comprised of nucleotide sequences of a total of 23 isolates. *Hepatozoon canis* (MH615005) was used as an "outgroup". The phylogenetic analysis and the construction of phylogenetic tree were performed with 1000-repeated bootstrap using "maximum likelihood (ML)" method in the MEGA 7.0 (21) software. The nucleotide sequences obtained in the study were submitted as the corresponding accession numbers of MN832695-MN832699 in GenBank.

Results

The microscopic analysis revealed that 42 (28%) of 150 minced meat samples were positive for *Sarcocystis* microcyst (Figure 2). As a result of the multiplex-PCR analysis, 145 (96.6%) of 150 minced meat samples were found positive for *Sarcocystis* species. *Sarcocystis cruzi* was determined in all (96.6%) positive samples. Moreover, *S. hominis*-like was found in 143 (95%) samples, whereas *S. hirsuta* was detected in only 4 (2.6%) samples. Expected fragments of ~284 bp for *S. cruzi*, ~180 bp for *S. hominis*-like and ~108 bp for *S. hirsuta* were successfully amplified (Figure 3). In the RFLP-PCR stage, bands of ~900 bp were first expected and successfully

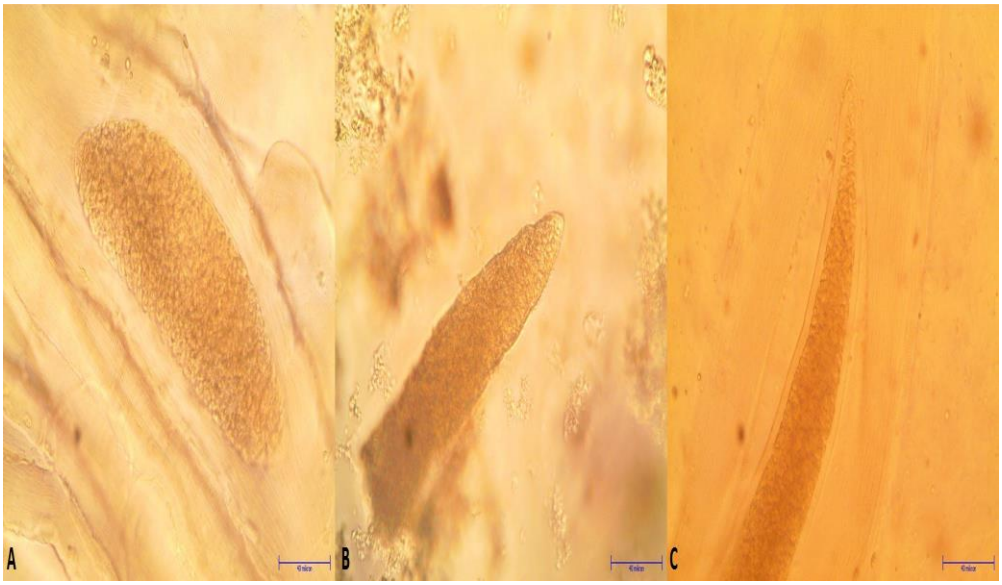

Figure 2. Microscopic determination of *Sarcocystis* microscyst. A: *S. cruzi*, B: *S. hirsuta*, C: *S. hominis*-like (*S. bovini* (unascertained information)), Scale bars: 40 µm.

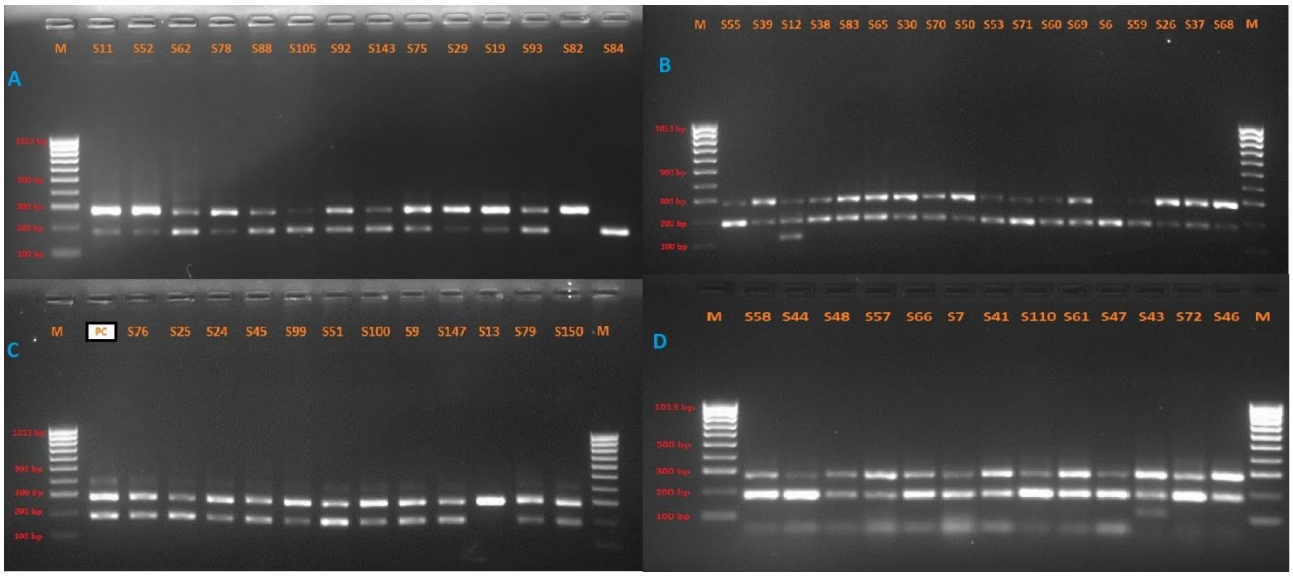

Figure 3. The analysis of multiplex-PCR products. M: 100 bp molecular size marker; PC (C): positive mPCR controls, S11(A), S55(B), S76(C), S58 (D): *S. cruzi*/*S. hominis-like*, S82(A) and S13(C): *S. cruzi*, S84(A): *S. hominis-like*, S12(B) and S43(D): *S. cruzi*/*S. hominis-like*/*S. hirsuta*.

amplified (Figure 4). According to PCR-RFLP analysis of the species identification of *Sarcocystis*, the digested 900 bp amplified fragments using BclI revealed that 145 (96.6%) of the 150 isolates were *S. cruzi*, 4 (2.6%) showed co-infection to *S. cruzi* and *S. hirsuta*, 143 (95%) was mixed infected to *S. cruzi* and *S. hominis-like*, 4 (2.6%) showed the pattern of mix infection to three species (Figure 5). The RFLP-PCR results were found to be compatible with the molecular prevalence rate (96.6%) as compared with the multiplex-PCR results.

Our *S. cruzi* (MN832695) isolates was found to be 100% similar to (MH681972) from China. In addition, the microcyst of *S. cruzi* obtained in the study of Imre et al. (18) was found to have similar morphological features with the microcyst taken in our study (Figure 1/A). Similarly, our *S. hirsuta* (MN832697) isolates showed 100% identity with the isolate (MH681973) from China. It is noteworthy that the microscopic photography of *S. hirsuta* obtained in the study of Murata et al. (28) was similar to the one in our study (Figure 1/B).

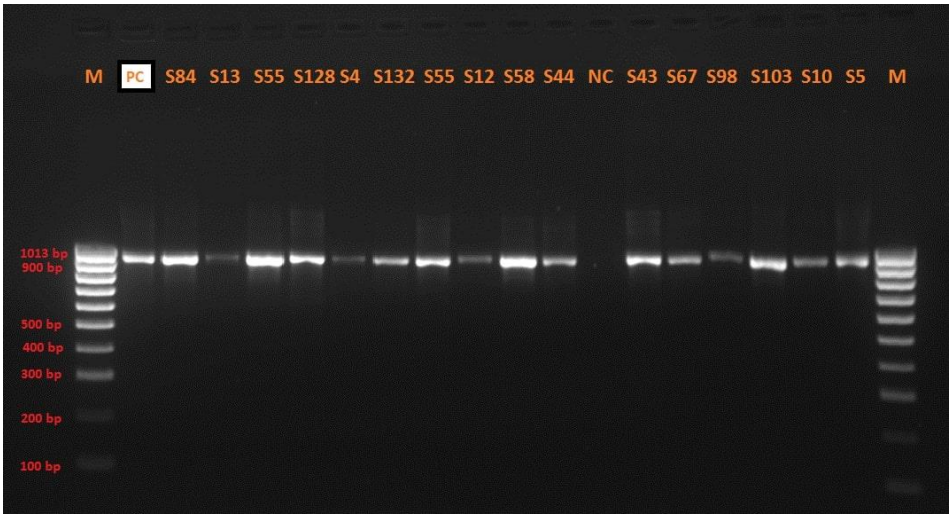

Figure 4. The single-PCR products of *Sarcocystis* species. M: 100 bp molecular size marker, PC: Positive PCR controls, NC: Negative PCR control, S84-S44/S43-S5: *Sarcocystis* spp. positives (~900 bp).

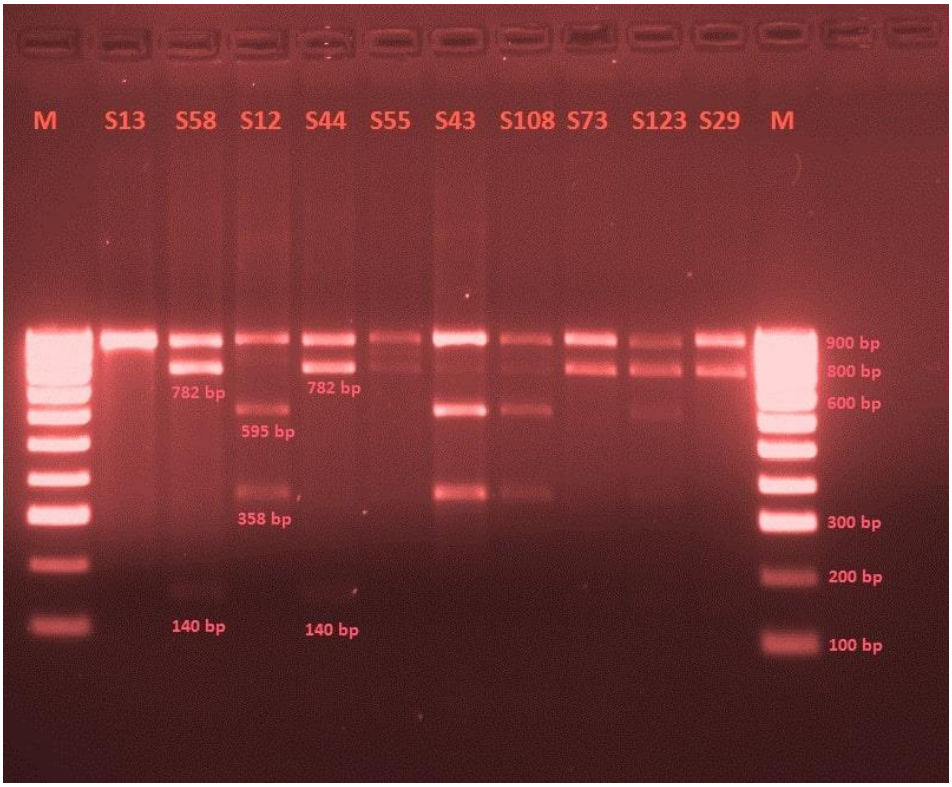

Figure 5. The BcII restriction endonuclease reaction of *Sarcocystis* spp. PCR products. M: 100 bp molecular size marker, S13: *S. cruzi*, S58-S44: *S. cruzi* mixed with *S. hominis-like*, S12: *S. cruzi* mixed with *S. hirsuta.*

The nucleotide percent identities among 18S rRNA sequence of *S. hominis*-like from Turkey (MN832696) showed 99.45% identity with *S. bovini* (KT901155) from New Zealand. Furthermore, our *S. hominis*-like from Turkey (MN832696) differed by one nucleotide (A to G) in the 18S rRNA sequence of *S. bovini* (KT901155) from New Zealand (Figure 6). Additionally, it is worth noting that the microscopic view of *S. bovini* obtained in the study of Murata et al. (28) was similar to the microcyst found in our study (Figure 1/C). Figure 7 shows the phylogenetic tree constructed with ML method (Kimura 2 parameter) of *Sarcocytis* isolates identified from minced meat in Van Province and some other *Sarcocytis* isolates from various parts of the world.

Download GenBank Graphics

Sarcocystis bovini isolate B7.2 clone 6 18S ribosomal RNA gene, complete sequence

Sequence ID: KT901155.1 Length: 1836 Number of Matches: 1

Range 1: 216 to 396 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
329 bits(178)	2e-86	180/181(99%)	0/181(0%)	Plus/Plus

```
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Query  61   GATCGCATTATGATCATATTATTATGATTGGCGATAAATCATTCAAGTTTCTGACCTATC  120
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
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Query  121  AGCTTTCGACGGTAGTGTATTGGACTACCGTGGCAGTGACGGGTAACGGGGAATTAGGGT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336  AGCTTTCGACGGTAGTGTATTGGACTACCGTGGCAGTGACGGGTAACGGGGAATTAGGGT  395

Query  181  T  181
            |
Sbjct  396  T  396
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Figure 6. DNA sequence alignment of 18S rRNA gene in isolated *S. hominis*-like samples compared with the published sequences of *S. bovini* on GenBank (Accessison no: KT901155).

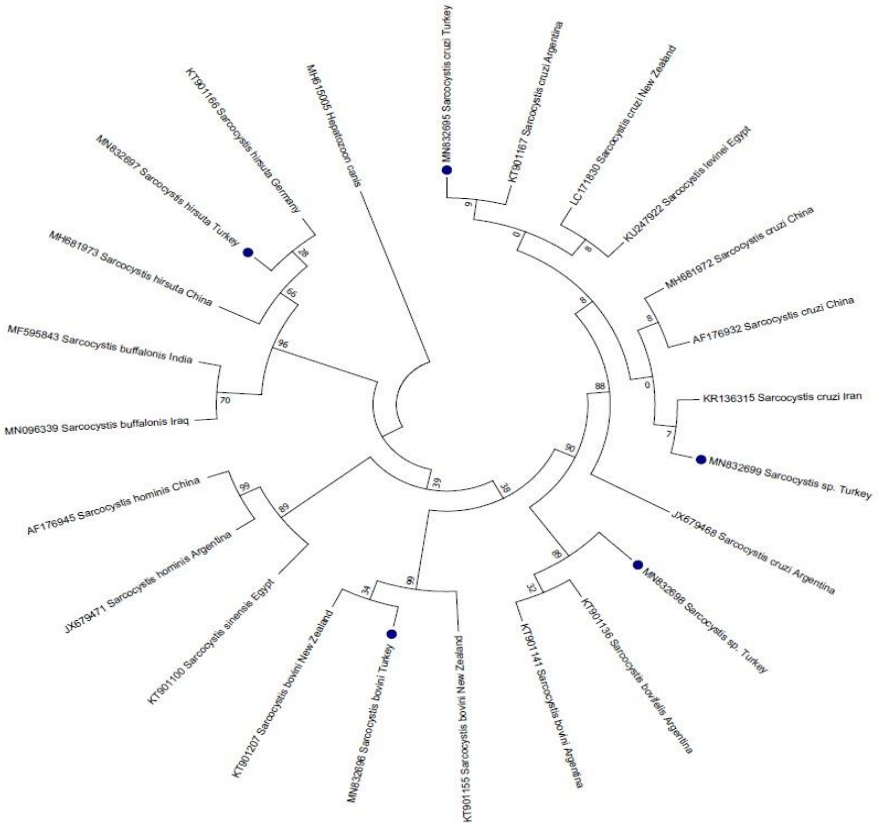

Figure 7. Maximum likelihood phylogenetic tree of *Sarcocystis* spp. 18S rRNA gene sequences with 1,000 bootstrap replicates. The evolutionary history was inferred by using the Kimura 2 parameter. Sequences were obtained from the GenBank database and GenBank accession numbers and country of origin from which the sequences were derived are included for each sequence. Isolates from this study are indicated with a blue round.

Discussion and Conclusion

Cattle are the most common intermediate host of seven *Sarcocystis* species, i.e., *S. cruzi*, *S. bovifelis*, *S. hirsuta*, *S. bovini*, *S. hominis*, S. *rommeli* (formerly known as *S. sinensis*-like) and *S. heydorni*, in their muscles (6, 8, 15, 18, 28, 35).

The results of the prevalence of *Sarcocystis* spp. infection reported in this study are consistent with the ones found in similar studies. The prevalence of infection in cattle in Turkey and other countries around the world has been reported to be high. For example, a 100% prevalence of infection in cattle has been reported in countries such as Brazil (38), Iran (29), the USA and Argentina (25). Studies conducted on *Sarcocystis* spp. in cattle in different parts of Turkey using the microscopic methods reported high prevalence rates of 100% in Bayburt region (30), 91.5% in Elazığ region (31), 100% in Bursa region (41), 92% in Van region (39), 95.3% in Kars region and 97.1% in Erzurum region (2). The prevalence of infected cattle were varies between 66.0% and 78.1% in Hungary (17), 57.5% and 83.6% in Estonia (22), 78.1% and 91% in Italy (4, 5), 64.6% and 100% in Portugal (11), and 90% and 100% in France (3). Lower prevalence of infected cattle, were determined in Germany and Romania, i.e., 26.4% and 17.9%, respectively (26). To sum up, it is difficult to carry out a detailed analysis and comparison of the data on prevalence of *Sarcocystis* spp. infection in domestic animals. For example, in a study in Romania, the researchers only performed the PCR analyses of the samples that they found to be positive under a microscope. If they had examined all the samples with the PCR method, the rate they detected would have been expected to be even greater (18). In other words, the methods used in *Sarcocystis* infections can change the prevalence rates considerably. The results of this study also prove this fact. It has also been reported that the prevalence of infection can vary significantly depending on the muscle groups. In a study on *Sarcocystis* in different muscle groups in cattle, the prevalence of infection was found to be 98.1% in oesophagus, while it was 44.9% in lumbar muscles (*M. longissimus dorsi*) (19). Considering the public health, the accurate data on the prevalence of *S. hominis* in cattle is needed, and nowadays reliable and fast molecular methods for the identification are preferred.

Techniques based on DNA sequence analysis have been successfully applied for the classification of foodborne pathogens, improving diagnostic methods and phylogenetic analyses (4, 10, 18, 23, 28, 35). In the current research, *Sarcocystis* spp. isolated from cattle was attempted to identify by using 18S rRNA gene region. The rate of *Sarcocystis* in minced meat was determined as 96.6% by applying two different molecular techniques. The prevalence rate of *Sarcocystis* was found to be close to the ones found in the studies previously conducted in Turkey (2, 30, 31, 36, 39). The primers used in the multiplex PCR analysis were taken from Chiesa et al. (4). They also reported in a different study they conducted five years later that the primer of *S. hominis* they synthesized was required to evaluate as *S. hominis*-like (*S. hominis*, *S. bovifelis*, *S. bovini* and *S. rommeli*) and these species could not be clearly distinguished by 18S rRNA analysis (35). *Sarcocystis cruzi* was found in all (96.6%) positive samples in the evaluation of our study results. *Sarcocystis hominis*-like was found in 143 (95%) samples whereas *S. hirsuta* was detected in only 4 (2.6%) samples.

Sarcocystis cruzi is the most commonly found species in *Sarcocystis* spp. that is found in cattle in the whole world and Turkey, as well (18, 23, 39). In this study, it was found to be 96.6% using the molecular methods. From an epidemiological point of view, infected carnivores are considered to shed *S. cruzi* sporocysts onto the nature at very high levels. These sporocysts can maintain their viability for long periods for various reasons (such as high temperatures or freezing). Pasture cattle can easily ingest these contaminated feces shedding to the environment. Sarcosporidiosis caused by *S. cruzi* in cattle is very difficult to diagnose in living animals. Several studies claim that eosinophilic myositis and sudden deaths in cattle are associated with *S. cruzi* (1, 9, 17). Microscopic or molecular methods are used in postmortem diagnosis of *Sarcocystis* species. Moussa et al. (27) compared the macroscopic, microscopic and molecular methods in a study conducted on 55 imported frozen buffalo meat in Alexandria, Egypt. In the examined samples, *Sarcocystis* spp. infection was found to be 23% macroscopically, 20% microscopically and 70.9% by molecular methods. The molecular analyses of cysts determined macroscopically and microscopically showed that the imported buffalo meat was infected with *S. fusiformis* and *S. cruzi* species, respectively. In a study conducted by Pritt et al. (33) in the USA, the histological and PCR methods were compared for the detection of *Sarcocystis* spp. on 110 beef meat samples. The PCR method was reported to be more sensitive than histological studies and gave an accurate result for species identification. In addition, in the sequence analysis result of the positive samples, *S. cruzi* was determined in all samples while *S. hominis* could not. In the study conducted on the hearts and oesophagus of cattle in Egypt, El-Kady et al. (7) reported that *Sarcocystis* spp., which were morphologically similar, were two different species (*S. cruzi* and *S. hjorti*) as a result of genetic analyses, and these species did not have host specificity. In this study, 18S rRNA gene region was obtained for *S. cruzi* in minced meat for human consumption. According to the *Sarcocystis* morphology, the sequences of the 18S rRNA gene region of the isolate identified as *S. cruzi* were verified on the basis of species as a result of BLASTN

analyses performed with *Sarcocystis* isolates submitted to GenBank database from different countries. The results of the analyses showed that our isolate named TRVBO1 (MN832695) was found to be 100% similar to *S. cruzi* isolate (MH681972) found in cattle in China.

In the current study, *S. hirsuta* was identified in only 4 (2.6%) samples. Murata et al. (28) isolated this species in one sample in Turkey. In the study conducted on 200 raw hamburgers in Kashan, Iran, Hooshyar et al. (16) found *S. hirsuta* in only two samples. The reason for the low prevalence rate in these studies can be attributed to lower frequency of encounters between cats and cattle. In addition, as a result of the phylogenetic analyses, *S. hirsuta* (MH681973) isolate obtained from China showed 100% similarity with our isolate (MN832697).

One of the positive samples determined as *S. hominis*-like was sent for sequence analysis. The sequence result of *S. hominis*-like (MH832696) was subjected to comparative analysis in the GenBank database and found to be 99.45% similar to *S. bovini* (KT901155) isolated from New Zealand. The genetic distance between the isolates was calculated at a rate of 0.011%. This finding constitutes the first scientific report for Turkey. However, recent studies confirm that the cox1 mitochondrial gene has a higher differential power for *Sarcocystis* species. Given the taxonomic confusion between *Sarcocystis* species, it is also reported that identification of a gene that can be used as a reference gene is very important for the clear identification of *Sarcocystis* species, which are very close phylogenetically, is of great importance (15, 28, 34, 35). Therefore, we believe that it would not be accurate to make a definitive diagnosis for our isolate without cox1 gene verification. For this reason, there is no definite finding for zoonotic *S. hominis* in minced meat in Van Province, Turkey. In fact, from a broad perspective, there is no substantial finding for Turkey. The studies date back to old times and most of them constitute microscopic data. In the future we think that it would be appropriate to carry out more detailed studies covering different gene regions for both *S. hominis* and *S. bovini.* In addition, in our bordering neighbour Iran, *S. hominis* was detected in a molecular study on raw hamburgers (24).

In conclusion, this is the first study combining classical morphological methods and advanced molecular diagnostic techniques on *Sarcocystis* species in minced meat in Van Province, and the molecular characterization of *S. cruzi* and *S. hirsuta* has been introduced for the first time in Turkey. The first data offering an insight into further studies on *S. bovini* have been also established in this study. In addition, species identified as *S. hominis*-like and *S. sinensis*-like need to be distinguished or verified with gene regions with high interspecific and low intraspecific variation. As expected, *Sarcocystis* species were found in high prevalence in minced meat. Although there is no definite finding on *S. hominis* related to public health in our study, it is recommended that meat and meat products to be consumed be frozen in deep freeze for 1 day or cooked over 70°C for 8-15 minutes (20, 37). Additionally, it is very important to control the disease in that the organs and tissues of the intermediate host should not be fed to cats or dogs raw or undercooked.

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Ethical Statement

This study was approved by the Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee (dated 28/06/2018 and numbered 06).

Conflict of Interest

The authors declared that there is no conflict of interest.

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The morphological characteristics of the Muradiye Dönek pigeon, a native Turkish genetic resource

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Abstract: This study was conducted to investigate the morphological characteristics of Muradiye Dönek pigeon. It is a local animal genetic resource and cultural heritage and has an important place among the genotypes of domestic pigeons in Turkey. The animal material of this study consisted of 100 pigeons (50 females and 50 males) from seven pigeon enterprises. In the study, morphological characteristics were evaluated and morphometric measurements were determined. Body plumage colors of pigeons were determined as black (70%), red (16%) and blue (14%) galaca. In some birds, the yellow beaks and nails may bear black spots, which pigeon breeders refer to as 'zikir'. The term 'biyik' (mustache) is used when black feathers appear among the white feathers on the head, and the term 'kemer' (belt) is used when black feathers appear in the tail. The differences between the mean values of body weight and wingspan ($P<0.001$), chest depth ($P<0.05$) values were statically significant for age groups, which were reported to affect flight characteristics in birds. As a result, when the morphological characteristics obtained in Muradiye Dönek pigeons are compared with other researchers, it is thought that the selection of these birds were carried out by the breeders in terms of the game behavior such as rotation movement during the diving and landing. Although the data obtained in the study support the hypothesis that Muradiye Dönek pigeons should be defined as a different breed, further studies with DNA analysis are required to confirm this situation.
Keywords: Muradiye Dönek, morphological characteristics, pigeon, Turkey.

Türkiye'nin yerli gen kaynağı, Muradiye Dönek güvercinlerinin morfolojik özellikleri

Özet: Bu çalışma, Muradiye Dönek güvercinlerinin morfolojik özelliklerinin araştırılması amacıyla gerçekleştirilmiştir. Bu güvercinler Türkiye'nin yerli hayvan gen kaynağı olup kültürel mirasıdır ve evcil güvercin genotipleri arasında önemli bir yere sahiptir. Araştırmanın hayvan materyalini yedi işletmede bulunan toplam 100 adet (50 dişi ve 50 erkek) güvercin oluşmuştur. Çalışmada güvercinlerin morfolojik özellikler incelenmiş ve morfometrik özellikler belirlenmiştir. Güvercinlerde tüy rengi siyah (%70), kırmızı (%16) ve mavi (%14) galaca olarak belirlenmiştir. Bazı kuşlarda sarı renkli gaga ve tırnaklarda güvercin yetiştiricilerinin 'zikir' olarak adlandırdığı siyah noktalar görülmektedir. Baş bölgesinde beyaz tüyler arasında siyah tüyler göründüğünde "bıyık", kuyrukta ise bu siyah tüyler "kemer" olarak adlandırılır. Kuşlarda uçuş özelliğini etkilediği düşünülen canlı ağırlık ve kanat açıklığı ($P<0,001$), göğüs derinliği ($P<0,05$) değerleri bakımından yaş grupları arasında farklılıklar önemli bulunmuştur. Sonuç olarak Muradiye Dönek güvercinlerinde elde edilen morfolojik özellikler yapılan çeşitli araştırmalar ile karşılaştırıldığında, bu kuşların dalma ve iniş esnasında dönme hareketi gibi sergiledikleri oyun davranışları yönünden yetiştiriciler tarafından seçildikleri düşünülmektedir. Bu çalışmada elde edilen veriler Muradiye Dönek güvercinlerinin farklı bir ırk olarak tanımlanması gerektiği hipotezini desteklese de, bu durumu doğrulamak için DNA analizleri ile gerçekleştirilecek gelecek çalışmalara ihtiyaç bulunmaktadır.
Anahtar sözcükler: Güvercin, Muradiye Dönek, morfolojik özellikler, Türkiye.

Introduction

Due to the wide variety of characteristics they present, pigeons have drawn the attention of fanciers and breeders around the world. These birds are believed to have been domesticated approximately 3000 years ago and have evolved into numerous varieties after intensive selective breeding (6, 14). While certain pigeon breeds are raised for their appearance (fancy/ornamental breeds),

some others (flying/sporting breeds) are bred for their performance and playful behavior (rolling, diving, tumbling, spinning).

In addition, pigeon breeds differ from each other in their specific movements, with some demonstrating playful behavior (maneuvering and tumbling or sudden diving during flight, rotating around their own axes when descending). Maneuverability is described as the coordinated rapid movement of the wings, body, tail, and head (24).

The Dönek pigeons are classified as diving-spinning pigeons. While flying at a certain altitude, upon observing an object of interest (e.g. a female pigeon or a shiny spinning object), these birds are capable of closing their wings and performing a sudden dive. Pigeon breeders refer to anything that triggers this sudden dive as a 'shine' or a 'dove'. When diving, the wings are flapped once or more around the tail axis with a propelling movement. When spinning, some pigeons pull their wings in, and some keep their wings slightly open (25). Dönek pigeons are classified based on the region where they are raised, their morphological characteristics, and this spinning pattern during dives. Thus, several varieties exist, including 'Dolapci' and 'Selanik Dönek' (33).

The Muradiye Dönek pigeons have a long history in Anatolia and are a valuable part of the local animal genetic resources. However, to the knowledge, no previous study on the morphological characteristics of this breed has been published. Therefore, due to the paucity of information available on this particular topic, this study is aimed to increase the recognition of the Muradiye Dönek as a distinct breed by determining its morphological characteristics.

Materials and Methods

This study was approved by the Local Ethics Board for Animal Experiments of Tekirdağ Namık Kemal University, Turkey (2017/09). The study was conducted between February 2018 and March 2019 on Muradiye Dönek pigeons raised by the local enterprises of the Muradiye district of the Balıkesir province, positioned between 39° 04′ and 40° 40 northern latitudes and the 26° 02′ and 23° 02' eastern longitudes.

The animal material of this study consisted of 100 pigeons (50 males and 50 females) from seven pigeon enterprises (including 12, 10, 22, 17, 12, 10, and 17 pigeons from each enterprise, respectively). The age and sex of the animals were determined on the basis of the records kept at the pigeon local enterprises. The pigeons were classified under the following age groups: 12-24 months (Group I), 25-35 months (Group II), 36-47 months (Group III), and ≥48 months (Group IV). The pigeons were managed and fed according to the routine program of their pigeon enterprise. Each pigeon was identified through a detailed examination of its plumage color, presence or absence of markings, head crest and foot feathers (muffs), head structure, eye color, numbers of tail and wing feathers, and body weight. The wing feathers were counted in the order of the primary-axial and secondary (p-a-s) feathers. The plumage colors of the pigeons were described with the specific local terms used by the pigeon breeders in the study region. The birds were weighed on a precision balance accurate to 0.01 g. The measurements of the body length were performed by using a metal ruler. Trunk length, wingspan, wing length, body length, tail length, and chest circumference measured with strip, while chest width and depth, head length and width, beak length and depth, and shank diameter measured with a digital caliper (1, 19, 23).

The effects of environmental factors such as sex and age on some morphological characteristics were calculated with the General Linear Model (GLM). Statistical analyses were performed using SPSS 17 for Windows. A value of $P<0.05$ was considered statistically significant. Tukey's multiple range test was used for multiple comparisons of the subgroups (21, 26). For this purpose, Tukey's multiple-range test was used to estimate some morphological measurements of the Dönek pigeon: $Yijkl = \mu + Ci + Dj + eijk$, where Y = the dependent variable, μ = the overall mean, Ci = the fixed effect of sex (i= female or male), Dj = the fixed effect of the age group (j=1,2,3,4), and eijklm = the random error.

Results

According to this study, the head type was determined to be round shaped. The Muradiye Dönek was observed to have neither a head crest nor foot feathers (muffs). The birds were examined for wing pigmentation patterns (T-check, checker, bar, and barless) as described by Vickrey et al. (31). In the pigeons in this study, three types of wing color patterns were determined as T-Check (Figure 4A), checker (Figure 4B), and barless (Figure 4C). The pigeons observed in the study had no bar wing marks.

It was also determined that the head, wing, and tail feathers were white, whilst the remaining body feathers were either black, red, or blue. This multicolored appearance (mottled color) is called 'galaca' by the local pigeon breeders (Figure 4A, 4B, 4C). Breeders refer to birds who present livid grey feathers among black body plumage color as 'striped' (i.e. 'tabby' or 'tekir') (Figure 4D). In this study, the pigeons with livid grey and black plumage were classified under the 'black mottled (black galaca)' group. The eye color was either black (60%), blue (25%) or white (15%). In general, the beak and nails are a light yellow color. In some birds, the yellow beaks and nails may bear black spots, which pigeon breeders refer to

as ‘zikir’ (Figure 1). The term ‘biyik’ (i.e. ‘mustache’) is used when black feathers appear among the white feathers on the head (Figure 2), and the term ‘kemer’ (i.e. ‘belt’) is used when black feathers appear in the tail (Figure 2). The differences among the mean values of body weight, head length, beak depth, chest circumference, wing length, and shank diameter (P>0.001); head width, beak length, and chest width (P>0.01); and tail length and chest depth (P>0.05) were statically significant for sex groups. Furthermore, the age groups were determined to statistically differ in body weight, head width, chest circumference, wing length and span, shank diameter (P>0.001); head length, tail length (P>0.01); and chest depth (P>0.05). The morphological characteristics of the pigeons and their rates of incidence are presented in Table 1. Statistical values of certain morphological characteristics of the age and sex groups are shown in Table 2. The plumage colors and markings observed in the pigeons are shown in Figures 1, 2, and 4. While the eye colors are shown in Figure 3.

Table 1. Some morphological characteristics in Muradiye Dönek pigeon.

Morphological characteristics	Ratio (%)
Plumage colour	
Black Galaca	70
Red Galaca	16
Blue Galaca	14
Eye Colour	
Black	60
Blue	25
White	15
Marks	
Zikir	7
Moustache	9
Belt	5
Unmarked	79
The number of wing feather	
10-1-12	25
10-1-10	48
10-1-9	27
The number of tail feather	
14 feather	75
13 feather	25

Figure 1. The black pigmentasyon on the beak and claw (Zikir).

Figure 2. Pigmentations on the edge of the beak (mustache) and the tail (belt).

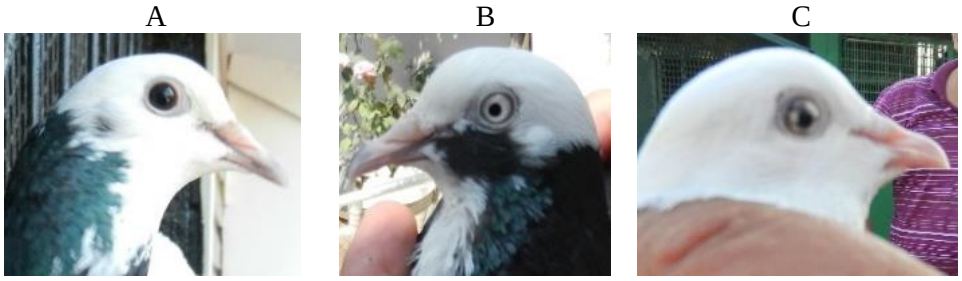

Figure 3. Eye colours of Muradiye Dönek pigeons (A: Black eye, B: White eye, C. Blue eye).

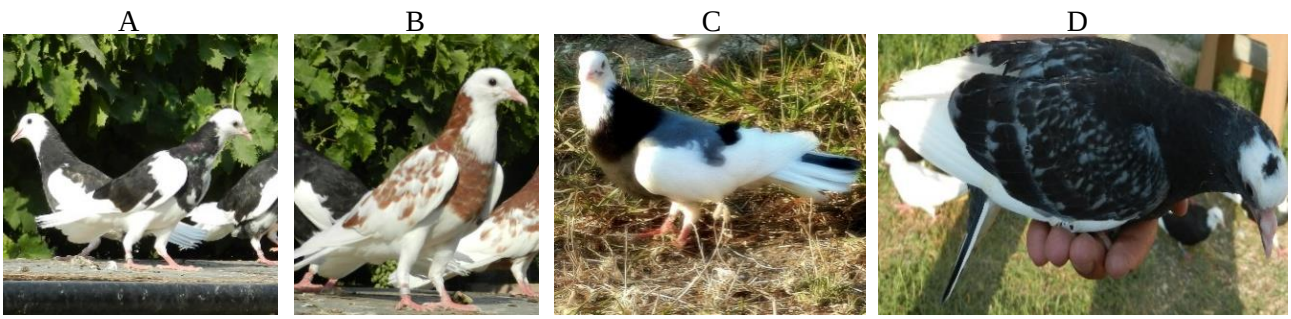

Figure 4. The body colours of Muradiye Dönek pigeons (A: Black galaca-wing structure, T-Check shaped; B: Red galaca-wing structure, checker-shaped; C: Blue galaca-garless; D: Tabby).

Table 2. The statistical values of the morphometric characteristics detected from Muradiye Dönek pigeons ($X \pm S_x$).

DÖNEK	n	Body weight (g)	Head length (mm)	Head width (mm)	Beak length (mm)	Beak depth (mm)	Trunk length (cm)	Tail length (cm)
Total	100	319.74±3.25	52.44±0.23	18.20±0.11	18.76±0.14	4.93±0.05	10.66±0.09	13.14±0.11
Sex		***	***	**	**	***	-	*
Female	50	306.34±4.45	51.43±0.27	17.84±0.13	19.16±0.18	4.64±0.06	10.71±0.10	12.86±0.11
Male	50	333.14±3.96	53.45±0.31	18.56±0.17	18.37±0.21	5.22±0.05	10.62±0.15	13.43±0.19
Age		***	**	***	-	-	-	**
Group I	25	318.63±5.19^{b}	52.68±0.39^{b}	18.19±0.23^{b}	18.38±0.37	4.94±0.08	10.47±0.19	12.75±0.18^{a}
Group II	20	318.71±7.42^{b}	52.37±0.60^{b}	18.23±0.20^{b}	19.25±0.38	5.09±0.09	10.98±0.18	13.57±0.20^{b}
Group III	22	285.13±4.86^{a}	50.94±0.34^{a}	17.15±0.20^{b}	18.68±0.26	4.77±0.12	10.45±0.15	12.68±0.12^{a}
Group IV	33	344.28±3.20^{c}	53.30±0.41^{b}	18.89±0.16^{c}	18.82±0.16	4.94±0.09	10.75±0.17	13.50±0.25^{b}

	n	Chest depth (mm)	Chest circumference (cm)	Chest width (mm)	Wing length (cm)	Wing span (cm)	Body length (cm)	Shank diameter (mm)
Total	100	57.81±0.35	19.34±0.13	49.94±0.35	29.30±0.16	67.57±0.60	35.10±0.27	3.51±0.04
Sex		*	***	**	***	-	-	***
Female	50	58.56±0.31	18.90±0.23	48.86±0.33	28.69±0.21	66.49±1.16	34.87±0.38	3.28±0.05
Male	50	57.05±0.62	19.79±0.11	51.02±0.58	29.91±0.21	68.66±0.24	35.32±0.40	3.74±0.06
Age		*	***	-	***	***	-	***
Group I	25	59.26±0.54^{b}	18.96±0.22^{ab}	50.14±0.74	28.92±0.29^{a}	68.00±0.37^{ab}	35.08±0.61	3.55±0.09^{b}
Group II	20	57.31±0.98^{a}	19.65±0.23^{bc}	50.54±1.12	29.40±0.31^{b}	68.30±0.30^{b}	35.03±0.54	3.62±0.07^{b}
Group III	22	57.41±0.82^{a}	18.57±0.43^{a}	48.18±0.25	27.81±0.35^{b}	67.16±0.28^{a}	34.57±0.51	3.08±0.07^{a}
Group IV	33	56.69±0.44^{a}	19.96±0.13^{c}	50.59±0.52	30.52±0.19^{c}	69.51±0.24^{c}	35.50±0.52	3.70±0.08^{b}

-: P>0.05; *: P<0.05; **: P<0.01; ***: P<0.001, a-c: means within a column with different letters are significantly different (P<0.05).

Discussion and Conclusion

Research shows that many domesticated pigeons raised today possess morphological and behavioral differences from their ancestors (3) because pigeons have undergone intensive selective breeding throughout history, resulting in the emergence of many varieties distributed across the world (6, 18, 28). In particular, the Muradiye Dönek pigeons have been breeding for many years in Anatolia. However, to the knowledge, no reports on this particular pigeon breed have been published. Therefore, this study is the first investigation into the morphological characteristics of this pigeon genotype.

In the present study, it was ascertained that the sex groups significantly differed in body weight, head length, beak depth, chest circumference, wing length and shank diameter (P<0.001); head width, beak length, and chest width (P<0.01); and tail length and chest depth (P<0.05). These findings agree with the results of previous research, suggesting the existence of sexual dimorphism in the pigeons (1, 2, 9). When morphological features were examined in age groups in pigeons, the average values of birds belonging to group III, excepting beak length and chest depth, were found lower than other groups. We think that the result may be explained with the sex distribution (18 females, 4 males) in group III. Wing size and bodyweight of birds are important factors affecting flight and racing performance in birds (12, 15, 17). In this study, wing size (length and span) and body weight average values were detected to be higher in group IV pigeons compared to other groups. This difference may be due to the selection of those animals by the breeder and the frequency of training applied to animals in this group. Through this research, it was also proved that the differences observed among the age groups for beak length and depth were not statistically important (P>0.05). In contrast to the opinion that the structure of a beak may change with its utility or the bird's diet (14, 26), this observation suggests that the development of beak structure depends on non-dietary factors, such as the development of the skull (4, 13).

The head width, beak length, and beak width values determined in this study were similar to the previously reported values for the ancestor of domesticated (Columba livia) given by Jonnston (10) and Jonnston and Janiga (11), whilst the head length, wing length, and chest depth values were greater. The body weight measurements of the Muradiye Dönek pigeons were similar to the Pencil Pouter, the fancy breeds Archangel and Mookie, the drummer pigeon breeds Kokah and Bokhara Trumpeter and the Parlor Roller, Lotan, and Indian Tumbler pigeon breeds, which reported in different regions of the world (22). The Muradiye Dönek can be classified as a medium-

sized pigeon breed when its body weight and some morphometric measurements (body length, chest width, head length) are compared to other pigeon breeds raised in Turkey and elsewhere, such as the Ankara Tumbler, Bursa Roller, Edremit Kelebek Roller, Thrace Roller, Dewlap (Adana), Scandaroon (Iskenderun) and fleet fliers (1, 2, 5, 19, 20, 22, 27, 34).

Pigeons have horizontally positioned eyes on two sides of their head. This position of the eyes allows these birds a panoramic vision of approximately 340º. The eye angle allows the pigeon to observe the detail of land and sky, thus escaping from predators (12). Research suggests that the position of the beak may affect the centering of the field of vision, the symmetry of the optic flow-field, direction of travel and time-to-contact (16). Pigeon fanciers who raise the Muradiye Dönek in Turkey expect these diving/spinning birds to immediately notice a 'shine' on the ground and perform a sudden dive towards it. The shape of the beak may play an important role in this ability to lock on a target and orient towards it. The mean beak length determined for the Muradiye Dönek (18.76 cm) was smaller than the mean beak lengths reported for Turkish roller pigeons (Thrace Roller and Bursa Roller) and Turkish diver pigeons (Edremit Kelebek Roller, Dewlap-Adana) and greater than the mean beak lengths reported for the Ankara Tumbler and Şanlıurfa Squadron flyer (1, 2, 5, 19, 20, 27). Furthermore, the mean beak depth determined for the Muradiye Dönek (4.93 cm) was smaller than the mean values previously reported for the Ankara Tumbler, Dewlap (Adana) and fleet fliers. This small beak depth and large beak length in the pigeon help to perceive the brightness more quickly and to direct towards it.

Another hypothesis addressed in this study stated that the phenotypic variety of pigeons is partly related to variances in plumage color. In the Muradiye Dönek, the plumage is 'mottled' and generally a combination of white feathers with red (16), black (70) or blue (14) feathers. Moreover, it was determined that the color combinations detected in the body, wing and tail in the pigeons showed random distribution. Some researchers have suggested that pigeons with different melanin pigmentation, in particular those with a dark plumage color, have developed different strategies to cope with feed restriction, including reducing their body mass index (7). The Muradiye Dönek may have developed such a strategy. To prevent the birds from flying, pigeon breeders do restrict the pigeons' access to feed. However, more detailed measurements and observations are needed before a conclusion can be drawn.

In previous research on the wing feather color patterns of pigeons, it was reported that a mutation in the Norrie Disease Protein (NDP) gene sequence, which impedes pigment production, results in the lightest 'barless' pattern and may also be associated with vision defects (31). In the Muradiye Dönek pigeons, darker wing pigmentation (86%) was found to be more common than lighter wing pigmentation (14%). Pigeon breeders may be responsible, as they attach importance to rapid reactions to shines and their Dönek pigeons with lighter pigmentation also possess vision defects, suggesting that this breed may have undergone selective breeding for visual acuity. However, the correlation between vision defects and genetic structure needs to be further investigated in more detail.

Previous research has also suggested that the flight capability and racing/sporting performance of pigeons can be affected by body weight, wing length, and chest depth and that birds with longer wings fly at a greater speed (8, 17). In the present study, based on morphometric measurements performed on the Muradiye Dönek, the mean body weight (319.74 g), mean wing length (29.30 cm) and mean chest depth (57.81 cm) values were found to be smaller than the values previously reported for other sporting pigeon breeds, including the Thrace Roller, Bursa Roller, Edremit Kelebek Roller, Ankara Tumbler, and Dewlap (Adana), as well as fleet fliers raised in Turkey (1, 2, 5, 19, 20, 27). A bird's wingspan affects the thermoregulation of the body during flight and its flying speed and maneuverability (30, 32), and the flying speed seems to increase with a reduced wingspan (30). In the case of the Muradiye Dönek pigeons, the mean wingspan (67.57 cm) was found to be smaller than that reported for the Ankara Tumbler, fleet fliers, and Scandaroon (68.82 cm, 67.67 cm, and 71.40 cm, respectively) and greater than the values reported for the Edremit Kelebek Roller, Thrace Roller and Bursa Roller (66.40 cm for males, 64.8 cm for females; 67.32 cm and 59.07 cm, respectively) (1, 2, 5, 19, 20, 27, 34). Thus, the general assessment of the body weight, chest depth, wing length, and wingspan data obtained in the present study suggests that fanciers of the Muradiye Dönek have subjected this breed to selective breeding not for flying speed, but for the playful behavior displayed by the bird when descending.

Animals, as an integral part of the local genetic resources of a country, are often investigated for their various morphological characteristics not only to shed light on phylogenetic research but also to develop more efficient production systems and to perform genetic improvement studies. The morphological characteristics determined for the Muradiye Dönek in the present study highlighted that this particular pigeon variety may be thought of as an individual breed native to Turkey. According to these results, the studies for registration as a new breed of these pigeons were started and these studies have been ongoing. Moreover, this morphological data needs to be supported with further DNA research.

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Ethical Statement

This study was approved by the Local Ethics Board for Animal Experiments of Tekirdağ Namık Kemal University, Turkey (2017/09).

Conflict of Interest

The authors declared that there is no conflict of interest.

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Phenotypic and molecular characterization of *Salmonella* Enteritidis isolates

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Abstract: *Salmonella* spp. is the most frequently isolated foodborne pathogens causing human and animal diseases. The aim of this study was to investigate antimicrobial susceptibility profiles and the molecular typing of 200 *Salmonella* Enteritidis strains isolated from the patients' stools between 2016 and 2019 in Turkey. The isolates were examined for antibiotic susceptibility patterns (21 antimicrobial agents) by Kirby-Bauer disc diffusion method or molecular typing by Pulsed-field gel electrophoresis (PFGE) and plasmid profiling. Although all isolates were susceptible to four antibiotics (suphamethoxazole/trimethoprim, chloramphenicol, streptomycin and trimethoprim), all were resistant to 15 different antibiotics. In the PFGE study performed with *Xba*I enzyme, all isolates were found to be related to each other according to similarity rates of 85% and above. There were two major clones, clone A and B. Clone A was divided into 6 pulsotypes (A1-A2-A3-A4-A5-A6) and clone B was divided into 3 pulsotypes (B1-B2-B3). Clone A had 87% similarity and Clone B had 90% similarity. The clustering rate was 86% (172/200). All isolates harboured 1-4 plasmid ranging in size from 2.5 to 57 kb and showed 6 plasmid profiles (P1-P6). All isolates carried the 57 kb plasmid individually or in combination with other plasmids. Most of the isolates 136 (68%) had P2 profile. Our findings indicate that the majority of all isolates were clonally related and had cross contamination problems. In this study, the importance of molecular typing methods in order to take more effective protection and control measures against *Salmonella* has been demonstrated and proposed to use such methods.
Keywords: Antimicrobial resistance, PFGE, plasmid profiling, *Salmonella* Enteritidis.

Salmonella Enteritidis izolatlarının fenotipik ve moleküler karakterizasyonu

Özet: *Salmonella* spp. insan ve hayvan hastalıklarına neden olan en yaygın gıda kaynaklı patojendir. Bu çalışmanın amacı, Türkiye'de 2016 ve 2019 yılları arasında hasta dışkılarından izole edilen 200 *Salmonella* Enteritidis izolatının antimikrobiyal duyarlılık profillerini ve moleküler tiplerini araştırmaktır. İzolatlar Kirby-Bauer disk difüzyon yöntemi ile antibiyotik duyarlılık profilleri (21 antibiyotik) ve pulsed-field jel elektroforezi (PFGE) ve plazmid profili ile moleküler tipleme açısından incelendi. Toplam 200 izolat hem 4 farklı antimikrobiyal ajana (sülfametoksazol / trimetoprim, kloramfenikol, streptomisin ve trimetoprim) duyarlı hem de 15 farklı antimikrobiyal ajana dirençliydi. *Xba*I enzimi ile yapılan PFGE çalışmasında, tüm izolatların %85 ve üzeri benzerlik oranlarına göre birbirleriyle ilişkili olduğu bulundu. İki ana klon vardı, klon A ve B. Klon A, 6 pulsotipe (A1-A2-A3-A4-A5-A6) ve klon B, 3 pulsotipe (B1-B2-B3) ayrıldı. Klon A ve B, sırasıyla %87 ve %90 benzerliğe sahipti. Kümeleme oranı %86 (172/200) idi. Tüm izolatlar, 2,5 ila 57 kb arasında değişen 1-4 plazmid barındırdı ve 6 plazmid profili (P1-P6) gösterdi. Tüm izolatlar 57 kb plazmidi tek veya diğer plazmidlerle birlikte taşıdı. İzolatların çoğu (n= 136, %68) P2 profiline sahipti. Bulgularımız, 200 *S*. Enteritidis izolatlarının çoğunun klonal olarak ilişkili olduğunu ve çapraz kontaminasyon problemleri olduğunu göstermektedir. Bu çalışmada, Salmonellosis'e karşı daha etkin korunma ve kontrol önlemlerinin alınmasında moleküler tipleme yöntemlerinin önemi gösterilmiş ve bu yöntemlerin kullanılması önerilmiştir.
Anahtar sözcükler: Antimikrobiyal direnç, PFGE, plazmid profili, *Salmonella* Enteritidis.

Introduction

Salmonella is Gram-negative facultative anaerobic bacteria which belongs to-*Enterobacteriaceae* family and a group of bacteria living in the intestinal tracts of many different domestic animals including birds, cattle (1, 11, 13). Humans usually become infected with *Salmonella* by eating foods contaminated with animals' feces (1, 11, 39). Salmonellosis is an infectious disease of humans and animals caused by these bacteria which are capable of having foodborne zoonosis importance (18). *Salmonella* spp. especially *S. enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) and *S. enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) that are known as non-typhoidal *Salmonella* serotypes, continues to be the most frequent cause of bacterial foodborne disease outbreaks (17-19, 44). In addition, products of animal origin, environmental contamination and indirect transmission through food and water are other causes of *Salmonella* outbreaks (4, 38). *Salmonella* infection develops in adults with contaminated foods, and newborns and children are more likely to develop infections with cross-contamination (34).

Foodborne diarrheal diseases caused by the significant ones like non-typhoidal Salmonella are an important cause of morbidity and mortality, and thus has emerged as a significant and growing public health and economic problem worldwide, especially industrialized countries (14, 27). *Salmonella* infections have a worldwide distribution and range clinically from the common self-limited uncomplicated gastroenteritis to enteric fever (9).

Antibiotics inhibit the growth of *Salmonella* spp., reducing the economic losses and public health problems (14, 18). However, multidrug-resistant *Salmonella* is increasing due to misuse and overuse of antibiotics in human and animals, which make it difficult to eliminate from its reservoir hosts (44). Multidrug-resistant *Salmonella* isolates have been associated with a considerable number of outbreaks worldwide (20, 44). The presence of antibiotic-resistant *Salmonella* in the human food chain requires the development of new-antibiotic to prevent the pathogens in reservoirs, including cattle, birds (25). The identification of *Salmonella* serotypes is important to monitor common source outbreak or origins, relationships among different isolates and to control future outbreaks of infectious diseases that transmit from animals to humans (4, 9, 11-13). The information to be obtained from typing methods such as Pulsed field gel electrophoresis (PFGE) and plasmid profile analysis (PPA) is very useful in preventing and controlling the spread of disease in animals and public (36). PPA is a molecular method used for subtyping (41). PFGE is also another molecular typing method used for typing outbreaks. This method provides information on the source and transmission pattern of the microorganism (19, 30). PFGE, which is used to determine the clonal and phylogenetical relationships between strains, is known to have high discrimination power (2, 4). Combined with PPA and PFGE provides a powerful discriminatory tool for the epidemiological analysis of *S*. Enteritidis isolates (29, 43). Most cases of Salmonellosis in humans are associated with the consumption of food contaminated with antibiotic-resistant *S.* Enteritidis from animals. Hence, it is possible to have a relationship between humans and animals regarding genotyping of antibiotic-resistant *S*. Enteritidis. This study aimed to investigate the antimicrobial susceptibility profiles and the molecular typing of 200 *S*. Enteritidis strains isolated from the patients admitted to the hospital by using PFGE and PPA and clarify the possible transmission rates and clonal relationships among these isolates.

Materials and Methods

Samples: *S*. Enteritidis isolates (n=200) were used in this study. Fecal samples of 200 patients who were sent to a public hospital microbiology laboratory (Cankiri province of Turkey) between 2016 and 2019 were examined. The study was performed by using *S.* Enteritidis obtained from the culture collection made from the fecal samples given by the patients who came to the outpatient clinic.

Salmonella **Isolation and Identification:** The samples were inoculated onto Eosin Methylene Blue (EMB) agar and Salmonella Shigella (SS) agar (Becton Dickinson, GmbH, Heidelberg, Germany) and into selenite F broth for enrichment (BBL Selenite F Broth, Becton Dickinson and Co., Sparks, MD, USA). They were incubated solid agars for overnight and selenite F broth for 8 hours under appropriate conditions. A single colony picked up and identified as *S.* Enteritidis using IMVIC test (citrate, methyl red, Voges-Proskauer, citrate, ornithine, urea, indole, Kligler iron agar media). Conventional methods (Triple Sugar Iron Agar, Simon's Citrate Agar, Urea Agar and nutrient broth.; Oxoid, Hampshire, UK) and Phoenix 100 (Becton Dickinson and Co., Sparks, MD, USA) automated system were used to identify lactose negative bacterial colonies that grow on EMB and SS agars and in selenite F medium. Serotypes of bacteria identified as *Salmonella* were determined by O and H antigens (Difco, Sparks, MD, USA). Somatic O antigens and flagellar H antigens were determined by slide agglutination using specific antisera. After the identification of the antigens, the name of strains was determined by using the Kauffman-White scheme (23, 24). Serological confirmation and serogroup of microorganisms identified as *S.* Enteritidis were

determined by using species-specific anti-sera (Difco Shigella Antisera Poly, Sparks, MD, USA).

Antimicrobial susceptibility test: Mueller-Hinton agar (Oxoid, Hampshire, UK) was used for antibiotic susceptibility testing. Kirby-Bauer disc diffusion method was used for antibiotic susceptibility according to the Clinical Laboratory Standards Institute guidelines (6) for the following antimicrobial agents (Oxoid, UK): Ampicillin (AMP: 10 µg), cephalothin (KF: 30 µg), gentamicin (CN: 10 µg), amoxycillin-clavulanic acid (AMC: 30 µg), cefuroxime sodium (CXM: 30 µg), cefoperazone (CFP: 30 µg), cefotaxime (CTX: 30 µg), ceftizoxime (ZOX: 30 µg), ceftriaxone (CRO: 30 µg), ceftazidime (CAZ: 30 µg), suphamethoxazole/ trimethoprim (SXT: 25 µg), chloramphenicol (C: 30 µg), tetracycline (TE: 10 µg), kanamycin (K: 30 µg), nalidixic acid (NA: 30 µg), ciprofloxacin (CIP: 5 µg), sulphonamides (S3: 300 µg), streptomycin (S10: 10 µg), trimethoprim (W: 5 µg), cefpodoxime (CPD: 10 µg), and amikacin (AK: 30 µg). *Escherichia coli* ATCC 25922 was used as a quality control strain in all tests. The plates were incubated for 24 hours at 35-37 °C. The diameters of the inhibition zones formed around the discs were measured in millimeters and evaluated as susceptible, intermediate, or resistant, according to the CLSI, 2012 (6).

Plasmid analysis: Plasmid DNA was isolated by alkaline lysis methods of Kado and Liu (15) and separated in 0.7% agarose gel (Serva, Heidelberg, Germany) prepared with 0.5xTris-Boric acid-EDTA buffer at 110 V for 3 h at room temperature and stained with ethidium bromide (0.5 µg). Plasmid size was determined by comparison with *E. coli* V517 (53.7, 7.2, 5.6, 5,1, 3.9, 3.0, 2.7, 2.1 kb) and *S.* Enteritidis strains (57, 5.8, 4.8 kb) and supercoiled DNA ladder (1 kb gene ruler DNA ladder fermantas, Litvanya).

Pulsed field gel electrophoresis: PFGE analysis was performed using *Xba*I according to the protocol described by Durmaz et al. (8). Briefly, single colony cultivation was performed on trypticase soy agar (Merck, Germany) from the bacteria that were identified as *S.* Enteritidis incubated at 37 °C for 20-24 hours under aerobic conditions. After overnight incubation, the purity was checked, and a single colony of each isolate was again passaged to SS agar (Merck, Germany) and incubated under the same condition.

The colonies were collected with a plastic loop and suspended in 4 ml of Cell Suspension Buffer (CSB; 100 mM Tris-HCL, 100 mm EDTA, pH 8.0). The cell suspension was centrifuged at 13000 rpm for 2 minutes at 4°C. The supernatant was discarded after centrifugation. 1 ml cold CSB was added to the pellet again and vortexed for a short time. The bacterial density was adjusted to 1 absorbance at 590 nm using a spectrophotometer (UV/Vis. Spectrophotometer, Shimadzu-1280, Japan). 2% low-melting agarose (LMA, Gibco BRL, Paisley, UK) was prepared in CSB buffer and the mixture containing CSB, LMA and 10% Sodium Dodecyl Sulfate (SDS, Merck, Germany) was transferred to plug molds (10mm x 5mm x 1.5mm, Sigma-Aldrich, Germany). The molds were allowed to stand for 10 minutes at +4 ºC until the agarose solidifies for quality DNA preparation. The agaroses containing the bacteria were removed from the plug mold and transferred into Cell Lysis Solution 1 (CLS-1, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 2.5 mg/ml lysozyme, 1.5 mg / ml proteinase K) and incubated for 1 hour at 37 °C. Then, the plugs were incubated in Cell Lysis Solution-2 (CLS-2, 0.5 M EDTA, 1% sarkosyl, 400 µg/ml proteinase K) at 55 ºC for 2 hours. After incubation, the plugs were treated 3 times successively with sterile ultrapure water (Reagent Grade Type 1) and TE buffer (10 mM Tris-HCL, 0.1 mm EDTA, pH 7.6).

Each of the agarose containing DNA was transferred into 100 µl *Xba*I buffer (1x) and incubated in a shaking water bath at 37 °C for 10 minutes. The restriction buffer was removed and fresh buffer (100 µl) containing *Xba*I restriction enzyme (Promega Corporation, WI, USA) was added to each plug in the tubes. Incubation was performed at 37 ºC for 2 hours. 1% agarose (pulsed-field certified agarose, Sigma-Aldrich, Germany) was prepared in 100 ml of 0.5xTBE (44.5 mM Trisma Base, 44.5 mM Boric acid, 1 mM EDTA, pH 8.0) solution and restricted DNA plugs were loaded on each tooth of tooth comb (USA Scientific, USA). The agarose solidified on the teeth was placed in a PFGE chamber containing 1900-2000 ml 0.5x TBE buffer. PFGE was performed via CHEF-DR® II system (Bio-Rad, Hercules, CA, USA). The electrophoresis condition was set as follows: Initial switch time; 2.3 s; final switch time, 65 s, run time, 19 h; gradient, 6V/cm^2; angle 120°; temperature, 14 °C. After electrophoresis, the gel was transferred into ultra-pure water containing ethidium bromide (5 µg/ml) and incubated to be stained for 20 minutes and visualized under UV light.

The DNA band images were photographed using Digi Genius Imaging System (Syngene, UK). Band profiles were analyzed using Gene Directory software (Syngene, Cambridge, UK). First of all, normalization was performed with the help of three control strains in each image. Clustering analysis was performed by creating a dendrogram of PFGE profiles. The relationship between the strains was determined according to the "Dice" similarity coefficient. Using the criteria developed by Tenover et al. (40), isolates were evaluated as indistinguishable, closely related, possibly related, or different.

Results

The *S.* Enteritidis isolates were isolated from clinical specimens (200 fecal samples) in this study. There were the multi-resistant *S.* Enteritidis (100%) which are resistant to a large diversity of antimicrobial agents (Figure 1). A total of 200 isolates were susceptible to four antimicrobial agents (SXT, C, S10 and W). The antibiotic resistance profiles were similar among *S.* Enteritidis isolates which are resistant to more antibiotics. The susceptibility and resistance patterns of the strains were found to be similar and were mainly divided into 3 groups as RI, RII and RIII (Table 1).

Molecular typing of the *S.* Enteritidis isolates were analyzed by using molecular techniques, PFGE and PPA. In the PFGE study performed for genotypic typing of *S.* Enteritidis, PFGE gel images were obtained after cutting *Salmonella* DNA with *Xba*I enzyme with restriction endonuclease activity. In the next step, dendrogram analysis was performed from gel images of *Salmonella* isolates showing PFGE band profiles. After the band profile analysis, PFGE profile dendrograms were established and the relationships between strains were determined. Cluster analysis was evaluated with the Dice similarity coefficient and UPGMA (Unweighted Pair Group Method Average) relationship rule parameters. When the dendrogram of 200 *Salmonella* strains were examined; according to the criteria of Tenover et al. (40), *S.* Enteritidis were found to be related to each other according to similarity rates of 85% and above. There were two major clones, but strains were divided into clones A and B. Clone A was divided into 6 pulsotypes (A1-A2-A3-A4-A5-A6) and clone B was divided into 3 pulsotypes (B1-B2-B3). Clone A had 87% similarity and Clone B had 90% similarity. The clustering rate was 86% (172/200) according to Tenover criteria (40). Clone A contained 172 strains and clone B contained 28 strains (Figure 1).

In this study, all of the *S.* Enteritidis isolates (n=200) were observed to have plasmid. It was determined that isolates showed 6 plasmid profiles (P1-P6) (Table 2). The 200 isolates carrying plasmids were found to carry 1-4 plasmids, ranging in size from 2.5 to 57 kb (Table 2). All isolates carried the 57 kb plasmid individually or in combination with other plasmids. Fourteen of the isolates carried at least one plasmid (57 kb). Most of the isolates (n = 136, 68%) had P2 profile.

Table 1. Antibiotic resistance profiles for *S.* Enteritidis isolates.

Resistance phenotype	Resistance pattern	Isolate number	%
RI	AMP, KF, CN, AMC, CXM, CFP, CTX, ZOX, CRO, CAZ, TE, K, NA, CIP, S3, CPO, AK	28	14
RII	AMP, KF, CN, AMC, CXM, CTX, ZOX, CRO, CAZ, TE, K, NA, CIP, S3, CPO, AK	121	60.5
RIII	AMP, KF, CN, AMC, CXM, ZOX, CRO, CAZ, TE, K, NA, CIP, S3, CPO, AK	51	25.5

AMP: Ampicillin; KF: Cephalothin; CN: Gentamicin (CN); AMC: Amoxycillin-clavulanic acid; CXM: Cefuroxime sodium; CFP: Cefoperazone; CTX: Cefotaxime; ZOX: Ceftizoxime; CRO: Ceftriaxone; CAZ: Ceftazidime; SXT: Suphamethoxazole/trimethoprim; C: Chloramphenicol; TE: Tetracycline; K: Canamycin; NA: Nalidixic acid; CIP: Ciprofloxacin; S3: sulphonamides; S10: Streptomycin; W: Trimethoprim; CPD: Cefpodoxime; AK: Amikacin.

Table 2. Plasmid patterns of *S.* Enteritidis isolates.

Plasmid profiles	Plasmids (kb)	Number of *S.* Enteritidis isolates (%)
P1	57	14 (7)
P2	57, 40, 3.0	136 (68)
P3	57, 40, 6.5, 4.5	29 (14.5)
P4	57, 5.8, 4.8	7 (3.5)
P5	57, 6.5, 4.5	7 (3.5)
P6	57, 2.5	7 (3.5)

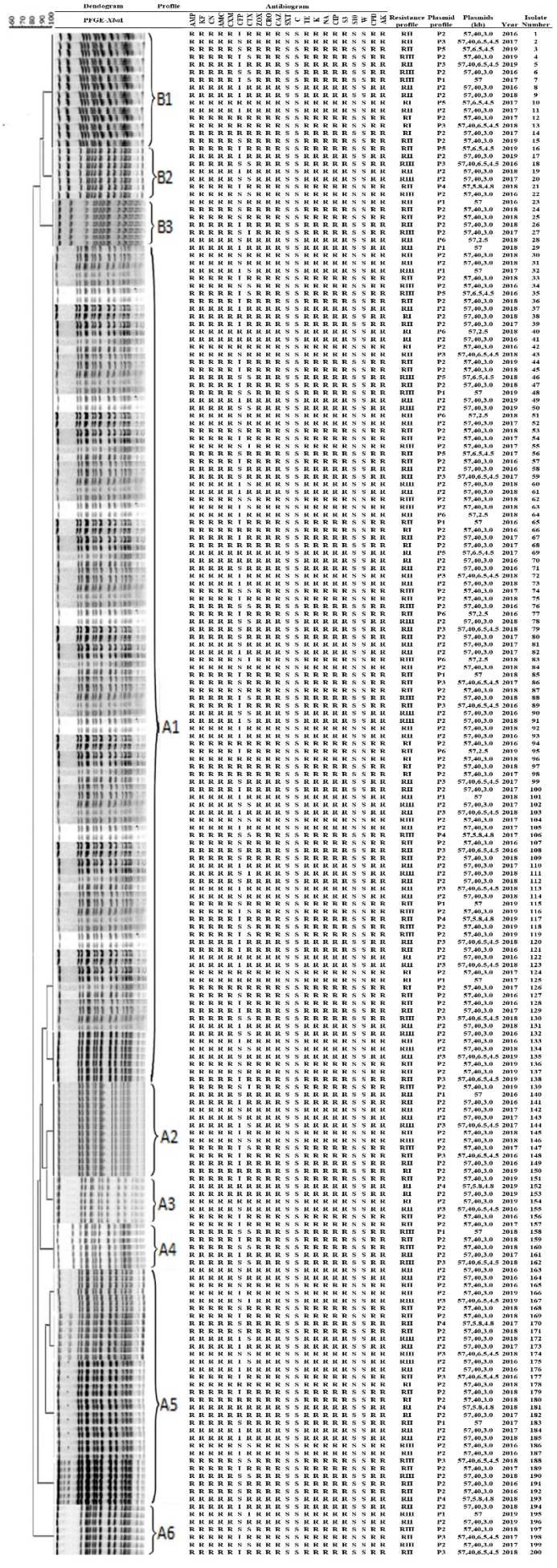

Figure 1. *Xba*I PFGE patterns, plasmid patterns and antibiotic resistant profiles of *S. Enteritidis* isolates.

Discussion and Conclusion

Salmonella infection in humans and animals continues to be a public health problem in Turkey, as it is in almost every region of the world (4, 17, 22). Phage typing and genotyping methods are accepted as the gold standard value for differentiation and subtyping of *Salmonella* isolates an epidemiological method (28, 35, 45). Genotyping methods such as PFGE, PPA, ribotyping, which rely on the comparison of multiple electrophoresis banding patterns, have been used for monitoring the epidemic evolution of *S.* Enteritidis strains (1, 37). *Salmonella* phage typing is a method that can be performed in several reference centers in the world and is not implemented in Turkey. Therefore, our limited knowledge about phage types and molecular characteristics of isolated *Salmonella* serotypes in Turkey is based on a few specific studies and there is insufficient information about phage types and molecular characteristics of multi-drug resistant (MDR) *S.* Enteritidis strains (1, 21, 43).

S. Enteritidis is the most common *Salmonella* serotype isolated from the human in Turkey (10). From the past to the present, the incidence of *S.* Enteritidis has been gradually increasing in humans (3). The occurrence of antibiotic-resistant strains of *Salmonella* spp. has become a serious health problem worldwide (5, 7). High rates of resistance against a large number of antibiotics in *S.* Enteritidis isolates from humans and animals in Turkey have been reported previously (3, 10, 12, 13, 21, 26, 33, 42). In this study, there were the multi-resistant *S.* Enteritidis (100%) and all isolates were susceptible to four antimicrobial agents (SXT, C, S10 and W). The antibiotic resistance profiles were similar among *S.* Enteritidis isolates which are resistant to more antibiotics. Erdem et al. (10) reported that there was resistance or decreased susceptibility to CIP in *Salmonella enterica* strains (n = 620) isolated from clinical samples in 10 provinces of Turkey between 2000 and 2002 years. After about 17 years, all of the isolates were resistant to CIP in this study. These results show that increasing incidence of multi-drug resistant *Salmonella* strains represent a risk to public health.

In this study, molecular typing of the *S.* Enteritidis isolates was analyzed by molecular techniques, PFGE and PPA. All strains were found to be related to each other according to similarity rates of 85% and above. There were two major clones, divided into clones A and B. Clone A had 87% similarity and Clone B had 90% similarity. The clustering rate was 86% (172/200). In a study, PGFE profiles of *S.* Enteritidis strains (n = 23) isolated from clinical samples from 7 provinces of Turkey between 2004 and 2010 have been determined using *Xba*I restriction enzyme. The findings demonstrated that 4 different PFGE profiles (type 1, 4, 9, and 10) were found among serotype *S.* Enteritidis and PFGE types had similarities below 85% and above 95% in strains within the same type (26). Us et al. (43) reported that a total of 122 *S.* Enteritidis strains (epidemic, n = 13; sporadic, n = 109) (103 stool, 16 blood and one bile, one urine and one cerebrospinal fluid) isolated from 10 different provinces of Turkey after 2000 year were investigated for PFGE profile. The PFGE analysis showed 11 different patterns (a to k) and PFGE pattern a and b consisted of 53 strains (43.4%) and 42 strains (34.4%), respectively, after digestion with macrorestriction enzyme *Xba*I. In mid-January 2008 (Isparta province of Turkey), after a large foodborne outbreak associated with eggs contaminated by *S.* Enteritidis in a military unit, *S.* Enteritidis was isolated from 276 stool samples and a blood sample of the hospitalized patients and a food item. Authors have reported that the PFGE patterns after *Xba*I digestion of these isolates were identical (19). Numerous studies in different countries reported that *S.* Enteritidis isolates were genetically similar and clonally highly related (16, 29-32). These data are consistent with our results of PFGE profile after digestion of with *Xba*I enzyme.

The plasmids carrying antibiotic resistance genes have an important role in transferring those genes to other strains or other species (25). In this study, all of the *S.* Enteritidis isolates (n = 200) were observed to have plasmid. The isolates carrying plasmids were found to carry 1-4 plasmids, ranging in size from 2.5 to 57 kb. In a study conducted by Aktaş et al. (1) in Istanbul between 2001 and 2004, plasmid profiling analysis of pediatrics *S.* Enteritidis isolates showed six plasmid profiles, ranging in size from 2.5 MDa to 38 MDa. High rate (92%) of the isolates harboured the same plasmid of 38 MDa. In another study in Ankara, although *S.* Enteritidis isolates harbored 1-4 plasmids with sizes ranging from 2.0 to 100 kb, 85 (69.7%) of isolates harbored the 57 kb plasmid (43). In these studies, the plasmid size harboured by *S.* Enteritidis isolates are similar to those found in our study.

In the present study, the occurrence of multi-resistance observed in 200 (100%) of the *S.* Enteritidis isolates from clinical human samples is of great publish health concern and suggests the successful implementation of surveillance and monitoring of antimicrobial use and continuing education on prudent antimicrobial agent use. The high level of resistance observed in the isolates was likely to the fact antibiotics were overused to the patients. While no correlation was found between resistance profiles and genotypic profiles, it was determined that isolates showing multiple resistance to antibiotics were distributed to different PFGE clusters and different plasmid profiles.

In addition, our results revealed that the majority of *S.* Enteritidis isolates in Cankiri province of Turkey is similar PFGE pattern and confirmed the clonal structure

of *S.* Enteritidis strains. Identification and typing of microorganisms at molecular level are very important. Investigating whether epidemiologically related isolates are genetically related may help to find the source and control spread of the epidemic in the community and hospitals (27).

In conclusion; combination of PFGE conducted with the restriction endonuclease *Xba*I and plasmid analysis was useful in detection of clonal relationship among *S.* Enteritidis. The importance of molecular typing methods in order to take more effective protection and control measures has been demonstrated and proposed to use such methods in this study.

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Ethical Statement

The study was approved by the ethic committee of Burdur Mehmet Akif Ersoy University (Ethic approval Code: GO 2020-258).

Conflict of Interest

The authors declared that there is no conflict of interest.

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Evaluation of the enrofloxacin excretion in Anatolian buffalo milk

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Abstract: Enrofloxacin (ENR) is a broad-spectrum fluoroquinolone antibiotic widely used in both human medicine and veterinary medicine. In this study, the milk-excretion patterns of ENR and its metabolite ciprofloxacin (CIP) were determined up to the 10th milking following the intramuscular administration of ENR (2.5mg/kg of body weight) to lactating dairy buffaloes. The milk of each animal was collected and the concentrations of ENR and CIP were detected in milk by LC-MS/MS. The detection limits of the method were determined for ENR and CIP as 0.35 µg/kg and 0.12 µg/kg, respectively. The highest total mean concentrations of ENR and its metabolite CIP was determined in the second milking after injection as 603 µg/kg. The residue level in all buffalo milk samples was found to be lower than the maximum residue limit (100 µg/kg) at the fifth milking. In addition, the employed method is successfully applied to evaluate the presence of ENR and CIP residue in 50 marketed buffalo milk samples and none of the samples contained these antibiotics. Consequently, the present study provided information on the milk excretion levels of ENR and CIP in Anatolian buffalo milks by an LC-MS/MS method.

Keywords: Anatolian buffaloes, ciprofloxacin, enrofloxacin, excretion, LC-MS/MS, milk.

Anadolu manda sütündeki enrofloksasin atılımının değerlendirilmesi

Özet: Enrofloksasin (ENR), hem beşeri hekimlikte hem de veteriner hekimlikte yaygın olarak kullanılan geniş spektrumlu bir florokinolon antibiyotiktir. Bu çalışmada laktasyondaki mandalara ENR'nin (2,5 mg/kg vücut ağırlığı) intramusküler uygulamasını takiben 10. sağımına kadar ENR ve metaboliti siprofloksasinin (CIP) süt ile atılımı belirlendi. Her bir hayvanın sütü toplanarak ENR ve CIP düzeyleri LC-MS/MS ile tespit edildi. Yöntemin tespit sınırları ENR ve CIP için sırasıyla 0,35 µg/kg ve 0,12 µg/kg olarak saptandı. ENR ve metaboliti CIP'nin en yüksek toplam düzeyleri, enjeksiyondan sonraki ikinci sağımda 603 µg/kg olarak tespit edildi. Tüm manda sütü numunelerindeki kalıntı seviyesinin, beşinci sağımda maksimum kalıntı limitinden (100 µg/kg) daha düşük olduğu belirlendi. Ayrıca, kullanılan yöntem, tüketime sunulan 50 manda sütü örneğinde ENR ve CIP kalıntı varlığını değerlendirmek için başarılı bir şekilde uygulandı ve hiçbir örneğinin bu antibiyotikleri içermediği saptandı. Sonuç olarak bu çalışma, LC-MS/MS yöntemi kullanılarak Anadolu mandalarında ENR ve CIP'in süt atılım seviyeleri hakkında bilgi sağladı.

Anahtar sözcükler: Anadolu mandası, atılım, enrofloksasin, LC-MS/MS, siprofloksasin, süt.

Introduction

Milk contains several major nutrients such as fat, carbohydrates, and proteins that are essential sources to the daily diet of human. In addition, significant minerals and vitamins including magnesium, calcium, pantothenic acid, riboflavin, and vitamin B_{12} are found to be in the milk content (1, 13). Buffalo milk provides also a very rich nutrient composition and commonly used in different types of milk products such as cream, cheese, yoghurt, and butter. In addition, this milk type is mostly produced after cow milk in the world (7).

Fluoroquinolones (FQs) exhibit a broad antibacterial spectrum by inhibiting bacterial DNA gyrase activity (15). They are frequently employed to treat many bacterial infections in human and veterinary medicine (20). However, improper or misuse of these antibiotics may

cause antibacterial resistance in clinically important bacteria including *Salmonella* spp. (6), *Escherichia coli* (23), and *Campylobacter* spp. (24). Official authorities have taken precautions to continue the usage of FQs as antibiotics and to reduce their potential food-caused health risks. Since enrofloxacin (ENR) is metabolized to its major active metabolite ciprofloxacin (CIP) by de-ethyletion and both antibiotics are found to be in animal tissues together (28), European Union has established maximum residue limits for ENR as the sum of ENR and its metabolite CIP in milk, which is 100 µg/kg (8).

To analyze FQs residues in a different matrix, many methods including capillary electrophoresis (27), immunoassays (2), high-performance liquid chromatography (22), liquid chromatography-tandem mass spectrometry (LC-MS/MS) were reported. Nevertheless, LC-MS/MS is considered as the most potent confirmatory method due to its high accuracy and sensitivity (3).

The purpose of the present study is to investigate the milk excretion of ENR in the Anatolian buffalo milk by a LC-MS/MS method. Some studies have evaluated pharmacokinetics of preventatives of FQs in farm animals such as cows (12, 18), pigs (14), goats (25), and sheep (9). However, the persistence of ENR in the milk of Anatolian buffaloes has not been evaluated.

Materials and Methods

Materials: Enrofloxacin, Ciprofloxacin, and formic acid of analytical grade were provided by Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and water of analytical grade were obtained from Merck (Darmstadt, Germany). All other chemicals and reagents were analytical grade and purchased from commercial sources.

Five healthy female Anatolian buffaloes, weighing 400-500 kg were chosen for the present study. The experimental animals were obtained from Afyon Kocatepe University, Veterinary Faculty Research and Application Farm. In addition, the ethical approval of the study was taken from Afyon Kocatepe University Animal Experiments Local Ethics Board (49533702/98). All the animals were kept under similar conditions having standard ration and free access to water. Each experimental animal was intramuscularly given a single recommended dose of Enrofloxacin 2.5 mg/kg with a commercial product (Ekoflox 10%, Ekomed ilaç, İstanbul, Turkey). Milk samples were collected during 5 days at the 0 (blank sample), 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120 hours. The chosen milking period was determined based on the withdrawal time of ENR and CIP 4 days (8 milking) for cattle. A blank sample was taken before drug administration from each animal. Collected milk samples were directly stored at -20ºC for further analysis.

A total of 50 buffalo milk samples were collected from Afyonkarahisar province between August and November in 2017. Milk samples were taken by both local markets and producers. All milk samples were transported to the laboratory immediately after sampling under cold conditions and stored at -20°C in a deep freezer for further analysis.

Enrofloxacin and ciprofloxacin stock solutions were prepared in methanol (1 mg/mL). To generate seven-point concentrations (0.5, 1, 2, 5, 10, 20, 50 ng/mL) of the calibration curve, calibration standard samples of respective antibiotics were prepared in milk by spiking with an appropriate volume serially diluted stock solution.

Methods: The buffalo milk samples were extracted as previously reported by Jank et al. (11) with some changes. 2 ml of buffalo milk sample was given to centrifuge tube and 4 ml of acetonitrile added to the tube. To purify milk samples, three times centrifugation was done (10 min, 4000 rpm and 4°C). After each centrifugation step, fat layer was discharged and the rest was re-centrifuged. The obtained supernatant was taken into a water bath (≤45°C) and the evaporation was performed under an N_2 stream. The volume of solvent was reduced to approximately 500 µl under this condition. The volume was achieved to 1ml then transferred to HPLC vials. For LC/MS/MS analysis of milk samples, Agilent Technologies 1200 series (Waldbronn, Germany), attached with a binary high-pressure gradient pump was employed. Zorbax Eclipse XDB-C8 (150 mm × 4.6 mm, 5 µm; Agilent Technologies) was used for LC separation at 45°C. The mobile phases consisted of solvent A (20 mM ammonium formate in 0.1% formic acid) and solvent B (acetonitrile). The gradient of LC separation was as follows: 0.0 min, A/B (100/0); 1.0 min, A/B (100/0); 3.0 min, A/B (20/80); 9.0 min, A/B (5/95); 10.00 min, A/B (5/95), 10.10 min, A/B (100/0). The injection volume was 20 µL and the mobile phase flow rate was 0.6 ml/min. Agilent 6460 LC/MS Triple Quadrupole instrument equipped with an ESI (Waldbronn, Germany) was used for mass spectrometry analysis. A nitrogen generator (Balston, Haverhill, MA, USA) was employed to produce nebulizer and drying gas (350°C). All MS parameters including sheath gas flow, nebulizer gas, capillary voltage, and sheath gas temperature were 10 L/min, 40 p.s.i., 4000 V, and 400°C, respectively. Positive ion mode was chosen for all MS analysis. Enrofloxacin retention time was determined as 5.017. Its molecular weights, precursor ions (m/z), and product ions (m/z) were 359.9, 341.8, and 315.8, respectively. Ciprofloxacin retention time was found to be as 5.004. Its molecular weights, precursor ions (m/z), and product ions (m/z) were 332.1, 314.1, and 231.1, respectively. The validation of the method was performed by spiking raw milk samples. The quality parameters were chosen as a limit of quantification (LOQ), limit of detection (LOD), linearity range, recovery, and intra- and inter-day precisions. The residual concentrations of the milk samples were measured based on the calibration curve for which a series of standard

solutions are prepared and calculated. The equation of calibration was established by fitting data on a line and the resulted equation is used to determine the antibiotic concentration of unknown samples. In addition, the coefficient of determination (r^2) was given to express the strength of the linear regression. The calculation of the above mentioned parameters was done using the software (Agilent MassHunter Workstation Software Version 7) of the related analytical instrument. The excretion values for ENR and CIP of five different buffaloes were given as mean ± SD for each milking time.

LOD values of ENR or CIP were described as the lowest concentration that the analytical process can confidingly differentiate from background levels (signal-to-noise ratio≥3) while LOQ values were defined as the lowest concentration that can be quantified (signal-to-noise ratio ≥10).

Results

The typical chromatograms for ENR and CIP were given Figure 1. Also, the validation of the present method was determined by recovery, accuracy, precision, linearity, LOQ and LOD. LC-MS/MS was employed to quantify the level of ENR and its metabolite CIP in buffalo milk samples. Chromatographic separation of the antibiotics was also done by an LC technique in line with Chui-Shiang et al. (4). The calibration curves for ENR and CIP were presented in Figure 2 and linearity of curves exhibited appropriate the coefficient of determination (r^2=0.997 and r^2=0.998) in the range from 0.5 to 50 µg/kg. Also, the method possessed high sensitivity and LOD and LOQ values were given in Table 1. The overall precision of the method was expressed by relative standard deviation (RSD%) which was lower than 6.9%. Intra-day and inter-day recoveries were used to express the accuracy of the method at three different levels of 45, 90, 135 µg/kg. Intra-day and inter-day recoveries and RSD values were given for ENR and CIP in Table 2 and Table 3, respectively. Some parameters of the developed instrumental methods for the determination of ENR and CIP residues in milk were given in Table 4.

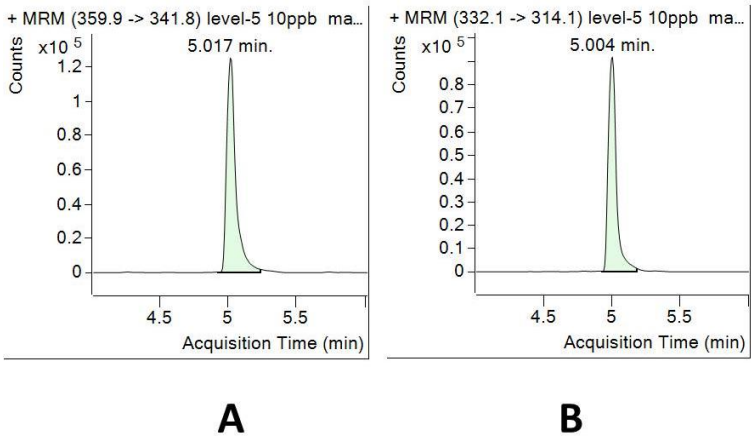

Figure 1. Chromatograms of enrofloxacin (A) and ciprofloxacin (B) standards.

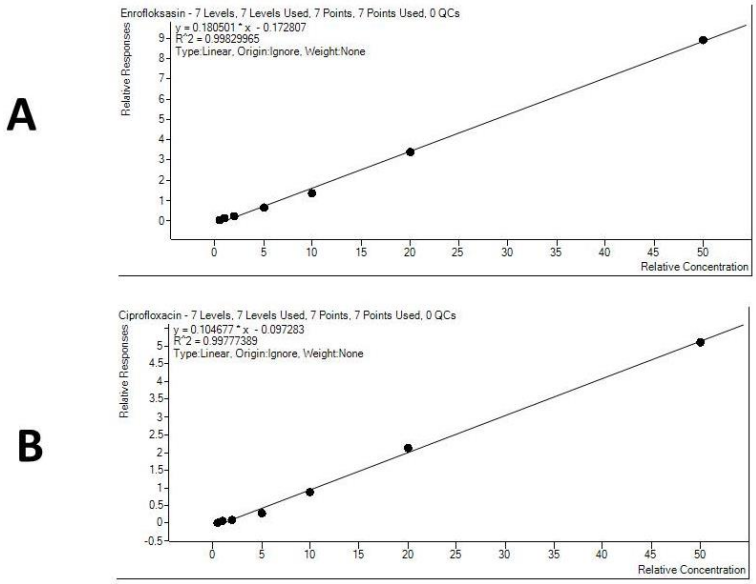

Figure 2. Calibration curves for enrofloxacin (A) and ciprofloxacin (B).

Table 1. Analytical parameters of enrofloxacin and ciprofloxacin in buffalo milk samples.

Antibiotic	LOD (µg/kg)	LOQ (µg/kg)	Coefficient of determination (r^2)	Data point	Linear range (µg/kg)	RT (min)
Enrofloxacin	0.35	1.16	0.998	7	0.5-50	5.017
Ciprofloxacin	0.12	0.40	0.997	7	0.5-50	5.004

Table 2. Intra- and inter-day precisions for enrofloxacin in buffalo milk samples.

	Intra-day assays (n=9)		Inter-day assays (n=9)	
Spiked (ppb)	Percentage Recovery±CV	RSD (%)	Percentage Recovery±CV	RSD (%)
45	84.73±4.43	5.23	84.42±4.60	5.58
90	80.52±3.44	4.27	75.69±3.45	4.56
135	93.28±3.34	3.58	79.02±5.16	6.53

Table 3. Intra- and inter-day precisions for ciprofloxacin in buffalo milk samples.

	Intra-day assays (n=9)		Inter-day assays (n=9)	
Spiked (ppb)	Percentage recovery±CV	RSD (%)	Percentage recovery±CV	RSD (%)
45	84.41±3.90	4.62	83.88±5.09	6.07
90	76.07±3.15	4.14	74.99±3.49	4.65
135	83.00±3.01	3.63	75.57±5.22	6.90

Table 4. Selective methods for the quantification of enrofloxacin and ciprofloxacin in milk.

Method Type	Analyte	Matrix	LOD µg/kg	LOQ µg/kg	Recovery (%)	Reference
LC-MS	ENR	Buffalo Milk	0.35	1.16	75-93	Current Study
	CIP		0.12	0.40	74-84	
LC-MS	ENR	Bovine Milk	0.3	1	77-84	26
HPLC-DAD	ENR	Goat Milk	15	20	81-86	5
	CIP		15	20	85-89	
HPLC-FAD	ENR	Bovine Milk	NA	2	91-107	10
	CIP		NA	2	95-101	
HPLC-UV	ENR	Bovine Milk	1.37	4.56	93-99	16
	CIP		3.74	12.5	89-97	

NA: Not available.

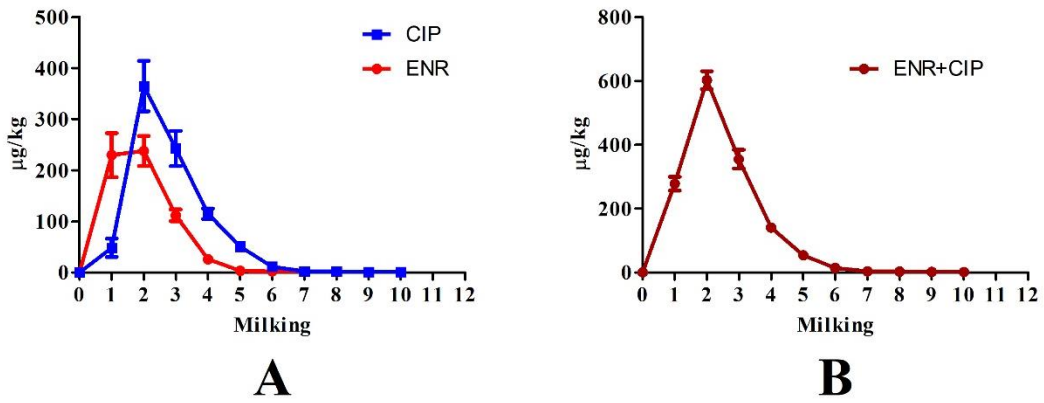

Figure 3. Excretion levels of enrofloxacin and its metabolite ciprofloxacin in milk of buffaloes. Separate excretion kinetics for each antibiotic (A), total excretion kinetics (B)[1].

[1]European Union has established maximum residue limits for ENR as the sum of ENR and its metabolite CIP in milk which is 100 µg/kg.

In this study, the highest concentrations of ENR and CIP were determined at the second milking with a mean concentration of 238.03±29.4 and 365.21±49.44µg/kg, respectively (Figure 3). Also, the total sum of ENR and its metabolite CIP in milk was decreased after the second milking consequently and their total concentration was found to be lower than the maximum residue limit (100 µg/kg) at fifth milking. The mean concentration of CIP in milk was measured at 10 milking post-treatment 1.63±0.12 µg/kg while the concentration of ENR was lower than the related LOD.

In addition, 50 buffalo milk samples marketed in Afyonkarahisar were tested regarding ENR and CIP residues and results of the analysis showed that none of the milk samples contained ENR and/or CIP.

Discussion and Conclusion

Fluoroquinolone antibiotics are commonly employed in human and veterinary medicine to treat bacterial infections based on their broad antibacterial activity (2). The LOD values of the present study for ENR and CIP were determined as 0.35 and 0.12 µg/kg, respectively while LOQ values were determined as 1.16 and 0.40 µg/kg for ENR and CIP, respectively. In a similar LC-MS method established for ENR analysis, LOD and LOQ values were reported as 0.3 and 1 µg/kg, respectively (26). Also, higher LOD (15 µg/kg) and LOQ (20 µg/kg) values were reported for ENR and CIP analysis by using HPLC-DAD method in goat milk (5). Recovery values of the present study ranged from 75% to 93% for ENR whereas these values were determined between 74% and 84% for CIP. These values are compatible with the Cinquina et al. (5) and Tian (26). However, they are lower than the values reported by Idowu and Peggins (10) and Lv et al. (16).

The present experimental research followed the passage of ENR and its metabolite CIP into buffalo milk and determined their milk excretion kinetics using LC-MS/MS. ENR and CIP were determined in milk from 0 to 10 milkings following intramuscular injection of ENR to dairy buffaloes. The highest concentrations of ENR which is described as a total sum of ENR and its metabolite CIP by EU commission regulation (8) were found to be at the second milking (603.24±27.97 µg/kg) and residue level in milk was found to be lower than the maximum residue limit of 100 µg/kg (8) at fifth milking. The persistence of ENR and CIP in the milk of livestock animals was reported by some previous studies. In one of these studies, Mahmood et al. (17) determined the persistence of fluoroquinolone antibiotics in cow milk. For that purpose, lactating animals were intramuscularly injected with Norfloxacin (NOR), ENR, CIP at the dose of 5, 2.5, and 5 mg/kg, respectively. Spectrophotometrically evaluation of milk samples showed that NOR was present in milk samples until the 5th days while ENR was washed out in 6 days. However, CIP was longer persistent in milk up to 8 days. In the present study, ENR was washed out at the 5 days. This may be attributed to employed method and/or animal species. In another study conducted by Kaartinen et al., (12), Five Ayrshcre cows were given enrofloxacin at the dose of 5 mg/kg body weight in three different ways including intravenous, intramuscular and subcutaneous and residue analysis of ENR and CIP was done with HPLC. The ENR found to be bounded to serum proteins at ratio of 36-45%. Approximately, 0.2% of the total ENR dose was detected in milk for the first 24 h and the transferred amount has not been affected by the administration route. The concentration of ENR in milk was determined in line with that in serum, whereas CIP was detected more concentrated in milk. Additionally, following the intravenous injection, the peak milk concentration of ENR and CIP was more distinctive comparing to intramuscular and subcutaneous routes. Also, intramuscular injection of ENR was resulted in similar residue level in milk in the present study. Haritova et al. (9) evaluated pharmacokinetics of enrofloxacin in lactating sheep by administrating ENR intravenously and intramuscularly at a dose rate of 2.5 mg/kg body weight. Their results showed that 24 h after the intravenous treatment of ENR, this antibiotic was found to be 10 times higher in the milk than the serum. Higher levels of ENR which is lipophilic drug in milk may be due to the low degree of binding of the ENR to serum proteins (29). After intramuscular treatment of ENR, its milk concentration was also more concentrated than those in the serum and these results indicate that the route of administration does not play a role in the good penetration of ENR into milk and its milk concentrations. In addition, the concentration of ENR was reached to peak level at the 24 hours after the application. Similarly, in this study the peak of ENR concentration was observed after 24 hours. The present study investigated for the first time, the persistence of ENR and CIP in the milk of Anatolian buffalos and results of the study regarding the milk concentration of ENR and CIP are comparable with the above-mentioned studies.

Also, a mini survey was conducted to screen the presence of ENR and CIP buffalo milk samples (n=50) marketed in Afyonkarahisar and results of the analysis showed that none of the milk samples contained ENR and/or CIP. Similarly, Nirala et al. (21) screened a total of 250 milk samples in India and reported that none of the samples were positive for ENR and CIP. In another study, 234 randomly milk samples from each of bulk tank, market milk, collecting centers, and UHT milk samples were tested for determination of the quinolones residues. Results revealed that quinolones were not detected in

collecting centers and market milk samples whereas was found to be in one sample bulk tank milk samples (19).

In conclusion, the present study reports here the milk excretion of ENR and its metabolite CIP for the Anatolian buffaloes by an accurate, precise, and reliable, LC-MS/MS method combined with easy sample preparation step. The LOD and LOQ values of this method are able to detect these antibiotics within the established MRL in milk. This developed method could be used for the analysis of ENR and CIP in buffalo milk. Also, the present study provided new information about the withdrawal period of ENR and its metabolite CIP in buffalo milk. This result showed that withdrawal period of ENR in buffalo milk is compatible with the withdrawal time established for cattle by the EU. In addition, real milk samples were analyzed and found to be safe regarding ENR residue risk in Afyonkarahisar Province of Turkey.

Financial Support

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Ethical Statement

This study was approved by Afyon Kocatepe University Animal Experiments Local Ethics Board (49533702/98).

Conflict of interest

The authors declared that there is no conflict of interest.

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Prevalence and molecular diagnosis of *Gongylonema pulchrum* in cattle and sheep in the Samsun region

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Abstract: This study was carried out between November-2017 and June-2019 to determine the distribution and molecular characterization of *Gongylonema pulchrum* in cattle and sheep slaughtered in local abattoirs and slaughtering areas during the feast of sacrifice. In this study, esophagi collected from 380 (358 young and 22 mature) cattle and 848 esophagi from sheep (816 young and 32 old) were examined. Esophagus taken after slaughtering was brought to the laboratory in plastic bag. In the laboratory, all the esophagi were cut open with scissors and all of the materials were examined using a loupe under a light source. Parasites were taken out using eye forceps and placed in 70% ethyl alcohol. Following the primary morphological examination, all the parasites cut into three parts, anterior and posterior parts were used for morphologic identification and mid part was used for molecular identification. BpCoxI-F1 and BpCoxI-R1 primers were used for molecular identification. Only two cows (older than four years) were infected and the infection rate was 0.53%. A total of 27 parasites were identified, 18 were male and 9 were female. Parasites were not found in the examined sheep. All the samples collected from the infected cattle were identified as *G. pulchrum* after morphological examinations and molecular confirmation and positive bands were detected at 400bp.
Keywords: Cattle, *Gongylonema pulchrum*, molecular, Samsun, sheep.

Samsun yöresinde sığır ve koyunlarda *Gongylonema pulchrum*'un yaygınlığı ve moleküler teşhisi

Özet: Bu araştırma, Samsun bölgesinde 2017 Kasım - 2019 Haziran tarihleri arasında, yerel mezbahalar ve kurban bayramında kesilen koyun ve sığırlarda *Gongylonema pulchrum*'un yaygınlığı ve moleküler karakterizasyonunu belirlemek amacıyla yapıldı. Çalışmada 380 sığır (22'si yaşlı, 358'i genç) ve 848 koyundan (32'si yaşlı, 816'sı genç) toplanan özefaguslar incelendi. Kesim sonrası alınan özofaguslar plastik torbalara konularak laboratuvara getirildi. Laboratuvarda tüm özefaguslar makas yardımı ile açılarak ışık altında ve büyüteç yardımı ile incelendi. Parazitler göz pensi ile çıkartılıp % 70'lik etil alkole konuldu. Parazitler üç parçaya ayrıldı, ön ve arka kısımlar morfolojik, orta kısımları ise moleküler teşhis amacıyla kullanıldı. Moleküler teşhis için Bp CoxI-F1 ve Bp CoxI-R1 primerleri kullanıldı. Yalnızca iki inek (dört yaş üstü) enfekte olmuş ve yaygınlık oranı % 0,53 bulunmuştur. Enfekte hayvanlardan 18 erkek ve 9 dişi olmak üzere 27 parazit toplandı. İncelenen koyunlarda parazite rastlanmadı. Enfekte sığırdan toplanan tüm örnekler, morfolojik incelemeler ve moleküler doğrulama sonrasında *G.pulchrum* olarak saptandı ve pozitif bantlar 400 bp'de elde edildi.
Anahtar sözcükler: *Gongylonema pulchrum*, koyun, moleküler, Samsun, sığır.

Introduction

Gongylonema pulchrum (gullet worm) is a nematode localized in the esophageal epithelium in variety of mammals and birds, particularly livestock, worldwide (31). In the genus *Gongylonema*, there are nearly 50 species identified in mammals and birds (6, 16, 32), however, *G. pulchrum* the most known and common species in Turkey (21, 23) and in the world (22, 31). The parasite had been identified for the first time in Turkey in 1922 by Tüzdil (30).

The parasite is located in shallow tunnels in the esophagus in a zig-zag pattern, and can sometimes be found in the rumen of the ruminants. The intermediate hosts are dung beetle and cockroaches (23). In Turkey, *G. pulchrum* has been detected in sheep, goats, cattle, buffalo, horses and wild boars and the prevalence rate was reported as 7.6 - 96% (4, 5, 8, 11, 28, 31).

Gongylonema pulchrum, a zoonotic parasite has been reported from many Asian, African, American and European countries, including Turkey, a large number of

human cases had been reported and have reached 200 to date (14, 33).

Morphological and genetic differences can be observed between *G. pulchrum* samples obtained from different animal species, as well as genetic differences between samples obtained from the same animal and it is suggested that there are at least two haplotypes for parasite (20, 27). The observation of genetic differences in the species has increased with the use of molecular studies, and the total mitochondrial genome of the parasite was determined in 2015 (18).

Morphological criteria are sometimes insufficient for the diagnosis of parasites and the distinction of closer species, and molecular diagnostic methods are used in such cases. For this purpose, the gene regions of the parasites such as the ribosomal DNA (ITS1, ITS2) and mitochondrial DNA (COX1) are used (20).

As a result of molecular studies, *G.nepalensis* species, which is morphologically very similar to *G.pulchrum* and separated from it only by the shorter left spiculum, has been identified in Nepal (27) and Europe (32).

Gongylonema pulchrum is considered to be insignificant when the number is low in ruminants. In severe infections, local inflammation, bloating, bleeding, mild pain, sometimes epithelial hypertrophy and acanthosis were observed in the esophagus (9), and although very rare, causes death in cattle and monkeys (15).

This study was carried out to determine the prevalence and molecular characterization of *G. pulchrum* in cattle and sheep slaughtered in the Samsun region, Turkey.

Materials and Methods

This research was carried out between November 2017 and June 2019 in Samsun, Havza (Temiz-Et Slaughterhouse) and Atakum (Florya Slaughterhouse) and sacrificial temporary slaughterhouse in Kavak district at 2017-2018. During this period, 380 (52 female, 328 male) cattle and 848 sheep (254 female, 594 male) esophagus were collected from slaughtered animals and brought to Ondokuz Mayıs University Veterinary Faculty Parasitology Laboratory.

After opening the esophagus under the light source with scissors in the laboratory, it was examined with the aid of a magnifying glass, and/or under a stereo-microscope. The parasites were removed from the esophagus with eye forceps, counted and macroscopic measurements were made and kept in 70% ethyl alcohol for each animal individually.

The parasites were morphologically divided into three equal parts and the middle parts of the body were stored in pure ethanol at -20 °C for use of molecular analysis. The front and back ends of the parasites were stored in 70% alcohol, and after making transparent with lactophenol, they were diagnosed under the microscope according to the related literature (23, 30, 31, 32). In the diagnosis, the important regions were measured and photographed.

DNA extraction: Genomic DNA was obtained separately from two worms using the commercial DNA extraction kit (Invitrogen PureLink Genomic DNA Mini Kit) according to the manufacturer's instructions and stored at -20 ºC until use.

Polymerase Chain Reaction (PCR): The COI gene region of genomic DNA was amplified using the BpCoxI-R1 (5'-ATGAAAATGTCTAACTACATAATA AGTATC-3') and BpCoxI-F1 (5'-TTTGG TCATCCTGAGGTTTATATT-3') primer pair as described in Makouloutou et al. (20) and Setsuda et al. (27).

The PCR mixture (50 µl) contained 10 mM Tris HCl, 50 mM KCl, 2.5 mM $MgCl_2$ (Thermo), 250 µM dNTP mix (Sigma), 0.5 µM each primer, 1.25 U Taq polymerase (Thermo), 2 µl template DNA, and distilled water. PCR conditions were as follows: first denaturation 94 °C for 3 min, 40 cycles at 94 °C for 45 sec, 52 °C for 1 min, 72 °C for 1 min and final cycle at 72 °C for 7 min. Amplicons were imaged and photographed under UV after electrophoresis in 2% agarose gels stained with ethidium bromide (Sigma).

Sequence and phylogenetic analysis: Purification of two PCR products obtained and sequenced for both strands with the same primers by Macrogen (Holland). The obtained sequences were verified by forward and reverse comparisons, assembled and edited with using Contig Express in Vector NTI Advance 11.5 (Invitrogen).

The blast analysis (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) of the sequences obtained in the GenBank database (1), the homologies and similarity percentages of the isolates among themselves and with other isolates in the world were determined. Multiple alignments of the nucleotide sequences of the haplotypes were performed using the Clustal W algorithm with the Mega X (17) and BioEdit (13) programs.

The genetic distances of the isolates (pairwise distance, Kimura 2 Parameter) were determined according to the Mega X program (17) and the maximum composite likelihood model (29). Nucleotide multiple alignments were converted to different file formats with the DnaSP version 6.10 program (26). In the jModelTest version 0.1 (25) program, the GTR model was found as the best DNA model using the Akaike information criterion (AIC).

The phylogenetic tree was created according to the maximum likelihood (ML) method with PhyML version 3.1 (10) program. In the bootstrap analysis, the value was set to be 100 reps, and the ≥50% bootstrap value was considered significant.

Figtree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree) program was used for tree drawing. Trees were created with the help of Inkscape 0.92 (http://inkscape.org/en/) program. The nucleotide sequence was submitted to GenBank under the accession number MK962623.

Results

Parasites were detected only in two elderly (over four years) animals from the examined 380 cattle, the prevalence rate was determined as 0.53% and gullet worms showing a typical epithelium-dwelling character (Figure 1A). Totally 27 parasites (18 male and 9 female) were collected from two infected cattle.

848 sheep (254 female and 594 male) were examined in the slaughterhouses in the Samsun region. The majority of the slaughtered sheep consisted of 816 (96.2%) young (under 1-year-old) and 32 (3.8%) old (over 2-years-old) sheep. No parasites were found in any of the sheep examined.

When female and male parasites were carefully examined on a black background, due to the difference in size and posterior ends, they can be easily differentiated with a simple magnifier (Figure 1B,C).

In the microscopic examination, there were cuticle ornaments on the one cm anterior of the parasites, which look as if they were adhered to the parasite and can spread to the outsides (Figure 2A).

It was observed that the developmental period of the parasites was different. The spicules of the three males were not yet fully developed, they were short, which were considered young (Figure 2B). The sizes of these young male nematodes were also nearly 10% smaller than the adults but remained within normal limits. All of the young and adult parasites were measured. The length of the males was, as an average, of 38 mm (29 - 47) and a thickness of 0.25 (0.21 - 0.28) mm.

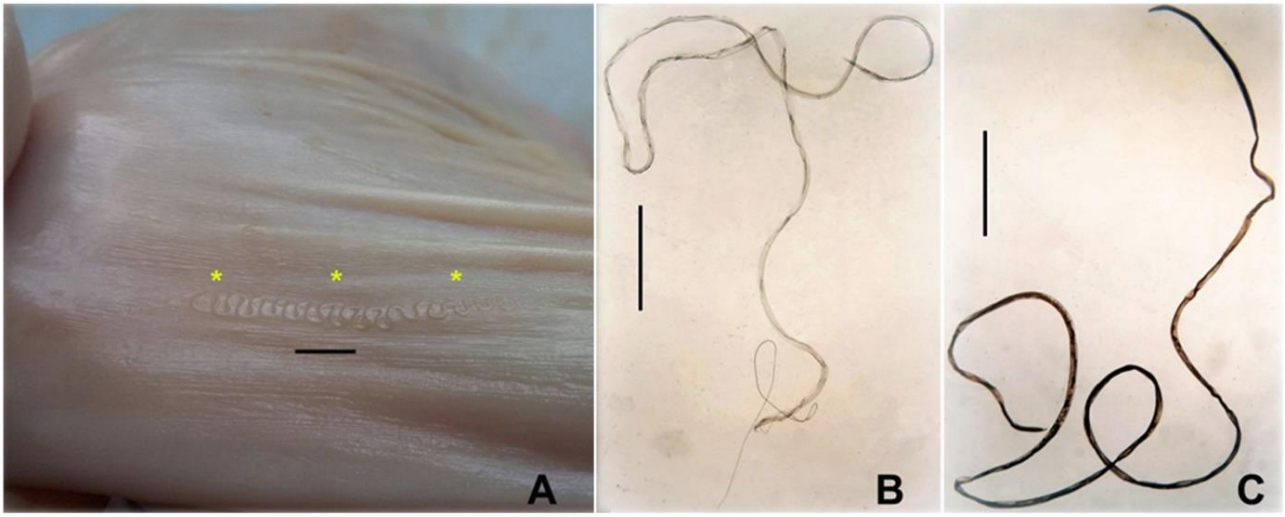

Figure 1. The natural and stereo microscopic view of *G.pulchrum*.
A. Zig-zag manner of parasite in the esophagus, B. Mature male, C. Mature female, Bar: 10 mm in all figures.

Figure 2. Microscopic view of the *G. pulchrum.*
A. Anterior end of the mature worm, B. Posterior end of the juvenile male, C. Posterior end of the mature male, D. Anal region of the female. E. Vulvar region of the female, F. Eggs in the uterus. Bar: 0.01 mm in all figures.

The spicules were asymmetrical, and the left spicule was very long and overflowing out of the bursa and it was 7.5 (5.6-12.1) mm long. The right spicule was very short and measured as 0.120 (0.100- 0.142) mm (Figure 2C).

The average length of the females was 72 (45-88) mm, and the thickness was 0.38 (0.32-0.44) mm. In females, the anus was prominent, the tail ends in a finger-like shape and the tail length was measured as 0.230 (0.190-0.270) mm (Figure 2D).

The vulva was located on the posterior half of the body and slightly protrudes outwards (Figure 2E). Thousands of eggs with a fully developed embryo were seen in the uterus of the adult female. The length of the eggs in the uterus was measured as 0.059 (0.057-0.061) mm and the width was 0.032 (0.030-0.036) mm (Figure 2F).

As a result of molecular analysis, all of the samples detected in both animals were detected as *G. pulchrum*, and positive bands were obtained at approximately 400 bp (Figure 3). The sequence result for *G. pulchrum* bovine isolate COI gene region is given in Table 1. The genetic distances of the isolates to each other (pairwise distance, Kimura 2 Parameter) are presented in Table 2 according to the maximum composite likelihood model.

The phylogenetic tree using the maximum likelihood (ML) method, which shows the phylogenetic relationships of the *G. pulchrum* bovine isolate COI gene region obtained in this study with other isolates and species in the world, is given in Figure 4. Nucleotide variations of the total alignment were shown in Figure 5.

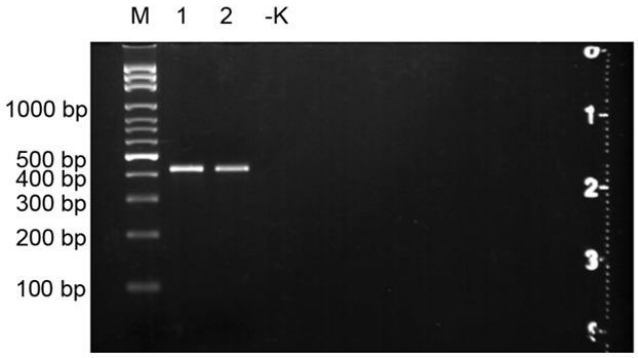

Figure 3. Electrophoresis image of the gene region of positive samples M: Marker, 1-2: Isolates, -K: Negative Control.

Table 1. The sequence result of *G. pulchrum* bovine isolate COI gene region.

>*Gongylonema pulchrum* voucher OMUPAR.32.18.01 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial
ATCCTGAGGTTTATATTATTATTTTACCTGCATTTGGTATTATTAGGGAATGTGTTTTATATCTAACTGATAAGGAAC
GATTATTTGGTCAAGCAAGTATGGTTTATGCTTCTATTTGAATTTCTGTTTTAGGTACGTCTGTTTGGGGCCATCATA
TATATACTGCAGGTCTTGATATTGACACTCGAACTTATTTTAGGGCAGCTACTGTTATTATTGCTATTCCTAGGGCGG
TTAAGGTTTTTAATTGACTTGGGACTTTATTTGGTTCTCGTCAATATTTACAACCTGTGTGATGTTGGACATATAGTT
TTATTTTTTTGTTTACTATTGGTGGTTTGAGCGGTATTATTTTGAGTACTGCTAGGTTGGATATTGTTTTACATGATAC
TTATTATGTAGT

Table 2. Genetic distances of isolates to each other.

Erişim No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
MK962623	-														
KM264298	0.00	-													
LC388895	0.00	0.00	-												
LC026040	0.00	0.00	0.00	-											
LC026035	0.00	0.00	0.00	0.00	-										
AB513728	0.00	0.01	0.00	0.00	0.00	-									
LC388914	0.02	0.03	0.02	0.03	0.02	0.02	-								
LC388905	0.02	0.03	0.02	0.03	0.03	0.02	0.00	-							
LC388909	0.03	0.03	0.03	0.03	0.03	0.03	0.01	0.01	-						
AB646115	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.01	0.02	-					
AB646122	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.00	0.01	0.01	-				
LC388893	0.10	0.11	0.10	0.11	0.10	0.10	0.10	0.10	0.10	0.10	0.10	-			
LC388892	0.10	0.11	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.00	-		
LC278393	0.10	0.11	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.00	0.00	-	
LC026046	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.14	0.14	0.13	0.14	0.13	0.13	0.13	-
LC331022	0.14	0.14	0.14	0.14	0.14	0.15	0.13	0.13	0.14	0.14	0.15	0.14	0.14	0.14	0.09

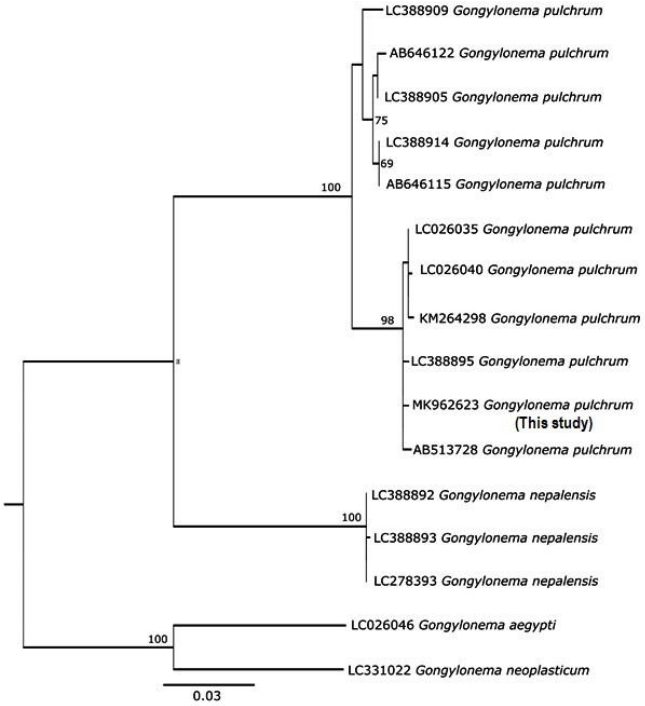

Figure 4. The phylogenetic tree made using maximum likelihood method for *G. pulchrum* cattle isolate COI gene region.

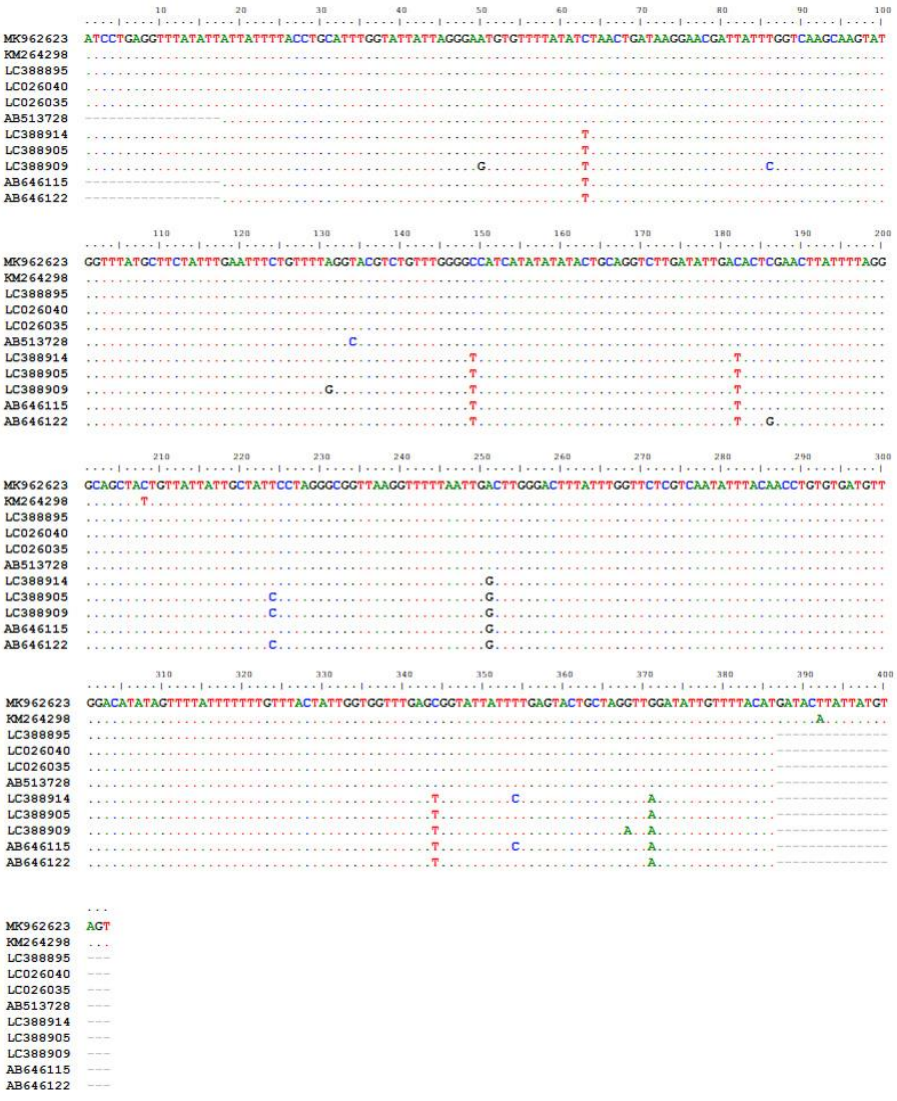

Figure 5. Nucleotide variations in mitochondrial COI gene regions of *Gongylonema pulchrum* isolate analyzed in the present study.

Discussion and Conclusion

Although the prevalence of *G. pulchrum* in cattle and sheep varies according to country and animal species recent reports (9, 15) indicated that the prevalence of the parasite was decreased. For instance, prevalence rate of the *G. pulchrum* in cattle in Iran was decreased to 16.2% in 2011 (15) compared to 49.7% in 1979 (3). Similarly, in Turkey, the prevalence was as high as 96% in Ankara region (30) while Celep et al. (5) found a significantly lower prevalence rate (6.3%) in the Samsun region close to prevalence recorded in Japan (5.3%) (20). However, in this study conducted in the Samsun region, we found the prevalence rate of the parasite as low as 0.53% in cattle.

Although there is no reliable and comparable information about the age and grazing conditions of the studies conducted, the prevalence rate of the parasite in the sheep, similar to cattle, decreases even in sheep fed more pasture. Even though Tüzdil (30), who carried out the first study on *G. pulchrum* in Turkey, reported a 60% prevalence in sheep and goats, Celep (4) found lower prevalence rate in sheep (14.7%) from Samsun region. On the other hand, the prevalence rate was recorded as 40% in sheep in Kars region (2), 42.8% (7) and 13.19% in Van region (11). In both studies performed in Van region, information about the age of the animals was not provided. This difference in the prevalence rate could be age-related. However, it should not be forgotten that the prevalence rates in the Eastern Anatolia regions of Turkey are still high and that it depends on some regional characteristics. In our opinion, the reason for this may be due to the inadequacy in veterinary services and the lack of drug use due to the widespread of nomadic livestock. Similarly, in foreign countries, in recent years, the prevalence rates in sheep are below 10%. For instance, its prevalence rate is 4.57% in Iran (9) and 5.5% in Algeria (24). In the present study, no parasites were found in 828 sheep esophagus examined.

As can be seen in the above publications, the prevalence rates are decreasing gradually both in foreign countries and in Turkey. In our opinion, the most important reason for the decrease in prevalence rates is the reduction in pasture livestock in our country, especially in the Samsun region. With the idea that it is more profitable, closed system integrated livestock farms are increasing. Since animals in such livestock farms are usually fed with commercial feeds, the probability of encountering intermediate host insects is reduced, thus the possibility of parasite occurrence is eliminated. In addition, the increase in the use of antiparasitic drugs and the restriction slaughtering of the rootstock females may also be effective.

The average length of the worms was measured 38 mm (29-47) in males, 72 (45-88) mm in females, the average thickness was 0.25 mm (0.21-0.28) in males, 0.38 mm (0.32-0.44) in females. In the adult male, the left spicula was very long and has extended beyond the bursa copulatrix and measured as 7.5 mm (5.6-12.1). The length of the eggs in the uterus of females was 0.059 mm (0.057-0.061) and the width was 0.032 mm (0.030-0.036). There was no significant difference between the body measurements in the present study as indicated in the literature (12, 23, 27).

In the present study, the number of parasites for per cattle was 13.5 (7-20). However, the parasite density in cattle in Japan was rather variable and recorded as 1-109 (20) while in Nepal the density was as low as 1-4 (19). Although no parasites were encountered in sheep in this study, the number of parasites per sheep was ranged from 10 to 100 in Iran (9, 22), and from 1 to 12 in Turkey (2).

The gullet worm which can be found all around the world has a wide selection of mammalian hosts, hence some minor morphologic (30) and genetic differences depending on the host species (20). Therefore morphological studies must be supported by molecular studies for definitive diagnosis. Although there are many studies on the morphological diagnosis of *G.pulchrum* both in our country (2, 5, 7, 11, 23, 28, 30) and in foreign countries (3, 6, 9, 14, 15), there are only a few molecular studies (18-20, 27, 32) in the world and none in our country.

As a result, *G. pulchrum* was not found in sheep in the Samsun region, and the prevalence rate was very low (0.53%) in cattle. The molecular diagnosis of the *G. pulchrum* was performed for the first time in Turkey and has been recorded in GenBank. According to the phylogenetic analysis, Turkey isolate was very close to the isolates of other countries, and the genetic distance between the isolates ranged from 0.00-0.03%. Some small nucleotide variations were observed between the nucleotides used in the study and the results of this study.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Effects of dietary supplementation of red ginseng root powder on performance, immune system, caecal microbial population and some blood parameters in broilers

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Abstract: The aim of the research was to determine the effects of red ginseng root powder on performance, immune system, caecal microbial population and some blood parameters in broilers. A total of 224 daily Ross 308 male broiler chicks were divided into one control group and three experimental groups each containing 56 chicks. Red ginseng root powder was added to the diets of groups at the level of 0, 75, 150 and 225 mg/kg, respectively. The experimental period was 38 days. There were no differences in growth performance. Addition of 225 mg/kg of the red ginseng root powder increased the relative weight percentages of spleen and gizzard ($P<0.05$). Ginseng root powder supplementation didn't affect biochemical parameters and antibody titers in blood serum. The values of haemoglobin, leukocytes, red blood cell distribution width and mean corpuscular haemoglobin concentration in group fed diet supplemented with 225 mg/kg ginseng were increased significantly ($P<0.001$). The count of *Lactobacillus* spp. in the caecum ($P<0.05$) was increased with 75 mg/kg ginseng root powder addition. As a result, improvements were provided in immune organ weight and some hematological parameters with the addition of 225 mg/kg and in *Lactobacillus* spp. count in caecum with the addition of 75 mg/kg red ginseng root powder to the diets in broilers. Further studies are required to evaluate the bioavailability of the active compounds of red ginseng root powder and to determine the effects of its various doses on performance, immunity, antioxidant potential and intestinal microflora under various stress conditions in poultry.
Keywords: Broiler, immunity, microbial population, performance, red ginseng root powder.

Broyler rasyonlarına kırmızı ginseng kökü tozu ilavesinin performans, immun sistem, sekal mikrobiyel populasyon ve bazı kan parametreleri üzerine etkisi

Özet: Bu araştırmanın amacı, broyler karma yemlerine kırmızı ginseng kökü tozu ilavesinin performans, immun sistem, sekal mikrobiyel popülasyon ve bazı kan parametreleri üzerine etkilerini belirlemektir. Toplam 224 adet günlük Ross 308 erkek broyler civciv her biri 56 civciv içeren bir kontrol ve üç deneme grubuna ayrılmıştır. Kırmızı ginseng kökü tozu grup yemlerine sırasıyla 0, 75, 150 ve 225 mg/kg düzeylerinde ilave edilmiştir. Deneme süresi 38 gündür. Gruplar arasında büyüme performansı bakımından farklılık gözlenmemiştir. Kırmızı ginseng kökü tozunun 225 mg/kg düzeyinde ilavesi dalak ve taşlığın relatif ağırlık yüzdesini artırmıştır ($P<0,05$). Ginseng kökü tozu ilavesi kan serumunda biyokimyasal parametreleri ve antikor titresini etkilememiştir. Yemine 225 mg/kg ginseng ilave edilen grubun kanında hemoglobin, lökosit, eritrosit dağılım genişliği ve ortalama eritrosit hemoglobin konsantrasyonu önemli derecede ($P<0,001$) artmıştır. Sekumda *Lactobacillus* spp. sayısı yeme 75 mg/kg ginseng kökü tozu ilavesi ile artmıştır ($P<0,05$). Sonuçta kırmızı ginseng kökü tozunun 225 mg/kg ilavesi broylerlerde immun organ ağırlığında ve bazı hematolojik parametrelerinde, 75 mg/kg ilavesinin ise sekumda *Lactobacillus* spp. sayısında iyileşme sağlamıştır. Kırmızı ginseng kökü tozu aktif bileşenlerinin biyoyararlanılabilirliğini incelemek ve farklı dozlarının çeşitli stres şartlarındaki kanatlılarda performans, immunite, antioksidan potansiyel ve bağırsak mikroflorasını belirlemek için yapılacak çalışmalara ihtiyaç duyulmaktadır.
Anahtar sözcükler: Broyler, immunite, kırmızı ginseng kök tozu, mikrobiyel popülasyon, performans.

Introduction

Ginseng (*Panax ginseng* C.A. Meyer) is a perennial plant that grows in shaded and humid areas throughout Korea, Japan and China. It is widely used as medicinal herbs, food and flavoring agent in the world (7, 10). Red ginseng is one of the categories of ginseng depending on

the manufacturing method (29). Ginseng has many biological activities such as antioxidant, antistress, antidiabetic, anticarcinogenic activities and immune modulator due to containing various bioactive compounds such as saponins, antioxidants, peptides, polysaccharides, alkaloids, lignans and polyacetylenes. Red ginseng has the most health benefits in all of the ginseng categories due to the high saponin content. Saponins have immune enhancer, anti-fatigue, antioxidant and hepato-protective physiological effects (24, 27). More than 30 different ginsenosides (saponins) having different pharmacological activities have been isolated and characterized (31).

Ginsan, a polysaccharide isolated from the root of *Panax ginseng* C.A. Meyer, has been shown to be a potent immunomodulator, producing several cytokines and stimulating lymphoid cells to proliferate (25, 30, 36, 39, 40).

Lim et al. (31) suggested that fine ginseng root fractions could have antioxidant and antimicrobial effects. Yan et al. (43) concluded that the use of 0.1% wild-ginseng root meal in the diets could increase growth performance and weight of immune organs, while decrease abdominal fat and serum cholesterol. However, some researchers reported that dietary supplementation with fermented red ginseng extract (3) and Korean ginseng root extract (44) did not influence performance and egg quality in laying hens. To the best of our knowledge, limited study has been published on the effects of red ginseng root powder in broilers. Therefore, this experiment was aimed to determine the effects of red ginseng root powder supplementation on performance, immunity, caecal microflora and some blood parameters in broilers.

Materials and Methods

All study were approved by the Animal Ethics Committee of the Ankara University (2015-4-71).

Animals and diets: A total of 224 daily Ross 308 male broiler chicks were divided to four groups and each group had 7 replicates of 8 chicks each. Each replicates were placed in separate floor pen having 80 cm width x 90 cm length x 80 cm height. Zeolite (ZETA, 1-2 mm of particulate size–Gördes Zeolite Madencilik Sanayi Tic A.Ş.-İzmir) was used as a litter. There were two nipples and one hanging suspended feeder in each pen. Water and mash feed were *ad libitum* during 38 days. Lighting was permanently applied. Temperature of room was 32±2ºC on the first week and then gradually reduced to 24-26ºC and this temperature was maintained upto slaughtering. The ingredients and chemical composition of the basal diets were given in Table 1. The diets were formulated to

Table 1. The ingredients and chemical composition of the basal diets (as-fed basis).

Ingredients (g/kg)	**Starter diet 0-21 days**	**Grower diet 22-38 days**
Corn	503.0	484.0
Soybean meal	240.0	172.0
Full fat soya	209.7	265.0
Sunflower seed oil	12.0	40.0
Limestone	9.0	9.0
Dicalcium phosphate	24.0	20.0
Methionine	3.7	2.5
Lysine	2.0	1.5
Sodium bicarbonate	1.0	1.0
Salt	2.5	2.5
Vitamin premix[1]	1.5	1.5
Mineral premix[2]	1.0	1.0
Salinomycine	0.6	-
Chemical composition (Analyzed)		
Metabolizable energy[3] (kcal/kg)	3010	3210
Crude protein (g/kg)	220.2	211.0
Ether extract (g/kg)	69.4	98.4
Crude fibre (g/kg)	48.2	41.0
Crude ash (g/kg)	56.2	53.4
Calcium (g/kg)	12.0	10.8
Total phosphorus (g/kg)	9.1	8.2

[1]: Provides 1.5 kg of premix: 11 000 000 IU vitamin A, 3 500 000 vitamin D_3, 100 g vitamin E, 3 g vitamin K_3, 3 g vitamin B_1, 6 g vitamin B_2, 35 g niacin, 15 g calcium D pantothenate, 1 g vitamin B_6, 20 mg vitamin B_{12}, 1 500 mg folic acid, 200 mg D-biotin.
[2]: Provides 1 kg of premix: 120 g Mn, 50 g Fe, 100 g Zn, 30 g Cu, 2 g I, 200 mg Co, 300 mg Se.
[3]: Metabolizable energy content of diets was calculated (5).

meet or exceed the nutrient requirements of broilers based on the management guide of Ross 308. Basal diets were supplemented with 0 (control), 75, 150 and 225 mg/kg Panax red ginseng root powder (Daedong Korea Ginseng Co. Ltd). Control group diet consisted of only basal diet.

Traits measured: Nutrient composition of basal diet was analyzed (4) for crude protein (CP, Method 968.06), ether extract (EE, Method 920.39), crude fiber (CF, Method 932.09) and ash (Method 967.05). Calcium (11) and total phosphorus (1) were analyzed. Metabolizable energy levels of diets were estimated (5).

All of the birds were weighed individually at day 1, 7, 14, 21, 28, 35 and 38 to determine weight gain. Feed intake was determined at these weighing days and feed conversion ratio (FCR) was calculated as kg feed per kg weight gain. Livability and European Production Efficiency Factor (EPEF) values of groups were calculated according to the following formula (28): Livability, % = (Number of live bird at the end / Number of birds at the beginning) x 100 and EPEF = ((Livability, %) x (Body weight, kg) x 100) / ((Age, day) x (FCR, kg feed/kg gain)).

Newcastle disease vaccine (Live attenuated, Lasota strain, Phibro Animal Health Products Corp.) was made at the begining and 14th day using the eyedrop method.

At the 28th day one bird from each replicate (7/group) was randomly selected, weighed and slaughtered. Blood samples were also taken from the wing vein into plain and EDTA-coated tubes. Blood samples from two birds of each replicate (14/group) were also taken from the wing vein into plain and EDTA-coated tubes at the 38th day of the experiment. Blood serum samples at the 28th and 38th day were used to make biochemical analyses and to determine the specific antibody titer against Newcastle Disease virus in vaccinated broilers using Hemagglutination Inhibition Test (2). Total protein, albumin, globulin, creatinine, urea, triglyceride, total cholesterol, LDL, ALT, AST and GGT levels were determined with an autoanalyzer (BT 3000, Biotechnica Instruments, Italy) using commercial kits of Randox RX series (Randox Laboratories Ltd., London, United Kingdom). Blood taken on EDTA-coated tubes on the 38th day was used to determine hematological parameters with automated hematology analyzer (Sysmex pocH-100iV, Sysmex Corporation, Japan).

At the 38th day two birds from each replicate (14/group) were randomly selected, weighed and slaughtered. Hot carcasses were weighed and carcass yields were calculated. Internal organs of bursa Fabricius, heart, kidney, liver, spleen, abdominal fat (only 38th day) and gizzard of the slaughtered birds at 28th and 38th day were removed, weighed and relative weights of internal organs were calculated by dividing these weights to slaughtering weight.

Caecum samples from slaughtering birds at the 38th day were collected in sterile containers for the determination of the number of total aerobic bacteria (12), coliform (17) and *Lactobacillus* spp. (16).

Statistical analyses: SPSS programme (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Kolmogorov-Smirnov test was applied for the normality of data distribution. One-way ANOVA was used to detect the effects of ginseng supplementation on different parameters. Comparisons among means were done by Tukey test. Polynomial contrasts were used to determine the linear, quadratic and cubic effects of ginseng supplementation on different parameters. Statistical significance level was accepted as $P<0.05$ (9).

Results

Effects of dietary ginseng supplementation on performance parameters were shown in Table 2. Different ginseng supplementation into broiler diets had no effect on body weight, body weight gain, feed intake, feed conversion ratio, livability and EPEF when compared to the control group. As shown in Table 3, no significant effects were observed in relative organ weight percentages on day 28, however relative weight percentages of spleen and gizzard of group fed 225 mg/kg ginseng on day 38 were found to be significantly higher than those of control group ($P<0.05$). Blood serum biochemical parameters were not affected with dietary ginseng supplementation (Table 4). Blood levels of haemoglobin, red blood cell distribution width (RDW), leukocytes (white blood cell, WBC) and mean corpuscular haemoglobin concentration (MCHC) of group fed 225 mg/kg ginseng were found to be significantly higher ($P<0.001$) than those of control group (Table 5). As shown in Table 6, dietary ginseng supplementation at the level of 0, 75, 150 and 225 mg/kg didn't affect hemagglutination inhibition levels in broilers. Dietary supplementation of red ginseng root powder at 75 mg/kg increased the count of *Lactobacillus* spp significantly ($P<0.05$) as given in Table 7.

Table 2. Effects of different levels of red ginseng root powder supplementation on performance of broilers.

Performance parameter	Red ginseng root powder, mg/kg				SEM	P-value			
	0	75	150	225		Combined	Linear	Quadratic	Cubic
Body weight, g									
0 day	44.20	44.11	44.24	44.39	0.072	0.620	0.313	0.421	0.785
38 day	2534.10	2515.17	2578.36	2582.24	18.359	0.509	0.223	0.762	0.403
Body weight gain, g									
0-21 day	773.47	732.06	777.17	755.57	9.784	0.358	0.922	0.615	0.091
21-38 day	1716.42	1738.99	1756.95	1782.28	15.599	0.517	0.140	0.966	0.933
0-38 day	2489.89	2471.05	2534.13	2537.85	18.367	0.513	0.225	0.764	0.404
Feed intake, g									
0-21 day	1133.10	1088.25	1110.78	1089.60	7.972	0.150	0.125	0.443	0.115
21-38 day	3008.41	2970.37	3021.01	3067.23	18.925	0.357	0.189	0.273	0.585
0-38 day	4141.51	4058.62	4131.79	4156.83	19.589	0.305	0.497	0.175	0.248
Feed conversion ratio, kg feed/kg weight gain									
0-21 day	1.466	1.490	1.435	1.447	0.014	0.561	0.389	0.858	0.266
21-38 day	1.754	1.709	1.721	1.725	0.011	0.550	0.464	0.291	0.514
0-38 day	1.664	1.643	1.632	1.641	0.009	0.683	0.353	0.446	0.911
Livability, %	97.96	95.92	97.96	97.96	1.053	0.886	0.838	0.648	0.540
EPEF	392.82	387.26	407.89	407.77	7.128	0.676	0.328	0.855	0.481

n=7, No significant differences among groups.

Table 3. Effects of red ginseng root powder supplementation on relative organ weights and carcass yield.

Item	Red ginseng root powder, mg/kg				SEM	P-value			
	0	75	150	225		Combined	Linear	Quadratic	Cubic
Relative organ weights on day 28, % (n=7)									
Liver	2.272	2.195	2.268	2.231	0.033	0.843	0.873	0.778	0.405
Heart	0.607	0.623	0.649	0.637	0.009	0.426	0.172	0.458	0.564
Gizzard	1.816	1.790	1.829	1.852	0.019	0.714	0.398	0.533	0.633
Bursa Fabricius	0.232	0.228	0.210	0.253	0.010	0.521	0.612	0.257	0.410
Spleen	0.089	0.087	0.106	0.080	0.005	0.327	0.824	0.260	0.147
Carcass yield on day 38, % (n=14)	70.46	70.43	70.73	70.19	0.150	0.667	0.706	0.403	0.399
Relative organ weights on day 38, % (n=14)									
Liver	1.959	1.949	1.935	1.962	0.023	0.978	0.979	0.699	0.831
Heart	0.464	0.490	0.500	0.503	0.006	0.088	0.019	0.323	0.861
Gizzard	1.340^{b}	1.417^{ab}	1.454^{ab}	1.484^{a}	0.017	0.016	0.002	0.472	0.808
Bursa Fabricius	0.213	0.219	0.196	0.199	0.005	0.240	0.116	0.817	0.193
Spleen	0.108^{b}	0.122^{ab}	0.115^{ab}	0.127^{a}	0.002	0.019	0.013	0.810	0.045
Abdominal fat	1.096	1.211	1.181	1.138	0.027	0.474	0.703	0.154	0.586

^{a,b}: Means within a row followed by the different superscripts differ significantly ($P<0.05$).

Table 4. Effects of dietary red ginseng root powder supplementation on blood serum parameters of broilers.

Blood serum parameters	Red ginseng root powder, mg/kg				SEM	P-value			
	0	75	150	225		Combined	Linear	Quadratic	Cubic
On day 28 (n=7)									
Total protein, g/dl	2.571	2.671	2.700	2.671	0.058	0.885	0.551	0.602	0.979
Albumin, g/dl	0.929	0.943	0.957	0.957	0.021	0.961	0.616	0.872	0.943
Globulin, g/dl	1.600	1.729	1.800	1.814	0.053	0.477	0.144	0.594	1.000
Creatinine, mg/dl	0.307	0.331	0.294	0.274	0.025	0.891	0.571	0.678	0.742
Urea, mg/dl	5.000	5.429	5.429	5.000	0.188	0.751	1.000	0.282	1.000
Triglyceride, mg/dl	63.43	60.71	53.86	70.29	3.310	0.384	0.646	0.160	0.362
Total cholesterol, mg/dl	106.43	101.29	95.86	109.43	2.220	0.140	0.851	0.036	0.316
LDL mg/dl,	22.89	23.37	22.80	20.17	1.166	0.785	0.428	0.526	0.927
AST, IU/l	333.57	346.14	338.86	298.00	15.608	0.726	0.437	0.415	0.925
ALT, IU/l	2.429	2.000	2.714	2.429	0.188	0.630	0.682	0.854	0.225
GGT, IU/l	20.14	20.86	21.86	21.57	0.681	0.830	0.413	0.728	0.807
On day 38 (n=14)									
Total protein, g/dl	2.764	2.607	2.657	2.607	0.036	0.364	0.191	0.455	0.339
Albumin, g/dl	0.957	0.900	0.921	0.864	0.022	0.533	0.209	1.000	0.441
Globulin, g/dl	1.871	1.657	1.750	1.743	0.044	0.395	0.458	0.243	0.304
Creatinine, mg/dl	0.266	0.299	0.212	0.229	0.017	0.257	0.185	0.813	0.135
Urea, mg/dl	6.714	7.286	6.429	6.214	0.230	0.390	0.256	0.396	0.318
Triglyceride, mg/dl	43.93	50.07	51.64	52.21	1.756	0.325	0.097	0.429	0.820
Total cholesterol, mg/dl	95.79	97.79	93.71	95.86	0.942	0.517	0.651	0.970	0.153
LDL, mg/dl	26.50	25.99	23.81	24.27	0.901	0.679	0.284	0.791	0.602
AST, IU/l	390.21	366.64	403.57	328.57	13.288	0.206	0.212	0.330	0.147
ALT, IU/l	5.500	4.429	4.214	4.071	0.548	0.799	0.372	0.679	0.876
GGT, IU/l	24.43	24.50	24.14	21.43	0.617	0.237	0.092	0.258	0.725

No significant differences among groups.

Table 5. Effects of dietary red ginseng root powder supplementation on some hematological parameters on day 38 in broilers.

	Red ginseng root powder, mg/kg				SEM	P-value			
	0	75	150	225		Combined	Linear	Quadratic	Cubic
RBC, 10^6/µl	3.236	3.297	3.291	3.420	0.084	0.893	0.481	0.846	0.795
WBC, 10^3/µl	162.05^b	163.25^b	189.74^a	199.42^a	3.833	<0.001	<0.001	0.514	0.150
Hematocrit, %	30.54	31.21	32.62	29.03	0.562	0.150	0.526	0.058	0.246
Haemoglobin, g/dl	7.169^b	7.386^b	7.696^b	8.855^a	0.152	<0.001	<0.001	0.071	0.512
Lymphocytes, %	56.82	55.55	59.43	54.07	1.337	0.551	0.718	0.452	0.238
Neutrophils, %	35.38	37.49	35.36	40.21	0.763	0.074	0.065	0.355	0.093
MCHC, g/dl	23.33^b	24.79^b	24.58^b	33.38^a	0.798	<0.001	<0.001	0.004	0.053
PDW, %	15.83	15.39	15.56	16.57	0.194	0.143	0.167	0.06	0.886
RDW, %	10.34^c	11.36^{bc}	12.31^b	15.51^a	0.348	<0.001	<0.001	0.026	0.280

n=14, ^{a,b,c}: Means within a row followed by the different superscripts differ significantly (P<0.05).
RBC: Erythrocytes, Red Blood Cells, WBC: Leukocytes, White Blood Cells, MCHC: Mean Corpuscular Haemoglobin Concentration, RDW: Red Blood Cell Distribution Width, PDW: Platelet Distribution Width

Table 6. Effects of dietary red ginseng root powder supplementation on hemagglutination inhibition levels (ND antibody level) on day 28 and 38 in broilers.

Day	Red ginseng root powder, mg/kg				SEM	P-value			
	0	75	150	225		Combined	Linear	Quadratic	Cubic
28 (n=7)	4.29	4.71	4.71	4.43	0.174	0.789	0.793	0.334	0.930
38 (n=14)	2.64	2.79	3.00	2.71	0.116	0.733	0.686	0.367	0.590

No significant differences among groups.

Table 7. Effects of dietary red ginseng root powder supplementation on caecum microflora (log10 cfu/g) on day 38 in broilers.

Microorganism	Red ginseng root powder, mg/kg				SEM	P-value			
	0	75	150	225		Combined	Linear	Quadratic	Cubic
Coliform	7.19	6.86	7.08	7.04	0.139	0.872	0.850	0.607	0.530
Lactobacillus spp.	6.49^{b}	7.14^{a}	6.75^{ab}	6.68^{ab}	0.072	0.010	0.768	0.010	0.026
Total aerobic bacteria	7.80	7.62	7.79	7.70	0.063	0.738	0.818	0.750	0.297

n=14, ^{a,b}: Means within a row followed by the different superscripts differ significantly (P<0.05).

Discussion and Conclusion

Dietary red ginseng root powder supplementation at the level of 75, 150 and 225 mg/kg did not affect body weight, body weight gain, feed intake and feed conversion ratio during the 38 day of the experiment. Similar to the present results, supplementation of 0.5 and 1% Sibirya ginseng leaf (37), 5% panax ginseng leaf (23), 1, 2 and 4 g/kg ginseng plant extract (3) did not affect body weight and body weight gain. Özcan (34) reported that body weight and body weight gain in Japanese quails were not affected with the dietary supplementation of 5 and 10 mg/kg panax ginseng root extract. According to the study of Yan et al. (43), body weight gain during weeks 3 to 5 and overall 5 weeks were improved with the dietary treatment of wild-ginseng adventitious root meal at 0.1%, but did not differ at the level of 0.2 and 0.3%. Muwalla and Abuirmeileh (32) reported that dietary supplementation of 0.25% Panax ginseng powder increased body weight gain.

Feed consumption was decreased with 5% Panax ginseng leaves (23) and not affected with the dietary usage of 0.5 and 1% Sibirya ginseng leaves (37) and 1, 2 and 4 g/kg ginseng plant extract (3). Similarly feed conversion ratio was not affected from the usage of 5% panax ginseng leaves (23), 0.2 and 0.3% wild-ginseng root meal (43), 1, 2 and 4 g/kg ginseng plant extract (3) and 2% fermented ginseng marc (8). However 1% Sibirya ginseng negatively affected feed conversion (37).

Chung and Choi (8) reported that no significant differences between the different treatments (basal diet, 2% red ginseng marc, 1% fermented red ginseng marc with red koji and 2% liquid red ginseng) were found for final body weight, feed intake and feed conversion. Chung and Choi (8) also concluded that weight gain and mortality was most enhanced in the groups fed diets supplemented with 1% fermented red ginseng powder combined with red koji.

The performance of broiler was also evaluated in terms of EPEF which includes body weight and livability. No significant effects of dietary red ginseng root powder supplementation were observed in livability and EPEF in the present experiment. Similar result was obtained in laying hens (44).

Dietary ginseng supplementation didn't affect the relative weight percentages of liver, heart, bursa Fabricius, gizzard and spleen on day 28 (Table 3). Carcass yield and the relative weight percentages of liver, heart, bursa Fabricius and abdominal fat was not affected by ginseng plant supplementation at the end of this experiment (Table 3). However the relative weight percentages of spleen and gizzard on day 38 were increased (P<0.05). Linear increases (P<0.05) were seen in the relative weight percentages of spleen and gizzard with an increase in the doses of red ginseng root powder. Measurement of immune organ weight is a method to determine the immune status of birds (15). For optimum immunoglobulin synthesis, the development of these organs are very important (13). In the present experiment relative spleen weight also increased with 225 mg/kg ginseng root extract supplementation and it was also observed that linear effects were seen with the dose of ginseng root extract. In the study of Yan et al. (43), 0.1, 0.2 and 0.3% wild-ginseng root meal (WGM) supplementation did not affect the relative weight of liver

and gizzard however relative weight of bursa fabricius increased in the groups supplemented 0.2 and 0.3%, relative spleen weight increased with all of dietary WGM and relative abdominal fat was decreased with 0.3% WGM supplementation. Similar to the present result, Kim et al. (23) indicated that 5% Korea Panax ginseng leaves did not affect carcass yield. In the study of Ao et al. (3), dietary supplementation of 1, 2 and 4 g/kg ginseng plant extract did not affect the weights of liver, heart, gizzard and abdominal fat but increased the weights of spleen and bursa Fabricius. Sohn et al. (37) reported that 0.5 and 1% Sibirya ginseng increased bursa Fabricus weight but did not affect the liver and spleen weight.

In the present study, ginseng root powder addition had no significant effect on blood serum biochemical parameters on day 28 and 38 (Table 4). A quadratic relationship of dietary ginseng root powder level was seen ($P<0.05$) with total cholesterol level on day 28. Similarly, some researchers (3, 14, 44) reported that ginseng root powder did not affect serum triglyceride and cholesterol levels. However some researchers (32, 35) reported that dietary ginseng supplementation impaired avian hepatic cholesterogenesis and therefore reduced serum total cholesterol and LDL cholesterol levels in avian species. Qureshi et al. (35) also indicated that ginseng supplementation reduced the β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) reductase activity and cholesterol 7α-hydroxyylase activity when compared with a diet without ginseng and reported that ginsenoside (saponins) are the bioactive agents for the suppression of cholesterogenesis and lipogenesis. Some researchers (18, 26, 34, 43) also found that serum triglyceride and cholesterol levels decreased with ginseng plant. Kang and Joo (20) concluded that ginseng saponin will partly recover the inhibited LDL biosynthesis in rabbits fed high cholesterol diet. The solubilizing effect of the saponin might stimulate the removal of lipids from the blood (19).

In a study with Japanese quails (33), Panax ginseng powder extract supplementation increased total protein, ALT, AST levels and did not affect urea, creatinine in serum of broilers. Ginseng plant supplementation increased AST, ALT and GGT levels in rats (22), did not affect AST and ALT levels in laying hens (21). Sohn et al. (37) reported that supplementation of 0.5 and 1% Sibirya ginseng leaf did not affect AST, ALT, albumin, total protein and increased triglyceride, cholesterol and glucose in blood serum. Similarly to the present study, Song et al. (38) concluded that supplementation of ginsan, polysaccharide isolated from the root of *Panax ginseng* C.A. Meyer, did not affect serum AST and ALT activities and albumin levels.

In the present study, ginseng plant supplementation did not affect erythrocytes (red blood cells, RBC), hematocrit, lymphocyte and platelet distribution width (PDW) and increased haemoglobin, leukocytes (white blood cells, WBC), red blood distribution width (RDW) and mean corpuscular haemoglobin concentration (MCHC) ($P<0.001$). A linear relationship of dietary red ginseng root powder level was seen with WBC, haemoglobin, MCHC and RDW (Table 5). These results show that Panax ginseng supplementation strengthen immune cellular defences of the organism (41). Şimşek et al. (41) reported that haemoglobin concentrations, RBC counts, WBC counts and lymphocyte numbers significantly increased with Panax ginseng supplementation to drinking water for 30 days in rats. Ao et al. (3) and Yan et al. (43) indicated that ginseng supplementation did not affect RBC and WBC counts and increased lymphocyte levels ($P<0.05$). In the study with laying hens (26), WBC, hematocrit and haemoglobin levels were not affected and RBC count was increased with ginseng supplementation ($P<0.05$).

In the present study, there were no differences among groups in hemagglutination inhibition levels (Table 6). This result is consistent with the findings of Catalan (6). Zhai et al. (45) reported that significantly increased serum heamagglutination inhibition titers against Newcastle disease virus when chickens were intranasally immunized with live Newcastle disease vaccine after drinking water supplemented ginseng stem-leaf saponins at the dose of 2.5 to 10 mg/kg for 3 d. Zhai et al. (46) indicated significantly increased serum hemagglutination inhibition titers against Newcastle disease virus and avian influenza virus when chickens were intramuscularly injected with inactivated Newcastle disease or avian influenza vaccines following drinking water supplemented 5 mg/kg ginseng stem-leaf saponins for 7 d. Zhai et al. (47) demonstrated that ginseng stem-leaf saponins provided a better protection against virulent infectious bursal disease virus (IBDV) challenge following vaccination than the control. Zhai et al. (47) concluded that oral administration of ginseng stem-leaf saponins enhances both humoral and gut mucosal immune responses to IBDV and offers a better protection against virulent IBDV challenge. According to Zhai et al. (47), the usage of ginseng saponins might be a potential oral adjuvant for vaccination against infectious diseases in the poultry industry. Kang et al. (21) indicated that red ginseng by-products can be utilized as an immunostimulant for laying hens. Different results can be due to the different adjuvant effects of saponins produced from ginseng plants.

In the current study dietary ginseng root powder supplementation increased the count of *Lactobacillus spp*. ($P<0.05$) and did not affect total aerobic bacteria and coliform number in caecum of broilers on day 38 (Table 7). Quadratic and cubic relationships of ginseng root

powder level were seen with the count of *Lactobacillus* spp. (P<0.05). Hassan et al. (14) observed that dietary fermented ginseng byproducts reduced *E. coli* and *Salmonella* in ileum but did not affect yeast and *Lactobacillus* spp. in ileum of broilers. Kang et al. (21) reported that the concentration of *Lactobacillus* was greater in the red ginseng byproduct groups than that of control group. The concentrations of *Salmonella* and *E.coli* in the caecum were not affected by inclusion of red ginseng byproducts in laying hens (21). From the results of present study and some literatures, ginseng plant may contribute to increase the number of *Lactobacillus* within intestinal microflora. *Lactobacillus* spp. uses carbohydrates such as inulin and oligofructose and evaluates fructoligosaccharides from these carbohydrates better than other group bacteria in terms of fermentation. *Lactobacillus* spp. is an indicator microorganisms for flora that mediates good digestive tract functions in poultry, these microorganisms produce short-chain fatty acids that form an acidic environment that suppresses the development of bacteria that emit stinking. In addition, *Lactobacillus* species are thought to inhibit *E. coli* toxic amin synthesis by secreting antienterotoxins against *E. coli*. Several ginseng constituents have been deemed responsible for the antimicrobial property of ginseng (42).

Different results can be due to the diet composition, ginseng sources, ginseng species, dosage of ginseng and biological active materials of ginseng.

As a conclusion, improvements in relative weight percentages of spleen and some hematological parameters with the addition of 225 mg/kg and in *Lactobacillus* spp. count in caecum with the addition of 75 mg/kg red ginseng root powder were provided in broilers. Further studies are required to determine the effects of its various doses on performance, immunity, antioxidant potential and intestinal microflora under various stress conditions in poultry.

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Ethical Statement

This study was approved by the Ankara University Animal Research Ethics Committee (2015-4-71).

Conflict of Interest

The authors declared that there is no conflict of interest.

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Investigation of the effectiveness of dehydrated corneal collagen barriers on corneal defects: An experimental rabbit model

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Abstract: The aim of this study was to evaluate the effect of collagen shield on epithelial wound healing in rabbit eyes. Adult New Zealand Albino rabbits were used in the study. All surgical procedures were carried out under general anesthesia. Superficial keratectomies of 6 mm in diameter were created in 40 eyes of 20 rabbits and they were separated into 3 groups as the control (CN), medical treatment (CA) and collagen barrier (CB) groups. In the CN group, 6 rabbits received 0.9% NaCl drops. In the CA group, 7 rabbits received ciprofloxacin and acetylcysteine. In the CB group, a collagen shield was placed on corneal defect for 72 hours in 7 rabbits. Central corneal thickness was measured using an ultrasound pachymeter. Corneal thickness was determined before and at 72 and 96 hours after surgery. There was a significant increase (CA group: $P<0.01$, CB group: $P<0.001$) in corneal thickness at 72 hours. The wound size was evaluated immediately after the surgery, then at 72 and 96 hours. There was a significantly greater healing response in the collagen shield group ($P<0.001$) compared to the other groups. The earlier wound closure in the CB group may be due to protection and lubrication of the epithelial cells in the margins of the fresh wound. These findings suggest that the collagen shield may be useful when treating corneal surface conditions in which de-epithelialization is a component.
Keywords: Collagen barrier, collagen shield, cornea wound, rabbit.

Kornea yaralarında dehidre korneal kollajen bariyerlerinin etkinliğinin araştırılması: Deneysel tavşan modeli

Özet: Çalışmada tavşanlarda korneal yaraların iyileşmesinde korneal kollajen bariyer etkinliğinin araştırılması amaçlandı. Çalışmada yetişkin Yeni Zellanda Beyaz Tavşanı kullanıldı. Tüm cerrahi prosedürler genel anestezi altında gerçekleştirildi. Yirmi adet tavşanın 40 gözünde biyopsi kornea merkezinde 6 mm çapında süperfisiyal keratektomi yapıldı. Tavşanlar; kontrol (CN), medikal tedavi (CA) ve kollajen bariyer (CB) olmak üzere 3 gruba ayrıldılar. Kontrol grubunda 6 tavşana %0,9 NaCl damlatıldı. Medikal tedavi grubunda 7 tavşana siprofloksasin ve asetilsistein damla uygulandı. Kollajen bariyer grubunda ise 7 tavşanda kornea üzerindeki yarayı kapatacak şekilde 72 saatlik kollajen bariyer yerleştirildi. Merkezi kornea kalınlığı ultrasonografik pakimetre kullanılarak ölçüldü. Kornea kalınlığı ameliyattan önce ve sonra 72 ve 96. saatlerde ölçüldü. Yetmiş ikinci saate kornea kalınlığında önemli düzeyde bir artış belirlendi (CA group: $P<0,01$, CB group: $P<0,001$). Lezyonlu bölgenin belirlenmesi amacıyla kornea hasarı oluşturulduktan sonra 0, 72 ve 96. saatlerde ölçüm yapıldı. Kollajen bariyer kullanılan gruptaki iyileşme düzeyinin ($P<0,001$) diğer gruplara kıyasla iyi olduğu belirlendi. Hızlı iyileşmenin; kollajen bariyerlerin yapısı gereği epitel göçüne engel olmaması, korneayı nemli tutması ve iyileşmeye destek olması gibi özelliklerinden kaynaklı olabileceği düşünülmektedir. Bu bulgular ışığında kollajen bariyerler, kornea hasarlarında epitelizasyonun sağlanmasında etkili ve kullanışlı bir sağaltım aracı olarak değerlendirilebileceği düşünüldü.
Anahtar sözcükler: Kollajen bariyer, kollajen kalkan, kornea yarası, tavşan.

Introduction

In the 1970s, collagen was applied as a therapeutic agent for wound healing in skin burns and ulcers (33). Collagen barriers developed in 1980 was used as a corneal bandage after radial and laser keratotomy operations (18). Rigid polymethylmethacrylate scleral contact lenses (28) and hydrogel bandage contact lenses have been used in a variety of clinical applications (9).

Eyelid movements prevent epithelial cell migration. Collagen barriers act as a barrier on the surface of the cornea, accelerates the migration of epithelial cells adjacent to the laceration area, reduces the healing time and may reduce stromal edema and leukocyte infiltration. Due to its transparency, it can slightly decrease vision (30).

Collagen barriers are used for ocular surface protection following surgical operations and traumatic and non-traumatic corneal wounds. Collagen shields have always been recommended for the use of collagen barriers in clinical ophthalmological practice (34).

Antibiotics are widely used usually as drops in corneal wounds, but they can be cytotoxic, delay epithelization and cause hyperemia and chemosis on the ocular surface. Furthermore, they can also induce bacterial resistance (3, 31).

This study aimed to investigate the efficacy of collagen barrier in the early healing phase of aseptic corneal wounds without antibiotic use.

Materials and Methods

The study material consisted of 20 adult New Zealand white rabbits, each weighing 2-3 kg. The rabbits were maintained at 23±5 ºC (relative humidity 60±10%) with food and water ad libitum. All rabbits were kept under observation for 3 weeks prior to the experimental study. After thorough physical and ophthalmological examinations animals without any health problems were included in the study.

The study was conducted in the Small Animal Clinics of the Department of Surgery of Veterinary Faculty of Aydın Adnan Menderes University with the approval of ADÜ - HADYEK No. 64583101/028.

Corneal thickness measurement: Ultrasonographic pachymeter (Pocket II, Quantel) medical was used to measure corneal thickness (Figure 1) and the corneal thickness was measured 4 times, i.e. before the lesion was created, and at 0, 72 and 96 hours after. The measurement result was obtained by averaging 5 measurements and the measurement records were transferred to the computer.

Fluorescein test: The corneal wounds were visualized using fluorescein and photographed at 0, 72 and 96 hours. Fluorescein sodium (Fluorescite 10% 100 mg / ml 5 ml ampoule, Alcon, USA) was dropped into the eye, and then washed with physiological saline (0.9% NaCl 500 ml solution, Biofleks pvc bag). The photograph was taken under cobalt blue light applied through slit-lamp (Kowa, SL-15 portable slit-lamp). A ruler was used for each measurement.

Anesthesia: The rabbits were anesthetized by intramuscular injection of xylazine hydrochloride (5 mg / kg Xylazinbio 2% 50 ml, Bioveta, Czech Republic) and ketamine hydrochloride (35 mg/kg mg/kg Alfamine 10% 10 ml, Alfasan, The Netherlands). Oxybuprocaine hydrochloride (Oxybuprocaine, 4.5 mg / ml, 10 ml, Benoxinate, Liba) was used as a local anesthetic.

Surgical procedure: The ocular surface was cleaned with 3% boric acid solution, the eyelids speculum was applied and a circular shaped defect of 6 mm in diameter was established on the center of the cornea each rabbit using biopsy punch, corneal blade (MVR 19 gauge) and Alger Brush under sterile conditions (Figure 2).

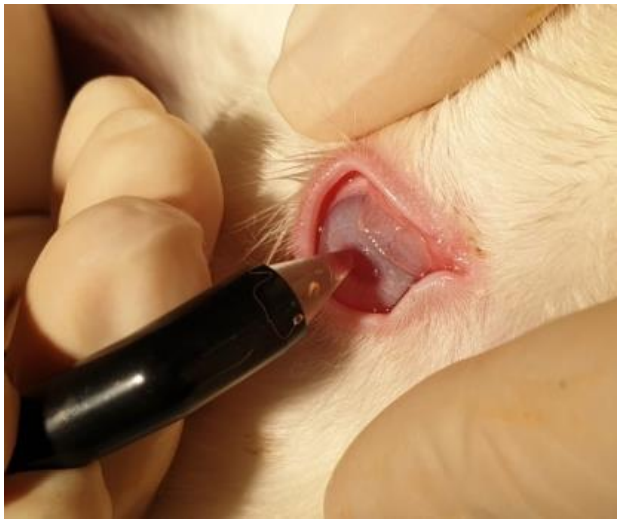

Figure 1. Measurement of corneal thickness.

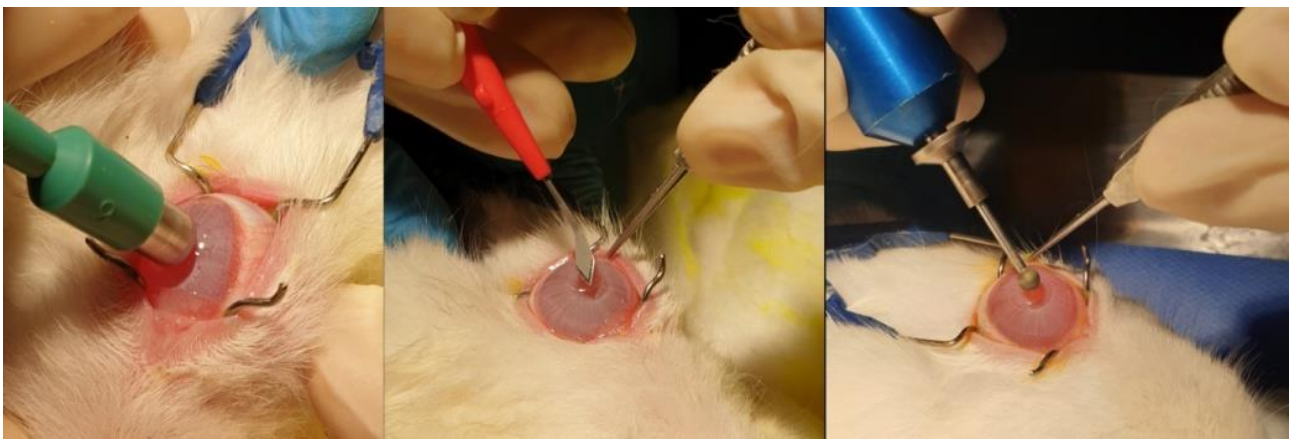

Figure 2. Making a circular defect area using 6 mm diameter biopsy punch, corneal blade (MVR 19 gauge) and Alger Brush.

Figure 3. The appearance of the lesion created in the center of the cornea and fluorescein test after surgical procedure.

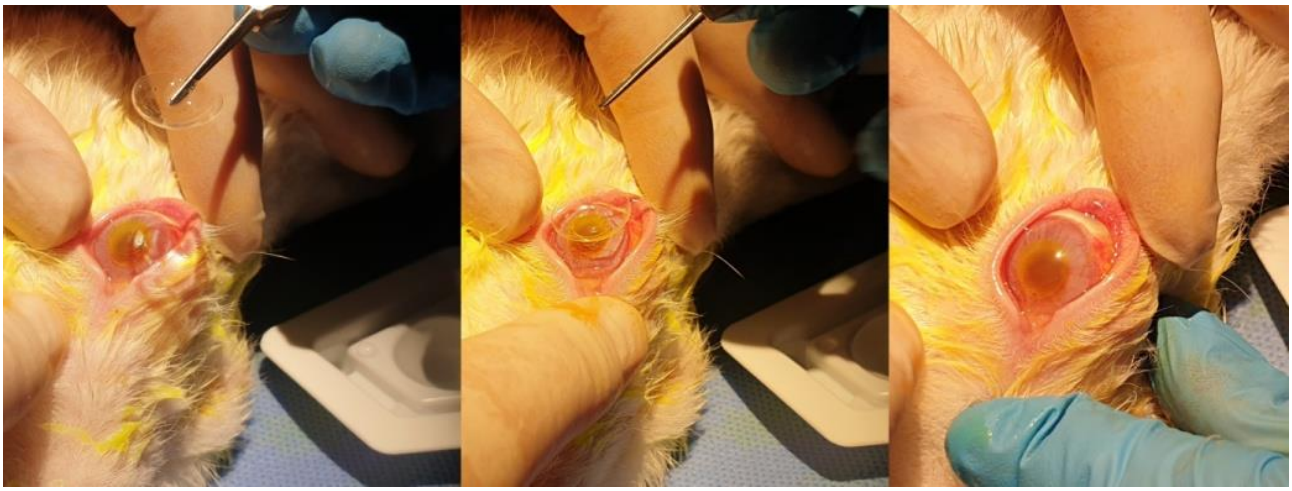

Figure 4. Placing the collagen barrier on the eye.

Fluorescein test: The corneal wounds were visualized using fluorescein and photographed at 0, 72 and 96 hours. Fluorescein sodium (Fluorescite 10% 100 mg / ml 5 ml ampoule, Alcon, USA) was dropped into the eye and washed with physiological saline (0.9% NaCl 500 ml solution, Biofleks PVC bag). The photograph was taken under cobalt blue light through slit-lamp. A ruler was used for each measurement (Figure 3).

Experimental design: The corneal lesion was created in both eyes of the rabbits as described previous reports (4, 7) because the traumatic lesion in the experimental model did not cover the whole eye and was not large enough to prevent the vision. So fewer rabbits were included in the study. During the trial period, all the animals had free access to food and water and the corneal defect did not cause any apparent discomfort. The rabbits were randomly assigned to the following groups: 1) CN group: 12 eyes of 6 rabbits received 0.9% NaCl drops 4 times a day, 2) CA group: 14 eyes of 7 rabbits received ciprofloxacin (4 drops a day, Ciloxan 0.3% 5 ml sterile ophthalmic solution, Novartis, Belgium) and acetylcysteine (2 drops a day, Brunac 5%, 5 ml sterile eye drops, Bio-Gen, Italy), 3) CB group: In all 7 rabbits, a 14.5 mm collagen shield (Vet Shield, OASIS® 72, ABD) was wet with saline and placed on the corneal defect (Figure 4).

At the end of the study, all rabbits were sterilized and owned as pet animals after they were fully healed.

Calculation of defect area: The photographs obtained from the corneal defects were transferred to a computer and the defect area was measured using the polygon method and was recorded in mm^2 using an image processing software (Image J 1.52a, Wayne Rasband National Institutes of Health, USA) (Figure 5).

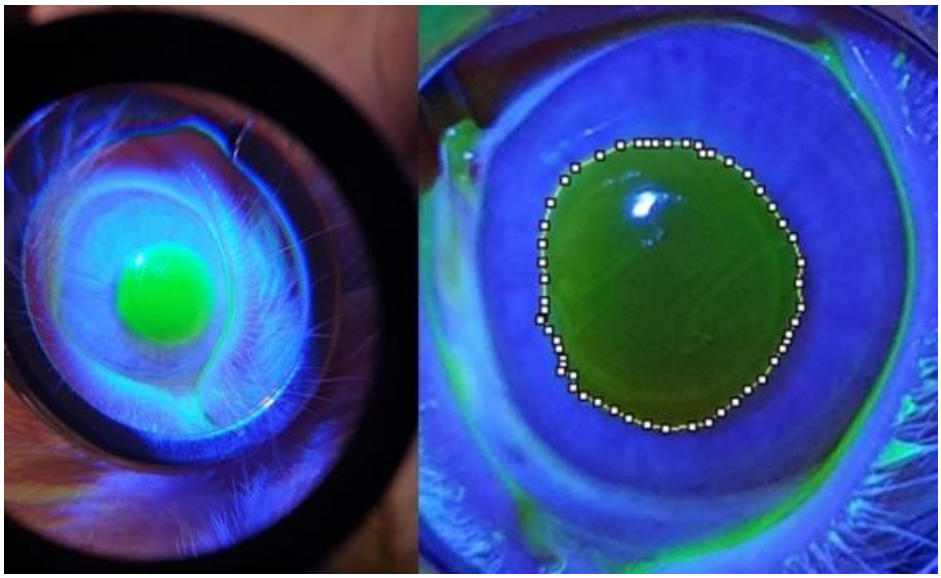

Figure 5. Measuring the lesion using Image J polygon method.

Table 1. The mean values of the corneal thickness according to time (µm, mean ± standard error).

	Groups					
	CN		CA		CB	
Hours	**n**	**$\bar{X}$± $S_{\bar{x}}$**	**n**	**$\bar{X}$± $S_{\bar{x}}$**	**n**	**$\bar{X}$± $S_{\bar{x}}$**
Preoperative	12	413.36±14.94	14	375.14±10.58^{a}	14	382.21±14.06^{ac}
0	12	424.18±27.66	14	388.71±14.46^{ac}	14	368.50±12.87^{a}
72	12	471.73±28.30	14	446.86±20.32^{b}	14	468.29±18.33^{b}
96	12	445.73±27.54	14	418.214±13.94^{c}	14	419.79±16.84^{c}
P				**		***

n: Number of data; a, b, c: The difference between times in the same column is statistically significant.
**: P <0.01; ***: P <0.001

Statistical analysis: Data obtained in the study were analyzed statistically using SPSS 22 (Statistical Package for Social Sciences) software. Sample size estimation determined achieves a power of 0.81. The Shapiro-Wilk and Levene tests were used to evaluate the assumptions of normal distribution and homogeneity of variances. Descriptive analyses were presented using mean ($\bar{X}$) and standard error ($S_{\bar{X}}$) values. Repeated measures ANOVA was used to determine the statistical significance of time distribution. Post-hoc comparisons were made using Duncan's test. A value of P<0.05 was regarded as statistically significant.

Results

Photophobia and epiphora ranging from mild to moderate were observed in all rabbits on the postoperative first day and these signs were not detected on the postoperative second day. Menace response, pupillary light, and dazzle reflexes were recorded as positive.

Mean corneal thicknesses are shown in Table 1. At 72 hours, corneal thickness increased in all groups compared to the first postoperative measurement (Figure 6). No significant difference was observed between the groups at the 72nd and 96th hours. Statistical differences were determined in the corneal thickness measurements at the different times in the CA (P<0.01) and CB groups.

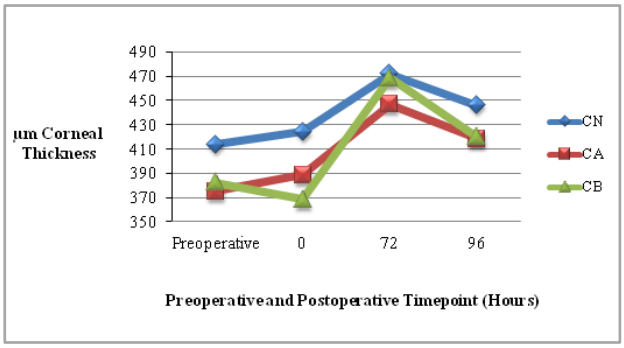

Figure 6. Change of corneal thickness obtained in groups over time.

Mean corneal defect areas are shown in detail in the tables (Tables 2-3). Mean corneal defect areas were statistically different in all groups between 0, 72, and 96 hours. The defect size percentages for each group were averaged for each time point (Figure 7). There was a significantly greater healing response in the CB group than in the other groups (P<0.001) (Figure 8).

It was observed that in some rabbits corneal defects recovered fully with a resultant of fluorescein negative opacity at 72 and 96 hours (Figure 9).

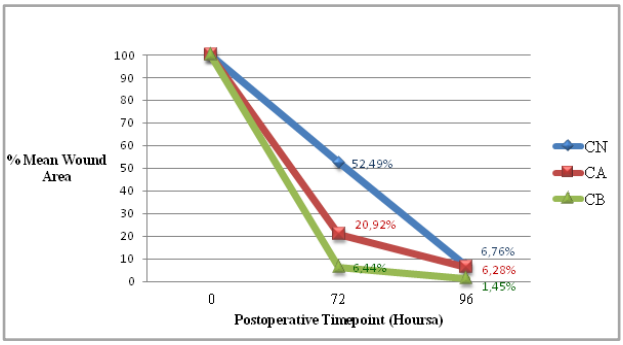

Figure 7. Mean wound areas (%) of the groups over postoperative time.

Table 2. Change of the defect areas formed in the cornea according to time (mm^2, mean ± standard error).

		Groups				
		CN		CA		CB
Hours	**n**	$\bar{X}\pm S_{\bar{X}}$	**n**	$\bar{X}\pm S_{\bar{X}}$	**n**	$\bar{X}\pm S_{\bar{X}}$
0	12	32.129 ± 1.58^{a}	14	31.41 ± 1.33^{a}	14	29.01 ± 0.92^{a}
72	12	16.87 ± 2.25^{b}	14	6.57 ± 1.63^{b}	14	1.87 ± 0.66^{b}
96	12	2.17 ± 0.46^{c}	14	1.97 ± 0.48^{c}	14	0.42 ± 0.18^{b}
P		***		***		***

n: Number of data; a, b, c: The difference between times in the same column is statistically significant.
**: P <0.01; ***: P <0.001.

Table 3. Change of defect areas formed in the cornea over time (mm^2, mean ± standard error).

		Hours		
		0	**72**	**96**
Groups	**n**	$\bar{X}\pm S_{\bar{X}}$	$\bar{X}\pm S_{\bar{X}}$	$\bar{X}\pm S_{\bar{X}}$
CN	12	32.129 ± 1.58	16.87 ± 2.25^{a}	2.17 ± 0.46^{a}
CA	14	31.41 ± 1.33	6.57 ± 1.63^{b}	1.97 ± 0.48^{a}
CB	14	29.01 ± 0.92	1.87 ± 0.66^{c}	0.42 ± 0.18^{b}
P			***	**

n: Number of data; a, b, c: The difference between groups in the same column is statistically significant.
**: P <0.01; ***: P <0.001.

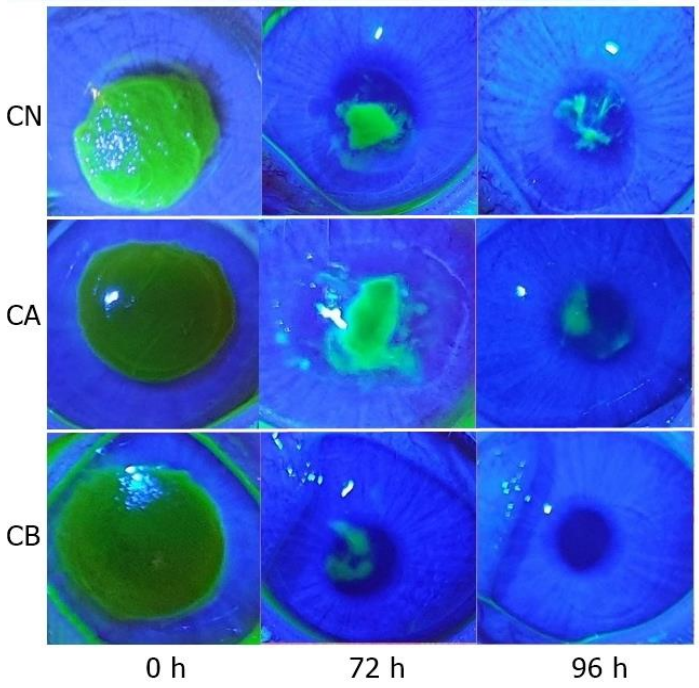

Figure 8. Corneal wound healing. CN: Control group; CA: Ciloxan and acetylcysteine group; CB: Collagen barrier; h: hour.

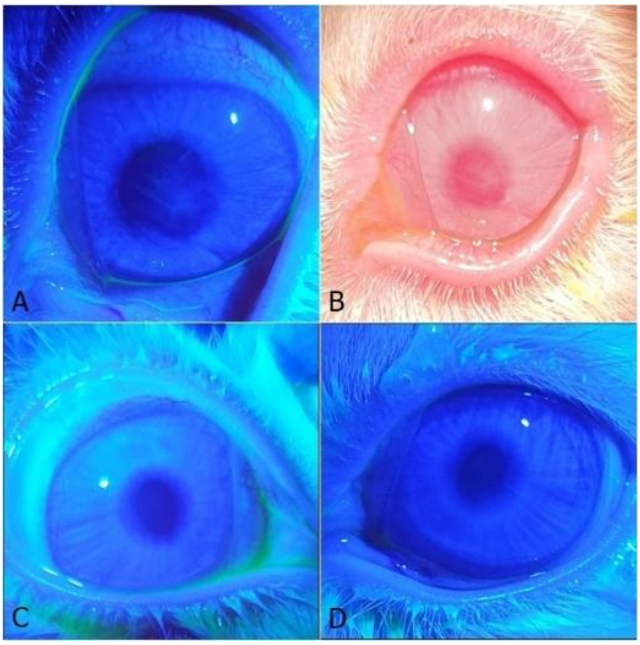

Figure 9. Corneal opacity, CN group, rabbit no 3, left eye, 96th hour (A and B). Healed cornea, CB group, rabbit no 4, right (C) and left (D) eye, 96th hour.

Discussion and Conclusion

Laboratory animals, such as rabbits, rats, and mice are widely used in ophthalmic research. The rabbit model is frequently preferred especially in experimental studies of the cornea (6, 8, 12, 15, 22). The presented study was planned to investigate the usability of the collagen barrier in veterinary medicine. Considering the indivisible structure of the collagen barrier, the rabbit eye was very suitable for the study.

There are different methods of creating corneal defects form linear to circular shape lesion (7, 15, 23, 24, 30). In the present study, the corneal epithelial defects were created with aids of punch biopsy, and cornea blade and Alger brush. This method was found to be cheap as well as simple, and especially the usage of the biopsy punch was quite beneficial and enables to create unique corneal defects in all subjects. Thus, it can be considered that this method will be preferred by researchers in future experimental studies.

Many studies (1, 23) showed that superficial corneal defects can recover within 48-72 hours due to epithelial proliferation and have also emphasized that the 72nd hour is critical in determining the morphological and pathological changes during the healing process of the cornea (2, 10, 15). In the current study, considering the previous studies, the defects induced were assessed using pachymeter and fluorescein and photographed at 0, 72 and 96 hours after the induction. These time intervals are found to be sufficient to monitor changes in corneal defect healing and corneal thickness.

In the literature, there are more data on rabbit corneal thickness. Researchers have reported the corneal thickness in rabbits to be between 338 and 430 µm at its center (29). Ultrasonographic pachymeter is generally used because it is cheap, easy to apply and repeatable. Contact with the cornea is the most important disadvantage (19). In this study, ultrasonographic pachymeter was applied by the same surgeon, and similar corneal thicknesses to those of previous studies were obtained and in this respect no difference between the groups (CN: 413.36; CA: 375.14 and CB: 382.21 µm) was also determined.

In the present study, corneal thickness reached the highest level at 72 hours and decreased the normal level at 96 hours. The increase in corneal thickness is thought to be the result of proliferation of epithelial cells at approximately 24 hours after injury, persists for up to 72 hours and returns to normal after the third day (10, 15). It has been reported that corneal thickness increased in measurements taken at 0, 15 and 30 minutes after removal of corneal epithelium (410.38 µm, 435.56 µm and 458.44 µm, respectively) (25). The changes in corneal thickness determined in the study are consistent with the literature data (10, 15). The increase in thickness is thought to be due to cell proliferation-migration and edema.

Despite many protection mechanisms, any trauma-related ocular surface damage or local or systemic immunosuppressions may induce the normal flora of the eye to become a source of infection. In treatment, it is necessary to select broad-spectrum antibiotics (17), thus when planning the experimental design, in CA group ciprofloxacin which is a widely used antibiotic in veterinary medicine was applied. No evidence of infection was observed in the CN group. In this case, it may be considered that antibiotics are not required in non-infectious, traumatic or non-traumatic cornea wounds.

Collagen-rich layers form the corneal tissue (21). Collagen, which has an important place in wound healing, collapses in corneal injuries due to its large concentration

in the cornea and prevents the destruction of deeper layers. Local collagenases which are released from the damaged epithelium, keratocytes, macrophages and polymorphonuclear leukocytes increase in corneal damage and cause severe destruction of collagen as well as the corneal structure. Acetylcysteine, which has an anti-collagenic effect and activates the formation of collagen fibrils in the treatment of corneal wounds (11, 20, 26) was used as 3% in addition to ciprofloxacin in the CA group. Previous studies in dogs have reported that this concentration alone is suitable for the treatment of different corneal lesions (11, 13). The healing rate was higher in the CA group compared to the CN group. It was used in combination with ciprofloxacin in the CA group. Therefore, the rate of recovery cannot be attributed just to the positive action of acetylcysteine.

The use of a bandage lens has long been the preferred method for the treatment of corneal wounds. In a normal open eye, the partial oxygen pressure in the tear film layer is 155 mm Hg, and while the eyelids are closed it reduces 40-50 mm Hg (14). Using lenses lowers oxygen pressure further and causes epithelial hypoxia, resulting in corneal neovascularization (27). Collagen barriers have sufficient oxygen permeability for the metabolism of the corneal epithelium compared to other lenses. In the study, no evidence of neovascularization was observed. Research on the use and effectiveness of dehydrated corneal collagen barrier is becoming increasingly widespread (20, 33). Infection is a potential complication in the use of hydrophilic lenses in patients with epithelial or stromal defects. The disposability and solubility of the collagen barriers eliminate this risk (14, 27). The present study was planned to investigate the potential benefits as well as the practical application of the collagen barrier alone in the early period (96 hours) for the repair of corneal defects compared to classical treatment. In the presented study, no infection and neovascularization were detected indicating that the collagen barrier does not have disadvantages seen in the other type lenses.

During the study period, it was observed that in CN and CA groups the repeated application of eye drops caused a loss of time and labor whereas in the CB group the collagen barrier had benefits of ease of application and time saving.

Collagen is preferred as a wound healing agent in wounds and ulcers and acts as a support for corneal epithelial cells. Collagen barriers have long been used in human medicine to protect the ocular surface in corneal lesions. Previous studies have shown that wound closure time was shorter in the collagen barrier groups compared to untreated control (7, 30). The studies on the effectiveness of collagen barriers and therapeutic contact lenses in mechanical post-corneal debridement have demonstrated that this barrier eases epithelial cells migrating to the region and also protect epithelial proliferation from the mechanical movements of the eyelid (7, 30, 32). Eshar et al. (5) reported that collagen barrier was successful in the treatment of a rabbit with a chronic bilateral ulcer. Collagen barriers are also used in surgically induced, traumatic or non-traumatic corneal wounds to protect the ocular surface. Some studies on rabbits showed that it increases re-epithelialization in epithelial defects and keratotomy wounds, keeps the cornea moist and reduces stromal inflammation and edema. Collagen barrier promotes stromal healing by limiting keratocyte loss and reducing potential subepithelial fibrosis (34). In a study of cats by Croix et al. (16) which used a 72-hour form of collagen barrier (Vet Shield, OASIS®), it was reported that the use of the collagen barrier after keratotomy increased the recovery rate.

In the present study, the classical treatment group (CA) and control group (CN) were compared with the collagen barrier group (CB). According to the fluorescein test performed at 72 hours, the defect areas were determined as 16.87 ± 2.25 mm^2 in the CN group, 6.57 ± 1.63 mm^2 in the CA group and 1.87 ± 0.66 mm^2 in the CB group. From these data, a statistically significant difference ($P<0.001$) was detected between the groups and this difference was found to be between CN and CB groups where the largest defect area was seen in the first and smallest in the later group. In the 96th hour measurements, defect areas were measured as 2.17 ± 0.46 mm^2 in the CN group, 1.97 ± 0.48 mm^2 in the CA group and 0.42 ± 0.18 mm^2 in the CB group. The size of defect reduction in the CB group was found to be significantly lower as compared to the CN and CA groups ($P<0.001$), which shows that the healing rate was faster in the CB group compared to the other groups (Figure 7 and Table 3). These findings show that the collagen barrier alone accelerates wound healing inconsistent with other clinical and experimental studies (5, 7, 16, 30, 32, 34).

In conclusion, the results of this study showed that collagen barriers support epithelial migration, reduce the corneal wound area, reduce keratocyte loss and sub-epithelial fibrosis, and promote stromal healing. It is clear that medical treatment of corneal injury is long-term and tiring for patients and animal owners. The collagen barrier can be applied readily, eliminating the need for frequent application of eye drops, which is especially important in busy owners and in aggressive animals. In cases of corneal ulcers/wounds, collagen barrier can be recommended as a practical and effective treatment tool instead of eye drops.

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Ethical Statement

This study was approved by Aydın Adnan Menderes University Animal Research Ethics Committee (ADÜ-HADYEK) (64583101/028).

Conflict of Interest

The authors declared that there is no conflict of interest.

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The effectiveness of intravaginal progesterone insert in Ovsynch and resynchronization protocols in cyclic and non-cyclic Holstein heifers

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Abstract: In the present study, it was aimed to compare of resynchronization with used progesterone (P4) releasing intravaginal device (PRID), with modified ovulation synchronization in cyclic and non-cyclic Holstein heifers. Animals were randomly divided into two groups; Group I (GI, n=87) and Group II (GII, n=88). Fixed-time artificial insemination (FTAI) following modified Ovsynch protocol with PRID insertion, was performed to all animals. In GI, used PRID was reinserted to the same animals, 15 days after the first FTAI (day 25) for 12 days. Pregnancies were detected by ultrasound in all groups on day 37. For resynchronization, prostaglandin (PG) $F_2\alpha$ was applied to non-pregnant animals on day 37. In GI, following 48 hours from $PGF_2\alpha$ injection, the gonadotropin-releasing hormone was administrated, and after 18 hours the second FTAI was performed. No additional treatments were applied to the non-pregnant animals found in GII, and heifers were taken to the routine service program. In both groups, the second pregnancy diagnosis was performed 27 days after inseminations by transrectal ultrasonography. Pregnancy rates (PR) following the first and overall inseminations in cyclic and non-cyclic heifers (GI and GII) were detected as 66.4% and 52.3% (P>0.05); 76.3% and 59.1% (P>0.05), respectively. In conclusion, the usage of PRID in the Ovsynch and resynchronization protocol resulted in higher PRs of non-cyclic heifers than cyclic heifers. Moreover, this study reveals that the initial P4 levels of non-cyclic animals should be used as a determinant for pregnancy success in dairy heifers.

Keywords: Heifer, pregnancy, progesterone, resynchronization.

Siklik ve siklik olmayan Holştayn düvelerde Ovsynch ve resenkronizasyon protokollerinde intravajinal progesteron ilavesinin etkinliği

Özet: Sunulan çalışmada, siklik ve siklik olmayan Holştayn ırkı düvelerde modifiye ovulasyon senkronizasyonu ve kullanılmış progesteron (P4) salan intravajinal araç (PRID) ile resenkronizasyonun etkinliğinin karşılaştırılması amaçlandı. Hayvanlar rastgele iki gruba ayrıldı; Grup I (GI, n = 87) ve Grup II (G II, n = 88). Tüm hayvanlara PRID eklenerek modifiye edilmiş bir Ovsynch protokolünü takiben sabit zamanlı suni tohumlama (SZT) yapıldı. Grup I'de, kullanılmış PRID, ilk SZT'dan 15 gün sonra (25. gün) 12 gün süreyle aynı hayvanlara tekrar yerleştirildi. Tüm gruplarda 37. günde ultrason ile gebelikler saptandı. Resenkronizasyon için, gebe olmayan hayvanlara 37. günde prostaglandin (PG) $F_2\alpha$ uygulandı. Grup I'de $PGF_2\alpha$ enjeksiyonundan 48 saat sonra gonadotropin salgılatıcı hormon uygulandı ve 18 saat sonra ikinci SZT yapıldı. Grup II' deki gebe olmayan hayvanlara ilave tedavi uygulanmadı ve düveler rutin tohumlama programına alındı. Her iki grupta da ikinci gebelik teşhisi SZT'dan 27 gün sonra transrektal ultrasonografi ile yapıldı. Siklik ve siklik olmayan düvelerdeki (GI ve GII) ilk ve tüm tohumlamaları takiben gebelik oranları sırasıyla %66,4 ve %52,3 (P>0,05); %76,3 ve %59,1 (P>0,05) olarak saptandı. Sonuç olarak, Ovsynch ve resenkronizasyon protokolünde PRID kullanımı, siklik olmayan düvelerin siklik olan düvelerden daha yüksek gebelik oranları ile sonuçlandı. Ayrıca, bu çalışma, siklik olmayan hayvanların başlangıçtaki P4 düzeylerinin, sütçü düvelerde gebelik başarısı için belirleyici olarak kullanılması gerektiğini ortaya koymaktadır.

Anahtar sözcükler: Düve, gebelik, progesteron, resenkronizasyon.

Introduction

Reproductive efficiency is one of the main determinants of economic benefit in dairy herds (24). It is reported that decrease in pregnancy rates (PR) heifers due to poor estrus behaviours, lower conception rates and high embryonic deaths (30). In addition, the time interval for pregnancy is getting longer because of inadequate managements in crowded herds and inefficient estrus detection (3, 6, 7). Various programs have been used for estrus synchronization in dairy cow and heifers (8, 18, 30). Thereby, estruses could be detected effectively and time spend for heat detection could be decreased in cows and heifers (18, 27, 31). Pharmacological control of estrus cycle to increase pregnancy rates has been achieved through synchronization of follicular development, control of corpus luteum regression and synchronization of ovulation (1, 15, 19, 20, 27, 28).

In current synchronization programs, no application has been considered for non-pregnant animals after insemination (2, 6, 24). Therefore, economical income obtained from non-pregnant animals is decreased. To overcome this issue, the resynchronization programs have been developed for recent years (2, 8, 13). Synchronization programs used for animals that are diagnosed as non-pregnant following the first insemination have been named as resynchronization programs (3) and various protocols were developed for resynchronization (6).

In resynchronization programs, it is aimed to initiate the subsequent cycle, regardless of whether animals become pregnant in the previous insemination (22). In the case of pregnancy, resynchronization is ceased. Otherwise, resynchronization processing is continued. Resynchronization protocols mainly include the use of progesterone (P4) and this hormone does not harm an ongoing pregnancy (14, 16). Indeed, P4 administration for resynchronization prevents embryonic deaths (10).

Nowadays, pregnancy rates following resynchronization protocols were insufficient in heifers. The objective of this study is to investigate the efficacy of the resynchronization program with the use of P4 based on pregnancy rates especially in dairy heifers. In synchronization protocols with the use of P4, P4 devices are discarded following synchronization. However, it has been demonstrated that PRID releases for at least 15 days which is longer than its usage in the vagina (10). Therefore, the second objective of this study is to investigate the effectiveness of the re-use of PRID for resynchronization process to increase PRs to the decrease cost of resynchronization, in heifer.

Materials and Methods

Animals: This study was carried out on healthy Holstein heifers (n=180) older than 15 months of age, and the body condition scores (12) all of the animals used were close to each other. All animals were fed and managed under the same conditions at the the Günta Agriculture and Livestock Inc. Kayseri, Turkey. Heifers were fed with alfalfa hay, corn silage and concentrated feed, accessed to water ad libitum basis in free-stall barns, throughout the study. All procedures on animals were carried out according to approval by the Local Ethics Committee for Animal Experiments of Erciyes Veterinary Medicine Faculty with the number of 2006/040 during this study.

Animals were randomly allocated to Group I (GI, Resynchronization; n=90) and Group II (GII, Control; n=90), respectively. The occurrence of oestrous cycles (cyclic vs. non-cyclic) before the onset of treatments was determined by measuring the P4 serum concentration level in the blood samples collected on day-10 and day 0. The detecting of serum P4 level as <1ng/ml in all animals at the days mentioned above was evaluated non-cyclic status. In addition, blood samples were collected from all heifers on day +7 and +10 to detect P4 concentration. All sera samples were stored at -20°C until measurements.

During the experimental period, three heifers in GI and two heifers in GII were excluded from the study due to the loss of their PRID.

Reproductive Management: In GI (n=87) and GII (n=88), intravaginal device (PRID; 1.55 g progesterone + 10 mg estradiol benzoate, CEVA-DIF, Turkey) was inserted at the time of the GnRH (Ovarelin; 50 µg/mL gonadoreline diacetate, CEVA-DIF, Turkey, 2 mL, IM) injection (day 0). $PGF_2\alpha$ (Estrumate; 250 µg/mL cloprostenol, CEVA-DIF, Turkey, 2 mL, IM) was administered on the day of PRID removal (day 7) and the second GnRH was injected 48 hours later (day 9). FTAI was performed to all animals, 18 hours after the second GnRH administration (day 10).

PRIDs removed from heifers in GI at day 7 were cleaned and disinfected with 0.1% chlorhexidine solution and kept individually in a refrigerator until they were re-used for resynchronization. Used PRIDs without estradiol capsule were reinserted to the same animals 15 days after the FTAI (day 25) and held during 12 days.

On day 37, pregnancies were detected by transrectal ultrasonography (Honda HS 1500V, Japan) equipped with a rectal linear probe (5 MHz), in all groups.

In GI, $PGF_2\alpha$ was applied to non-pregnant animals on day 37. The GnRH was administered 48 hours after $PGF_2\alpha$ injection (day 39), and the second FTAI was performed 18 hours following GnRH injection (day 40). No additional treatments were applied to the non-pregnant animals found in GII and heifers were taken to the routine service program. In GII, heat patches KAMAR (Rumitech, USA) attached to tail head were used to detect estrus. Heifers detected estrus in GII were inseminated after 12 hours following estrus detection.

In both groups, the second pregnancy diagnosis was performed 27 days after inseminations by transrectal ultrasonography.

Hormone Assays: The blood P4 levels were determined by ELISA described as previously (23). For sera P4 analyses, intra-experimental and inter-experiment variation coefficients were 0.09 and 0.14 for low control and 0.11 and 0.17 for high control, respectively.

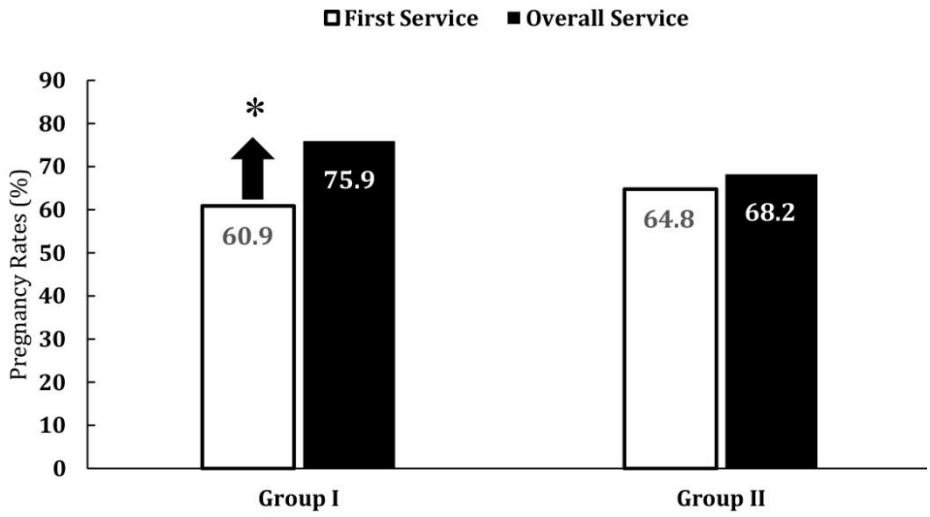
Statistical Analysis: Pregnancy rates and rate of non-cycling heifers between groups were analysed with the Chi-square test. The significance control of the differences in P4 levels between cyclic and non-cyclic heifers in GI and GII was performed using the Student T-test. The statistical significance control of the change in the PR in the first insemination according to the total PR was done with the Mantel-Haenszel test. NCSS 9.0 package program was performed in all statistical analyses.

Results

The rate of non-cycling heifers was numerically lower in GI (20.7%; 18/87) compared to that in GII (29.5%; 26/88) (P>0.05).

Pregnancy rates following the first insemination did not differ between GI (60.9%; 53/87) and II (64.8; 57/88) (P>0.05). The pregnancy rates in GI after the second FTAI were detected as 38.2% (13/34). Only four of 31 heifers were inseminated after the detection of estrus in GII. Three of four animals inseminated were diagnosed as pregnant (9.68%; 3/31). Pregnancy rates following the first and second inseminations (overall pregnancy rate) did not differ between GI (75.9%; 66/87) and GII (68.2%; 60/88) (P>0.05). Although the increase of between first and overall PRs were significant in GI (P=0.034); no significant result was detected in GII (P=0.634); (Figure 1).

The pregnancy rates of cyclic and non-cyclic animals in GI and GII were given in detail in Table 1. In non-cyclic heifers of GI and GII, the blood P4 levels at day -10 and day 0 were detected as 0.21±0.13; 0.35±0.26 ng/mL (P=0.038) and 0.18±0.19; 0.37±0.28 ng/mL (P=0.017), respectively. Progesterone concentrations of cyclic and non-cyclic animals in GI and GII at day -10, 0, +7 and +10 were summarized in Figure 2.

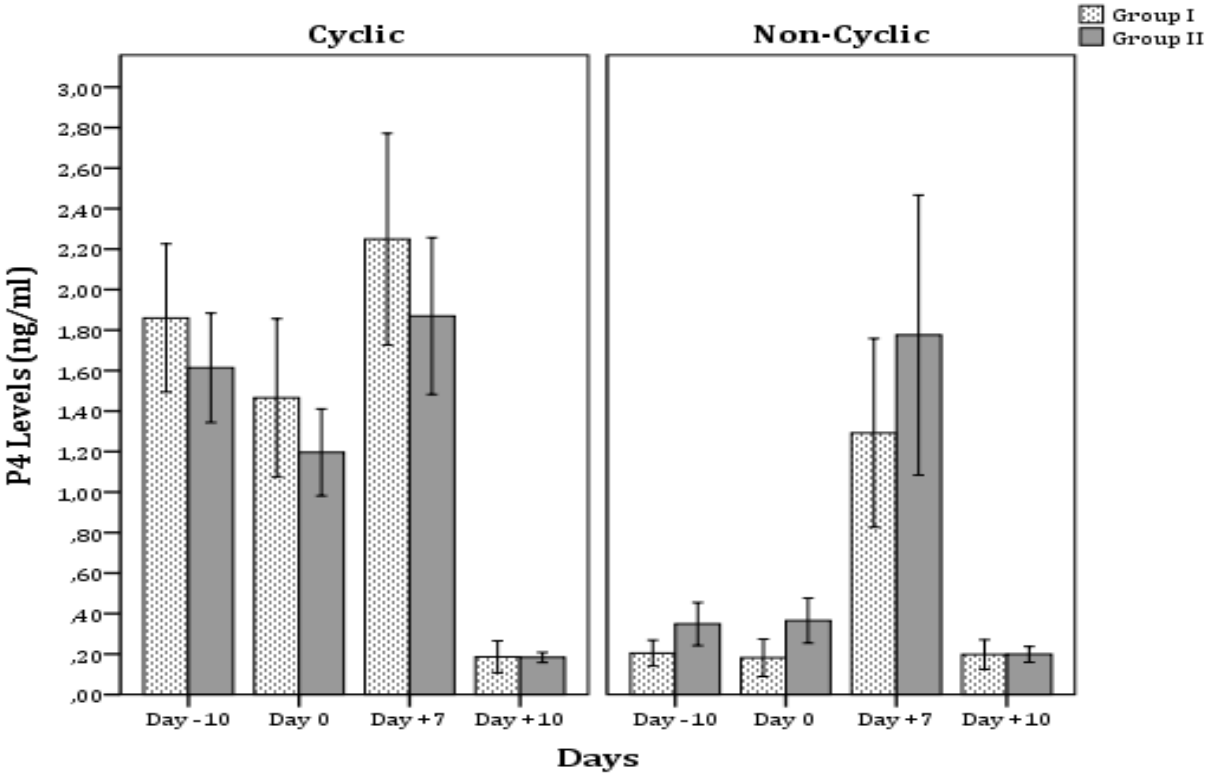


* The increase in the PRs within the group is statistically significant (P<0.05).

Figure 1. The PRs following first and overall services in both groups.

Table 1. The PRs after first and overall inseminations of cyclic and non-cyclic dairy heifers in and between GI and GII.

		Non-Cyclic		Cyclic		
	Groups	**Pregnant n (%)**	**Non-pregnant n (%)**	**Pregnant n (%)**	**Non-pregnant n (%)**	**P Value for Cyclicity**
First Insemination	GI	7 (38.9)	11 (61.1)	46 (66.7)	23 (33.3)	0.060
	GII	16 (61.5)	10 (38.5)	41 (66.1)	21 (33.9)	0.868
	P Values for Groups		0.241		0.948	
Overall Insemination	GI	9 (50.0)	9 (50.0)	57 (82.6)	12 (17.4)	0.010
	GII	17 (65.4)	9 (34.6)	43 (69.4)	19 (30.6)	0.909
	P Values for Groups		0.479		0.115	



In cyclic dairy heifers, no statistical difference between serum P4 levels in all days (P>0.05).
* In non-cyclic dairy heifers, the statistical difference between serum P4 levels at days -10 (P=0.021) and 0 (P=0.017) is significant.

Figure 2. The mean serum P4 levels (ng/mL) of cyclic and non-cyclic dairy heifers in GI and GII at days -10, 0, +7 and +10.

Discussion and Conclusion

The effect of cyclicity of beef and dairy heifers on the success of PRs obtained after modified ovulation synchronization (OVS) protocols have been recently questioned by some researchers (17). In accordance with the results of Lopez Helguera et al. (17), we found that the cyclicity of the dairy heifers also affects the success in modified synchronization protocols numerically.

While the average PRs of non-cyclic and cyclic animals were found as 53.27% and 66.41% following to first FTAI (P>0.05), the overall PRs were increased to 59.09% and 76.34%, respectively at the end of our study (P>0.05). The PRs obtained after the first FTAI with OVS (with P4 insertion) indicate that non-cyclic animals decrease the PRs in dairy heifers. Also, the overall PRs detected in GI indicate that the resynchronization of the heifers with used PRID after OVS increases the PRs in both non-cyclic and cyclic animals. As the results reported in a previous study (29), the overall PRs obtained from GI and GII presented that the application of the resynchronization protocol let us have an economically acceptable value than routine service programs in our study. This result also revealed that resynchronization using P4 is an effective method to increase PRs in non-cyclic animals.

In non-cyclic heifers, it's also so interesting that while the blood P4 levels of GI at day -10 and 0 were detected lower than those of GII (P<0.05), the PRs of GII were higher than that of GI (P>0.05). Considering these results together, in non-cyclic animals, even if the P4 levels were below 1 ng/mL at the beginning of OVS affect the PRs. The data obtained in this study were considered to be compatible with the studies conducted previously (4, 5) in which it was reported that the level of P4 was important before and during synchronization applications in terms of follicular development, ovulation, and embryonic development in cows. Since the number of relevant literature is limited in dairy heifers compared with dairy cows, therefore it is thought that the results of the current study were important.

Moreover, it's obvious that the numerical differences of PRs between GI and GII could be interpreted in consequence of the P4 administration in resynchronization protocol. Also, the overall PRs obtained in the study reveal that the negative effect of heifers' cyclicity at the beginning of the study could be decreased to an acceptable level by resynchronization with P4. The results that reported by Stevenson et al. (29) go along with our results.

The PRID is generally used once in animals and the duration of use varies between 5 and 12 days in field

conditions. However, it has been reported that short term used PRIDs could be re-used (11, 33). Our study showed that re-using of the PRIDs for 19 days was found to be successful in PRs obtained. In addition to all these, it was demonstrated that the resynchronization protocol used had advantages such as inseminating 2 times in a short period of 40 days in heifers.

Moreover, the expression of estrus in some non-pregnant heifers following the Ovsynch protocol integrated with P4 administration in this study could be due to the induction of cyclicity in those heifers previously non-cycling. Similar findings were reported by Chebel et al. (7).

Pregnancy rates obtained following the first insemination in this study were slightly higher than the results of previous reports in dairy (58.7%; (28)) and beef (44.1%; (20)) heifers following the Ovsynch protocol with P4 administration. It has been reported that PRs could be differ due to the cyclicity status of animals and the stage of the estrous cycle at the beginning of the synchronization protocols (26, 28). The present study revealed that serum P4 levels in non-cyclic heifers at the beginning of the study also affected PRs. This slightly higher PR in the current study could be attributed to a lower rate of non-cyclic heifers than the previously reported rate of anestrus (20-40%) in dairy cows at the beginning of synchronization protocols (21). Although P/AI for the second service in GI was lower than GII, PR to the second service was higher in GI compared to GII. This discordance between P/AI and PRs was basically due to the higher submission rate in GI.

Colazo et al. (9), reported that a once or twice-used CIDR for resynchronization resulted in the majority of non-pregnant heifers detected in estrus over a 4-day interval, with acceptable conception rates. In this regard, 78.5% PR following resynchronization has been reported, and twice-used CIDR for resynchronization resulted in 70-80% PRs in beef heifers. In this study, similar PR (75.9%) was obtained following resynchronization in dairy heifers. Colazo et al. (11) reported 54.1% PR following resynchronization with a used intravaginal progesterone-releasing insert in beef heifers. In the current study, the PR (75.9%) was higher than that (54.1%) reported by Colazo et al. (11) in beef heifers. Differences in PRs reported in the studies could be attributed to the synchronization protocol, type of rearing, and the breed of the animals. As a matter of fact, in our study, P4-supported OVS was used in dairy heifers. However, Rivera et al. (25) reported that PR was detected 79% following resynchronization with intravaginal progesterone releasing insert after AI in Holstein heifers. Similarly, we found that PRs were diagnosed as 75.9%.

It has been reported that PRs for resynchronization could be lower following the insertion of PRID after the first service compared to the first service PRs due probably to the development of persistent dominant follicles during P4 insertion (6, 10, 32). A lower PR obtained following resynchronization (38.2%) compared to the first service (60.9%) in this study could be attributed to the development of the persistent dominant follicle.

In conclusion, first of all, it was detected that PRs in dairy heifers can be increased by resynchronization applications with P4. As a secondary result, it was determined that PRID effectiveness continues up to 19 days in the resynchronization protocols. The most important of all, it was revealed that P4 levels had a significant effect on PRs before ovulation synchronization in non-cyclic dairy heifers. Therefore, further investigations regarding the level and effectiveness of P4 in terms of follicular development.

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Ethical Statement

This study was approved by the Local Ethics Committee for Animal Experiments of Erciyes Veterinary Medicine Faculty (2006/040).

Conflict of Interest

The authors declared that there is no conflict of interest.

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The effects of different storage conditions and periods on mould-yeast, aflatoxin, *E. coli* and *E. coli* O157 in wet sugar beet pulp

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Abstract: This study was designed to determine the effects of storage condition and period on mold-yeast, aflatoxin, *E. coli* and *E. coli* O157 in the wet sugar beet pulp (WSBP). For this aim, samples were taken from the first production of WSBP from the sugar factory (Control) and the newly packaged sacks from the packaging company (Packaged Control). Afterwards, additional two samples were taken from WSBP stored as packaged (Packaged) and as surrounded by a nylon cover outside near the barn (Non-packaged) from the predetermined farms with one-month interval. The pH value of the Packaged group was significantly lower than the Non-packaged group at the end of the 1st and 2nd months (P<0.01). The yeast-mold count of Non-packaged and Packaged groups at the end of the 1st and 2nd months was determined to be 4.61, 1.60 and 4.83, 1.26 log10 CFU/g, respectively (P<0.01). However, aflatoxin (B1, B2, G1 and G2) was not detected in groups. The *E. coli* was detected as 1.48 (in the 1st month) and 1.53 (in the 2nd month) log10 CFU/g in only Non-packaged group. It was worked for the identification of *E. coli* O157 from *E. coli* positive samples of Non-packaged group, but it was not detected. Consequently, it was determined that the use of packaged WSBP was better than the Non-packaged in terms of microbiological analysis.
Keywords: Aflatoxin, *E. coli*, mold-yeast, storage conditions, wet sugar beet pulp.

Yaş şeker pancarı posasında farklı depolama koşulu ve periyodunun küf-maya, aflatoksin, *E. coli* ve *E. coli* O157 üzerine etkileri

Özet: Bu araştırma, yaş şeker pancarı posasında (WSBP) küf-maya, aflatoksin, *E. coli* ve *E. coli* O157 üzerine depolama koşulu ve süresinin etkilerini belirlemek amacıyla planlanmıştır. Bu amaçla, fabrikadan WSBP'nın ilk üretimi (Kontrol) ve paketleme işletmesinin yeni paketlediği çuvallardan (Paket Kontrol) örnekler alınmıştır. Daha sonra, birer ay aralıklarla önceden belirlenmiş çiftliklerden paketlenmiş (Paketlenmiş) ve ahırın yakınında dışarıda bir naylonla çevrili olarak saklanan (Açık) WSBP'ndan iki numune daha alınmıştır. Paketlenmiş grubun pH değeri, 1. ve 2. ayların sonunda açık grubunkilerden önemli derecede daha düşüktü (P<0,01). 1. ve 2. ayların sonunda açık ve paketlenmiş grupların maya-küf sayısı sırasıyla, 4,61; 1,60 ve 4,83; 1,26 log10 CFU/g olarak belirlenmiştir (P<0,01). Bununla birlikte, gruplarda aflatoksin (B1, B2, G1, G2) tespit edilmemiştir. *E. coli* sadece açık grupta, 1,48 (birinci ayda) ve 1,53 (ikinci ayda) log10 CFU/g olarak belirlenmiştir. Açık grubun pozitif *E. coli* örneklerinde *E. coli* O157 identifikasyonuna çalışılmış, ancak tespit edilmemiştir. Sonuç olarak, mikrobiyolojik analizler açısından paketlenmiş WSBP kullanımının paketlenmemişten daha iyi olduğu belirlenmiştir.
Anahtar sözcükler: Aflatoksin, depolama koşulları, *E. coli*, küf-maya, yaş şeker pancarı posası.

Introduction

A great majority of livestock enterprises operate in the structure of small family-owned enterprises in Turkey. The enterprises should give importance to roughage production by using their equity capital in order to earn an economic income from animal husbandry. Since our farmers have started to produce particularly cereals (wheat, barley) and industrial crops, the high-quality roughage problem has been getting bigger (21). It is important to use the cheap feed resources efficiently in the animal feeding. Wet sugar beet pulp (WSBP), a by-product of sugar industry, is a cheap and safe diet component. It does not cause metabolic diseases since it has a high amount of pectin and a high level of

digestibility and is rich in cellulose (11). However, it may spoil rapidly if it is not stored under good conditions (13). The nutritive value of the spoiled feed decreases and several acute or chronic diseases develop in the animals. Feed is exposed to a waiting period from production stage until consumption stage. Even if several metabolic changes are observed in feed during this period mainly, microorganisms are responsible for feed spoilage. The types and amount of these microorganisms in feed vary based on the resource and type of feed. Also, they may be also affected by the factors such as the applications performed to prevent from formation of microorganism and the storage conditions of feed (6, 14). There are many microorganisms causing contamination in cereal kernels, feed, and vegetable substances. Mold and yeast can reproduce in 2-9 pH range, in the environments at 10-35^{o}C and with a water activity of 0.85 and above (17). As a result of fecal contamination, feeds may be contaminated by *E. coli*, *Listeria* and *Salmonella* species in the fields or Aspergillus species in humid environments depending on storage conditions (15). Some species of *E. coli* are dangerous. Especially, *E. coli* O157 which is known the most dangerous species may be formed in the environments at 37^{o}C and pH 7.2 (30). This species is found in cattle and, spreads through feces. Especially, the fertilizers contaminated by *E. coli* O157 carry the bacteria to the agricultural lands, and contaminate the products in these lands. Also, it may be seen in feed due to the irrigation of the cultivation areas with the contaminated water (8). The feed contaminated by aflatoxins negatively affects the health of animals. As aflatoxins are durable, they cause problems not only in the cultivation stages of products but also in their storage, transport and packaging stages. The toxicity of aflatoxins varies based on their species and, the most toxic ones are B1, G1, B2 and G2, respectively (25).

To determine which method is better in this study, samples were collected from businesses stored WSBP in packaged form or in open area at two different times (at one-month intervals). The samples were examined in terms of yeast-mold, aflatoxin, *E. coli*, *E. coli* O157 and pH.

Materials and Methods

The ethics committee approval was obtained based on the decision (04.05.2017 -08/01) of Fırat University Noninvasive Ethics Committee for this study.

Feed material and sample groups: For the first method, the samples were collected from the enterprises storing WSBP on the ground or covering with a tarpaulin or nylon cover. For the another method, the samples were collected from the enterprises providing of WSBP in the air-tight sacks. In order to compare of these two methods, the samples were taken from the same enterprises with one-month intervals. For this purpose, the samples were taken from the first production of WSBP in the sugar factory (Control with 5 pieces) and from the sacks newly packaged by the packaging enterprise (Packaged Control with 5 pieces). Two more times with one-month intervals, the samples of WSBP were taken from the enterprises storing in packages (Packaged; 10 enterprises, 10 samples) or from the enterprises surrounded with a nylon cover next to barn (Non-packaged; 10 enterprises, 10 samples). Since the number of the enterprises using package was few, the number of samples was limited to 10. The sampling method was applied based on the feed sampling regulation (4).

pH measurements of the sample groups: Twenty-five grams of WSBP samples and about 100 ml distilled water were placed in glass jars and, stirred in the mixer for about 10 minutes. Then, the pH of WSBP was measured with the calibrated digital pH meter (Orion star A111).

Microbiological analyses:

Yeast and mold count: The inoculation was performed using the pour plaque method in a dual-parallel manner. The samples were incubated at 25±1^{o}C for 5 days by using Dichloran Rose Bengal Chloramphenicol (DRBC) (Merck, Darmstadt, Germany) Agar. At the end of the incubation period, all the colonies developing in the media were counted as total yeast-mold. The results were given in CFU/g (3).

Aflatoxin analysis: Service procurement from a private company (Nanolab) working with a Shimadzu Prominence HPLC device for the determination of aflatoxin was realized. All the chemicals and the mobile phases in the analyses were HPLC grade. In accordance with the method, 25 grams of each sample were taken for aflatoxin analysis (B1, B2, G1 and G2) and, processed for HPLC analysis. The final solution was taken to the 1.5 ml HPLC vial. Afterwards, 100 µl of the solution was injected into the HPLC system whose sensitivity was increased by Kobra Cell (set to 100 µA). By using HPLC column (Spherisorb ODS-3, 250x4.6 mm, 5 µm), the samples were read in turn by using the fluorescence detector adjusted to 1 ml/minute mobile phase containing 350µ 4M Nitric acid. Then, 119 mg KBr added Methanol/ Acetonitrile/Water (20:30:50) in 1 liter (excitation:362 nm and absorption: 425) (5).

Before reading the samples, the HPLC device was calibrated by drawing the standard curves. The peaks of the extracted sample in their chromatogram were compared with the retention time of the standard peak. In case of determination of toxin in the sample, the amount of aflatoxin (B1, B2, G1 and G2) in the injected sample was calculated based on the following formula in the standard graphic (5).

Formula C = A / (M/Vext) x (Vcol / Vfin) x Vinj.

A= The amount calculated in the calibration graphic of the peak of the sample extraction (ng), M= Sample amount (g), Vext= Extraction volume (ml), Vcol= Solvent volume passing through the immunoaffinity column (ml), Vinj= Volume of the injected sample (µl), Vfin= The last volume at which the obtained eluate is dissolved (ml).

Escherichia coli count: It was taken twenty-five gr of each sample brought to the laboratory for *E. coli* count, and dilutions were prepared up to 10^{-2} and 10^{-3} from the dilution of 1:10 in the 225 ml peptone water. Each dilution was inoculated to TBX agar. After 24-hour incubation at 44ºC, it was counted (16).

Escherichia coli* O157:H7 *isolation: In the feed samples, FDA method (16) was used for *E. coli* O157:H7 isolation. 25 gr of each feed sample was subjected to enrichment (for 24 hours at 37ºC) in Tryptone Soya Broth containing 225 ml Novobiocin (TSB+n). The dilutions were prepared in Peptone Water up to 10^{-3}. 100 µl of each dilution was inoculated in Sorbitol Mac Conkey Agar containing Cefixime-Tellurite (CT-SMAC) by using spread plate method. After the inoculation, the petri dishes were incubated at 35-37ºC for 24-48 hours. Then, it was taken at least 10 typical colonies (colorless-transparent) from the petri dishes in which growth took place and, put in Tryptic Soya Agar with Yeast Extract (TSAYE) agar and, incubated at 35-37ºC for 18-24 hours. Those with indole test positive among the growing colonies were transferred into Levine's Eosin Methylene Blue (L-EMB) agar and, incubated at 35-37ºC for 18-24 hours. After the incubation, the metallic green or dark red brown colonies were inoculated in the 100 mg/L in SMAC agar containing 4-methylumbelliferone glucuronide (MUG) by using the spot-on lawn method and, incubated at 35-37ºC for 18-24 hours. Afterwards, the colonies not reflecting blue color (MUG negative) were taken under 365 nm UV light source and they were subjected to *E. coli* O157:H7 latex test. The agglutination strains were sought (16).

***Statistical analyses*:** SPSS software was used to determine the differences between the pH levels of the study groups (20). In the data assessment, the normality analysis was performed, then One Way Anova test was applied for pH analysis. The T-test was applied for mold-yeast analysis. Duncan multiple comparison test was applied for determining the significance between the pH levels. T-test was used for determining the pH, mold-yeast and *E. coli* density between 1st and 2nd months. In the statistical assessment of the results, the significance was accepted as $P<0.05$.

Results

Table 1 shows the pH levels of the groups measured at the end of the 1st and 2nd months. The pH levels of the Control and the Packaged control groups were measured as soon as the samples were obtained. The pH levels of the experimental groups at the end of the 1st and 2nd months were compared with the baseline pH levels of the Control groups. The pH values of the Packaged group significantly decreased compared with the other groups at the end of the 1st and 2nd months ($P<0.01$). The pH level of the Non-packaged group was significantly lower than the Control groups at the end of the 2nd month ($P<0.01$; Table 1). No difference was found between the pH results of the Non-packaged and Packaged groups in the 1st and 2nd month.

Table 2 shows the mold-yeast analyses of the experimental groups. Mold and yeast were not found in the control groups. When the examination of the mold-yeast analyses of the experimental groups, it was found that the mold-yeast levels of the Non-packaged group at the end of the 1st and 2nd months were significantly higher than the Packaged group ($P<0.01$). No difference was found between mold-yeast results of the Non-packaged and Packaged groups in the 1st and 2nd month. No aflatoxin (B1, B2, G1 and G2) was detected in all groups.

Table 1. pH levels of experimental groups (mean±SE).

Storage period, month	Control	Packaged Control	Non-packaged	Packaged	P
1	4.06±0.03^{a}	4.00±0.09^{ab}	3.84±0.05^{b}	3.41±0.06^{c}	P<0.01
2	4.06±0.03^{a}	4.00±0.09^{a}	3.80±0.06^{b}	3.37±0.07^{c}	P<0.01
t	1.36	0.388	0.539	0.445	
P	NS	NS	NS	NS	

$^{a-c}$: Mean values with different superscripts within a row differ significantly. NS: non-significant.

Table 2. Mold-Yeast count (log10 CFU/g) of the experimental groups (mean±SE).

Storage period, month	Control	Packaged Control	Non-packaged	Packaged	t	P
1	ND	ND	4.61±0.29	1.60±0.09	9.70	P<0.01
2	ND	ND	4.83±0.36	1.26±0.30	7.69	P<0.01
t			-0.472	1.11		
P			NS	NS		

ND: Not detected; NS: non-significant.

Table 3. *E. coli* and *E. coli* O157 counts (log10 CFU/g) of the experimental groups (mean±SE).

Storage period, month	Control	Packaged Control	Non-packaged	Packaged
1	ND	ND	1.48±0.13	ND
2	ND	ND	1.53±0.08	ND
t			-0,292	
P			NS	
E. coli O157			ND	

ND: Not detected; NS: non-significant.

Table 3 shows the results of *E. coli* analysis in the groups. In the analyses of the aforementioned groups, *E. coli* was found in the Non-packaged group. At the end of both 1st and 2nd months. *E. coli* counts were performed, but no significant difference was determined between the storage periods. *E. coli* O157 was not identified in the *E. coli* bacteria in the Non-packaged group.

Discussion and Conclusion

The pH values of the Non-packaged and Packaged groups at the end of the 1st and 2nd months were determined to be 3.84, 3.41 and 3.80, 3.37, respectively (P<0.01). Çerci et al. (10) determined that the pH value of WSBP silage was 4.36. Owing to the pH of the main material in the present study was low, the pH values of the Non-packaged and Packaged groups were deceased slightly (P<0.01; Table 1). This situation was attributed to the fact that easily soluble carbohydrate content of WSBP is high and therefore, an acidic environment was formed due to the fermentation in feed. In another study (29), it was reported that the pH value of WSBP was 4.36 and, decreased pH values of the silages prepared with different additives (P<0.05). In another study (31), the pH value of WSBP silage was determined to be 3.76. Especially, the pH is prime importance in terms of aflatoxin and some microorganisms in feed. Aflatoxins grow within 3.5-8 pH range (27, 28). In the present study, pH values in both Non-packaged and Packaged groups did not show significant differences to change the growth of microorganisms, compared with the main material. However, a significant decrease was observed in pH value of the Packaged group depending on a better fermentation, compared with the other groups. In the present study, the mold-yeast rate of the Packaged group was found to be significantly lower compared with the Non-packaged group (P<0.01). This was associated with the fact that WSBP is contacted with air much and is exposed to rain in the open environment (19). When the animal breeders store the pulp taken from a factory in bulk, 40-60% of the nutrients of pulp get lost due to the undesired fermentation events (2). The nutrient loss in this study was related to the storage conditions. It has been reported that some yeast species and, to a lower extent, some mold species cause a thermal breakdown in the structure of WSBP as they produce hemicellulosic enzymes (24). Similarly, it has been reported that the storage duration and storage method of sugar beet affects mold growth (19). In the present study the mold yeast counts at the end of the 2nd month did not have a significant difference in both the Non-packaged and Packaged groups. This indicated that factors such as contact with air increasing the mold-yeast counts emerged in the 1st month and, did not cause a statistically significant effect in the following period. Indeed, it is known that aerobic fermentation forms in the earlier stages in silage production, oxygen consumption occurs in the first day in normal fermentation, and the mold-yeast counts are affected by the contact of feed with air (14).

As a result of mold-yeast growth, aflatoxin risk rises in feed. But, as a result of the analyses performed in this study, aflatoxin was not detected in all the groups. As known, aflatoxins are produced at 12-40°C and pH 3.5-8.0 (27, 28). It was observed that the pH values of the groups varied between 4.06 and 3.37 in the present study. Those pH values were within the limits required for the development of aflatoxin. However, low pH values of the study may be effective in preventing aflatoxin production. In a study conducted to determine the levels of aflatoxin contamination in corn silage, Karakaya and Atasever (22) reported that aflatoxin B1 was found in 95.84% of the examined silage samples, but none of them had aflatoxin B1 greater than the maximum tolerance limit. If anaerobic conditions are maintained well, the pH remains around 4 and mold growth is no problem. However, if there is any change in anaerobic conditions (e.g. air and water entering the silo), molds can grow in the silage and, cause aflatoxin formation (22). It has been reported that mold and yeast grow in feed under inappropriate storage conditions and, form toxin (1, 6). The fact that aflatoxin was not found in all the groups in the present study indicated that the storage conditions of the Non-packaged and Packaged groups were not very bad. The Non-packaged group was more likely to mold growth due to air and water contact but this mold growth did not cause aflatoxin production. Indeed, in a study (23) in which a simple guideline was published for the identification of some common mold spoilage, it was reported that the white mold growth did

not produce toxin, but the yellow-green, pinky-red and purple, brown-black mold growth is an indicator of toxin production. In this study, the absence of aflatoxin production was associated with the mold growth in white color (23). However, absence of aflatoxin production did not eliminate the negative effects caused by mold growth. Mold growth causes losses especially at the energy level of feed (6, 23).

In the present study, *E. coli* was found in Non-packaged group. However, *E. coli* O157 was not found. In a study conducted in Diyarbakır region to determine the presence of *E. coli* in feed (6), it was determined to be $6x10^2$, $1.6x10^2$, $3.3x10^2$ and $1.4x10^2$ CUF/g in the feeds of dairy cattle, beef cattle, calf and lamb, respectively. As seen in the results of this study, *E. coli* was not found in the mixed feed types. The *E. coli* was not detected in the Control and Packaged groups in the present study. This was indicated that there was no contamination. However, *E. coli* was found in the Non-packaged group even if not detected at a high rate. This revealed the presence of contamination in the environment. However, *E. coli* O157 was not found at the end of the 1st and 2nd months. It has been reported that *E. coli* O157 has an acid resistance mechanism although it is known to grow in 7.2 pH under normal conditions (12, 18, 30). If *E. coli* O157 is present in the environment with 4.5-5 pH for a long time (18 hours), it is able to gain acid resistance and, may be resistant in more acidic environments (pH 3-3.5) (7, 9). Also, it has been reported that *E. coli* O157 can continue its activity during cold storage (26). In the present study, it was observed that *E. coli* O157 did not grow in the storage environment under cold winter conditions.

It was determined that the Non-packaged and Packaged groups, when compared with the control groups, were cleaner than expected in terms of the bacteria examined microbiologically. This, the pH levels under both storage conditions were associated with the fact that it was quite lower than the optimal pH conditions for the growth of the examined microorganisms.

Consequently, the growth of mold and yeast may be reduced to very lower levels by preventing air inlet in the outside storage. Also, contamination of *E. coli* may be prevented by attention to contamination from the environment such as feces. It was determined that packaging was partially better than the conventional method (Non-packaged group). However, hydration may occur in case of long-term storage. Therefore, packaged pulp is recommended to be consumed in maximum 2 months and stored in cold environments.

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Ethical Statement

This study was approved by the Fırat University Noninvasive Ethics Committee (04.05.2017 and 08/01).

Conflict of Interest

The authors declared that there is no conflict of interest.

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Detection of Israel variant 2 (IS/1494/06) genotype of Infectious Bronchitis Virus in a layer chicken flock

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Abstract: The aim of this study was to determine an infectious bronchitis (IB) infection, caused by an Israel variant 2 (IS/1494/06)-like IBV, in a layer chicken flock regularly vaccinated with vaccines containing IBV H120 and 4/91 strains. Mild respiratory symptoms, drop in egg production and soft-shelled eggs and eventually death were observed in a layer chicken flock. Clinical samples from four diseased chickens were examined for the detection and genotyping of IBV by virus isolation, a commercial real time reverse transcription polymerase chain reaction (rRT-PCR) and nucleotide sequencing. Both Israel variant 2 (IS-Var2) and 793/B serotypes were detected from samples by rRT-PCR, but sequencing results of a 345 bp part of S1 gene revealed that our IBV isolate, HFT-IBV, was IS/1494/06 (IS-Var2)-like with the 97.7% genetic similarity. These results suggested that immunity against vaccination with a combination of different genotypes (H120 and 4/91) could not be protective for IS-Var2 IBV field infection. In addition, identification of genotypes from the clinical samples, such as swabs and organ samples by commercial rRT-PCR assays failed to find correct IBV genotype responsible for the IB infection, whereas analysis of nucleotide sequencing of S1 gene of IBV as a gold standard test undoubtedly detected causative genotype of the infection. Also, the findings indicated that there is an urgent need for consider genotype- or protectotype-match vaccination strategies in the field to prevent vaccine- and IB-dependent economic losses of the poultry sector and logically protect chickens from IBV infection.

Keywords: Genotype, Infectious Bronchitis Virus, Israel variant 2, Layer chicken, vaccination.

Yumurtacı bir tavuk sürüsünde İnfeksiyöz Bronşit Virüsünün İsrail varyant 2 (IS/1494/06) genotipinin tespiti

Özet: Bu çalışmanın amacı, IBV H120 ve 4/91 suşları içeren aşılar ile düzenli olarak aşılanan bir yumurtacı tavuk sürüsünde, İsrail varyantı 2 (IS / 1494/06) benzeri IBV'nin neden olduğu infeksiyöz bronşit (IB) enfeksiyonunu belirlemektir. Yumurtacı tavuk sürüsünde hafif solunum yolu semptomları, yumurta veriminde düşüş, kabuk bozuklukları ve bir süre sonra ölüm gözlendi. Hastalıklı tavuklardan alınan klinik örnekler virüs izolasyonu, ticari gerçek zamanlı RT-PCR (rRT-PCR) ve nükleotid dizileme yöntemleri IBV'nin belirlenmesi ve genotiplendirilmesi açısından incelendi. Örneklerin rRT-PCR ile analizi neticesinde hem İsrail varyant 2 (IS-Var2 hem de 793/B serotipleri tespit edilmesine karşın, S1 geninin 345 bp'lik kısmının dizileme sonuçları IBV izolatının, HFT-IBV, % 97,7 oranında genetik benzerlik ile IS/1494/06 (IS-Var2) benzeri olduğunu ortaya çıkardı. Bu sonuçlar farklı genotiplerin (H120 ve 4/91) kombinasyonu ile aşılamaya karşı oluşan bağışıklığın, IS-Var2 enfeksiyonu için koruyucu olamayacağını göstermektedir. Ayrıca, svab ve organ örnekleri gibi klinik örneklerden ticari rRT-PCR testi ile genotiplerin tanımlama işleminin IB enfeksiyonundan sorumlu olan genotipin bulunmasında yetersiz kaldığı, buna karşılık altın standart test olarak kabul edilen IBV'nin S1 geninin nükleotid dizileme analizinin enfeksiyona neden olan genotipi doğru olarak tespit ettiği belirlendi. Buna ilaveten, çalışmanın bulguları kanatlı sektörünün aşı ve IB'ye bağlı ekonomik kayıplarını önlemek ve dolayısıyla tavukları rasyonel bir şekilde IBV enfeksiyonundan korumak için, sahada genotip veya protektotip uyumlu aşılama stratejilerinin dikkate alınmasının gerekliliğine işaret etmektedir.

Anahtar sözcükler: Aşılama, genotip, İnfeksiyöz Bronşit Virüs, İsrail varyant 2, yumurtacı tavuk.

Introduction

Avian infectious bronchitis (IB) is a highly contagious disease and, causes respiratory tract disorders, nephritis and reduction in egg production, and egg quality of chickens. IB is caused by Avian Coronavirus Infectious Bronchitis Virus (AvCoV-IBV) found in Gamma coronavirus genus belonging to *Coronaviridae* family. Genome of IBV is a single strand of positive-sense RNA. IBVs are made up of spike (S), envelope (E), membrane (M), and nucleocapsid (N) structural proteins (10, 11). The S1 subunit of S glycoprotein located outside of the virus plays a role for the fusion between the virus envelope and the host cell membrane (13). The epitopes in this subunit are protective antigens which are responsible for the production of neutralizing antibodies. In the S1 subunit, there are some hypervariable regions (HVR) determining the genotypes and serotypes, which are identified by molecular analyses of these regions (8). Genetic variation caused by mutations in the IBV S1 gene sequence is responsible for the worldwide diversity of IBV isolates (13).

Vaccination is the most important and indispensable way to control IB, but novel S1 gene mutants of IBV continue to emerge due to point or recombination-related variations in the field. Therefore, vaccination with current vaccine strains in the market may be insufficient to produce the protective neutralizing immunity to these novel serotypes and variants. Thus, continuous monitoring of the IBV genotypes from IB cases in the field and designing vaccination strategies with genotype-matched IB strains according to the monitoring results is necessary to improve protection (4-7).

The aim of this study was to determine an IB infection, caused by an Israel variant 2 (IS/1494/06)-like IBV, in a layer chicken flock regularly vaccinated with vaccines containing IBV H120 and 4/91 strains.

Materials and Methods

Samples: In November 2019, four dead chickens from a layer flock were submitted to the Department of Pathology at Fırat University in Elazığ province located in eastern Turkey. The history of respiratory problems such as gasping, sneezing and bronchial rales in addition to production losses was noted in this flock which had the capacity of 4000 Isa Tinted breed type of chickens at the average age of 52 weeks. According to the records, all the chickens in the flock were regularly vaccinated with Mass. (H120, Ma5 and Ma41) and 4/91 (793/B) variant strains, and the mortality rate was about 5-6%. Trachea, lung, kidney and cecal tonsils from those IBV suspected chickens were tested for IBV and variants (IS-Var2, Mass, 793/B, QX and D274) in Poultry Diseases Diagnosis Laboratory at Bornova Veterinary Control Institute, İzmir.

Necropsy: All the dead chickens were subjected to post-mortem examination. At necropsy, two chickens were detected to have thin-walled and fluctuant cysts containing 4-5 ml of clear liquid, attached to isthmus serosa of the oviduct. In only one of the chickens, kidneys showed mild pale appearance. No significant gross lesions were observed in the respiratory organs. Tissue samples for histopathology were taken from sinus, larynx, trachea, lungs, air sacs, kidneys, ovary, and infundibulum, magnum and isthmus sections of the oviduct, then fixed in 10 % neutral formalin solution. After processing routine procedures, the prepared paraffin blocks were cut into 5 µm thick, stained with haematoxylin and eosin (H&E) and, were evaluated by light microscopy.

Virus isolation: Each chicken was evaluated separately for virus isolation. Pooled organs were mixed with Phosphate Buffer Saline (PBS) (Sigma-Aldrich) containing penicillin (2000 units/ml), streptomycin (2 mg/ml) and gentamicin (50 µg/ml) antibiotics and, Mycostatin (1000 units/ml). The organs were homogenized using a MagNA Lyser (Roche) according to the manufacturer's instructions, followed by centrifugation at 3000 rpm for 10 min. The supernatants were filtered through a 0.45µm filter membrane and used for virus isolation and screened by real time reverse transcription polymerase chain reaction (rRT-PCR).

0.2 ml of the supernatants were inoculated onto the chorioallantoic cavity of ten 9-11 day-old specific pathogen free (SPF) eggs and incubated at 37°C. Inoculated eggs were checked twice daily. Those that died within 24h after inoculation were discarded. Deaths between 2 and 7 days post inoculation (PI) were considered to be virus specific. The chorioallantoic fluid was harvested aseptically from embryos that died between 48 and 72h PI, providing that the fluid showed no Hemagglutination (HA) activity. Dead embryos were examined for the presence of embryo stunting, curling, urate in the mesonephros, or focal necrosis in the liver. On day 3 PI, five live embryos were also removed from the incubator and were placed at 4°C for 24h and the chorioallantoic fluid of the embryos was collected for the next passage.

RNA extraction and cDNA synthesis: The chorioallantoic fluids collected on day 4 PI were serially diluted and used in Reverse Transcription-Polymerase Chain Reaction (RT-PCR). High Pure Viral Nucleic Acid Kit (Roche) was employed to extract total RNA from 200 µL of the chorioallantoic fluids according to the manufacturer's instruction. The extracted RNA was stored at -40 °C until PCR was performed. Viral RNA was

reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) and the obtained cDNAs were stored at −20°C until use.

Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR): The Real-Time Reverse Transcription-Polymerase Chain Reaction (rRT-PCR) was performed on LightCycler480 (Roche, Mannheim, Germany) by using the Kylt IB-aCo Kit for the detection of Avian Coronaviruses, and the Kylt IBV-Variant O2 Kit for the detection of IBV Middle-East GI-23 lineage (Var2-like), Kylt IBV-Variant IB-aCoV, Kylt IBV-Variant Massachusetts, Kylt IBV-Variant 4/91 (793B), Kylt IBV-Variant D274, Kylt IBV-Variant QX (all were purchased from AniCon Labor, Hoeltinghausen, Germany). rRT PCR tests were performed according to the manufacturer's instruction.

Partial sequencing of S1 gene: The cDNA samples which were detected as positive for IBV by rRT-PCR were subjected to partial sequencing of S1 gene by using two pairs of primers SX1: CACCTAGAGGTTTGY TWGCATG and SX2: TCCACCTCTATAAACACCY TTAC; SX3: TAATACTGGYAATTTTTCAGATGG and SX4: AATACAGATTGCTTACAACCACC (1). The first pair of primers (SX1 and SX2) were selected for use in the initial PCR and the other pair (SX3 and SX4) for nested PCR. First round amplification was performed in a final volume of 20 µL (2 µL D.W, 13 µL Norgenbiotek 2X PCR master mix (Canada), 2 µL of SX1 and SX2 primers and 3 µL of cDNA) with a thermal profile of one step denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 15 sec, 58 °C for 30 sec, 72 °C for 30 sec, and a final step of synthesis at 72 °C for 10 min. Amplifications were performed in an Eppendorf master cycler gradient thermocycler (Eppendorf, Hamburg, Germany). Nested-PCR reactions (total volume: 20 uL) were performed using 1 µL of the first PCR product. The reaction mixture was the same as the abovementioned PCR with the addition of nested primers (SX3 and SX4). The amplification products were analyzed by electrophoresis in 1.5% agarose gels in Tris-Acetate-EDTA (TAE) buffer, stained with GelRed™ (Biotium, USA) and visualized under UV light.

Nucleotide sequencing, alignment analysis and phylogenetic tree: rRT-PCR products were purified and sequenced by ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, USA) in a forward direction using primer SX3 and in a reverse direction using primer SX4. The sequences obtained (345 bp) were compared with the IBV sequences in GenBank database and similarities were analyzed by BLAST. Multiple sequence alignments were carried out with Clustal W and phylogenetic tree was constructed with MEGA software (version 7; Biodesign Institute, Tempe, USA) using the Maximum Likelihood tree method with 1000 bootstrap.

Results

Histopathological findings: Mild epithelial degeneration and desquamation were seen in the magnum of the oviduct of the four chickens. A mild to moderate salpingitis characterized by lymphocytes, macrophages and a few heterophils was present in the propria mucosa of the magnum and isthmus. Also, degenerated ova were seen in the lumen of the magnum. Tubular degeneration of individual cells and mononuclear cell infiltration were multifocally present in the interstitium of the kidneys. In the lungs, there was moderate congestion and oedema, and mild peribronchial lymphoid hyperplasia. No significant microscopic lesion was seen in the other organs.

Egg passage: After the seven passage in SPF embryonated eggs, no specific lesions for IBV such as stunting, curling and uric acid deposition in the kidneys and ureter were observed. The chorioallantoic fluid of inoculated eggs were found to be negative for Newcastle disease virus and Avian Influenza Virus by Hemagglutination (HA) Test.

PCR and rRT-PCR: PCR analysis of the infected chorioallantoic fluid samples could not yield any positive results. rRT-PCR analyses showed positive results, with a threshold cycle value of 29.97 for Avian Coronaviruses, 32.58 for IBV Middle-East GI-23 lineage (Var 02), and 34.33 for IBV 4/91 (793/B). Although the animals were vaccinated with IB 4/91 vaccine against 793/B three times (14, 42 and 84th days), the detection of this serotype put forward that the efficacy of the vaccine against this serotype was questionable or that the field strain obtained here was different from the vaccine strain.

Partial sequencing and alignment analysis of the IBV S1 gene: In the partial sequencing of IBV S1 gene, we obtained 345 nucleotide base in length and the similarity level of our isolate with the other strains deposited in the GenBank of NIH was compared (National Institute of Health, USA). A 345 bp length nucleotide sequence of S1 gene segment of our isolate (HFT) showed great similarity (approximately 99%) with those of IBV Eg/CLEVB-2/IBV/012 (Accession No: JX173488.1), IS/1494/06 (Accession No: EU780077.2) and TR8 (Accession No: KP259312.1). A few nucleotide deletions at the position of 1050, 1088, 1089, 1098, 1099 and 1107 of S1 gene were detected in our isolate (Figure 1). These deletions observed, except in the position of 1050, are probably due to reading errors that occurred in sequencer because of low level of fluorescence signal. The other

selected sequences of IBV strains from the GenBank were significantly different from our isolate HFT IBV.

Phylogenetic analysis of S1 sequence of our HFT IBV isolate also revealed a close genetic relation with IS/1494/06 isolate (GenBank Access No: EU780077.2), Eg/CLEVB-2/IBV/012 (GenBank Access No: JX173488.1) and with our previous laboratory isolate TR8 (GenBank Accession No: KP259312.1) (Figure 2).

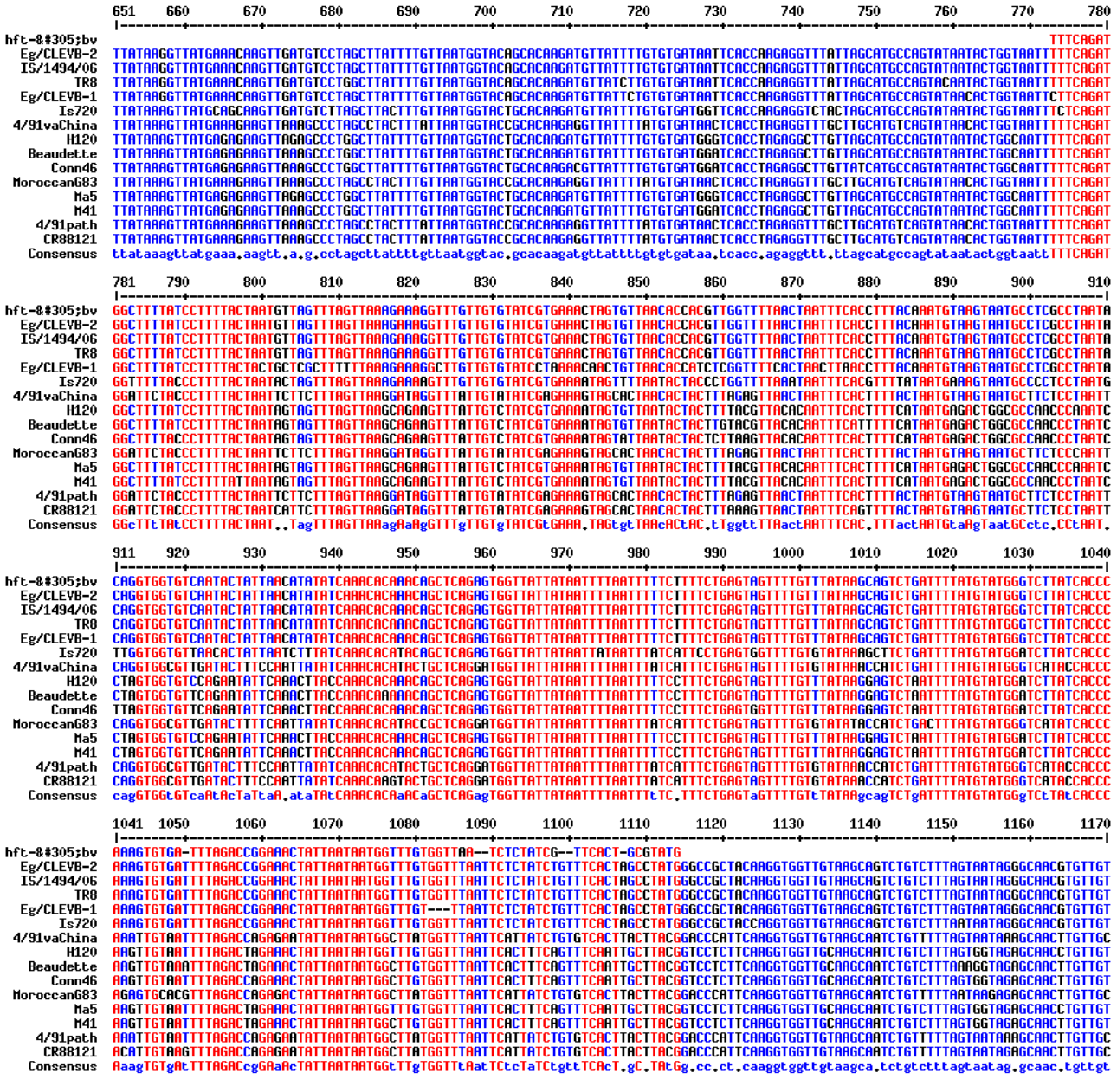

Figure 1. Alignment analysis of a selected 345 bp length S1 gene part of our HFT-IBV with some important IBV isolates from GeneBank, NIH.

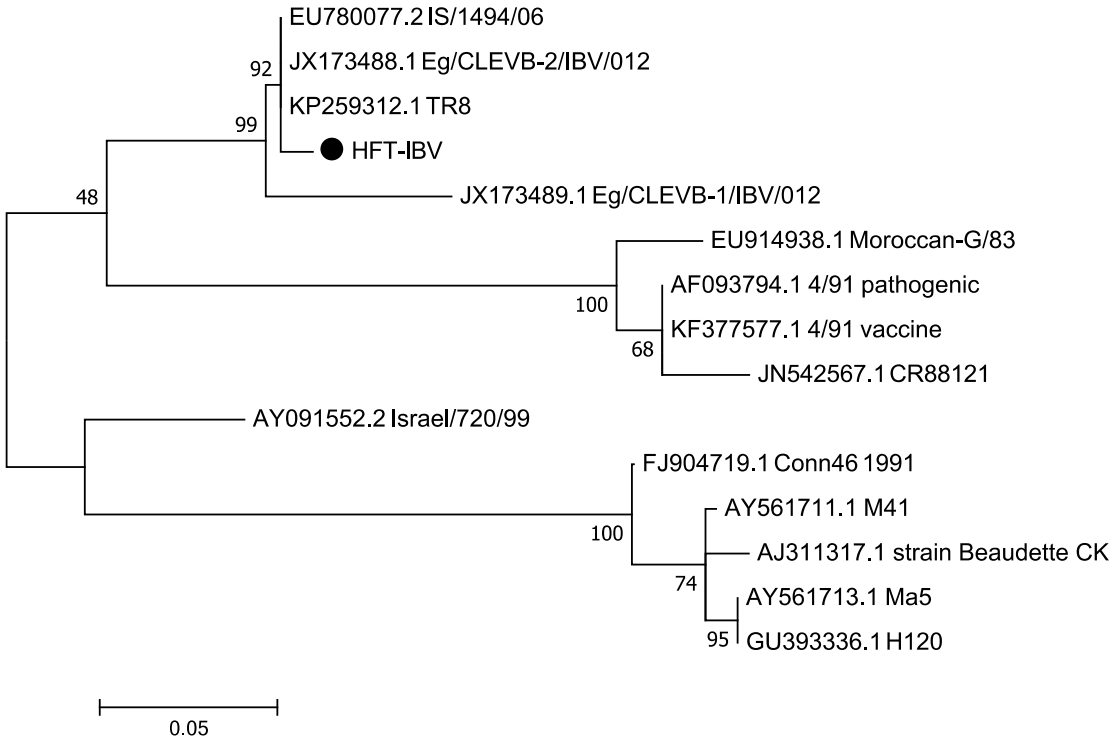

Figure 2. Phylogenetic tree showing genetic relations of S1 sequence of HFT-IBV with the other selected IBV strains from GenBank, NIH.

Discussion and Conclusion

In this study we isolated a IS/1494/06-like IBV (GI-23 lineage) named as 'HFT-IBV' from pooled organs (trachea, lung, kidney and cecal tonsils) of diseased layer chickens which had previously been vaccinated with H120 and 4/91 vaccine strains. Although information about protective antibody levels of this flock after vaccination is missing and some cross protections between H120, 4/91 and IS-Var2 IBV strains were previously reported, it is well known that protective immunity acquired from immunization with H120 and/or 4/91 vaccine strains could not yield sufficient protection against IS-Var2 IBV infections (2, 3, 12). This report can, therefore, be considered as an additional data to strengthen the knowledge that vaccinations with H120 and/or 4/91 IBV strains may not provide sufficient protection against IS/1494/06-like field virus infections. This finding was logically supported by the alignment and phylogenetic analysis of our HFT-IBV isolate. In the BLAST analysis, we found about 97.7% nucleotide sequence similarity with IS- Var2-like isolates, while this similarity with M41-related (H120, Ma5, M41) and 4/91 (793/B, Moroccan G/83 and CR88) strains was below 90% and reason for classifying the genotypes into the different genetic lineages. Because of this large difference in S1 gene, IS-Var2-like genotypes have been classified into GI-23 lineage, while M41 and 4/91-like viruses have been put into GI-1 and GI-13 lineages, respectively (13).

The findings of this study revealed that there was a huge genetic difference in the S1 gene between our HFT IBV isolate and the vaccine strains used in the layer flock examined. The genetic difference in S1 gene with above 10 % generally implied that immunity developed against IBV vaccine strains used in the layer flock was almost totally ineffective against clinical infection caused by IS/1494/06-like field strains such as our isolate HFT IBV. The clinical and pathological findings indicating the presence of IB infection within this flock supported this.

Recent studies carried out in Turkey have shown that IS-Var2 was circulating in the national chicken population and had great economic importance and losses particularly in layer flocks. The presence of this variant has been proved by Kahya et al. (9) who isolated and genotyped it in broiler and breeder chicken flocks. Yilmaz et al. (15) conducted a field study in broiler and layer flocks located in different regions of Turkey to investigate the presence of IBV and phylogenetic analysis of S1 gene, and reported that they detected IS-Var2 in addition to serotypes/variants similar to vaccine strains (Ma5, H120 and M41). The authors also noted that the IS-Var2 showed high similarity with those reported in the Middle East countries. In another study carried out by the same researchers in 2017 (14), a positivity of approximately 81% was reported in the real time RT-PCR analysis of the samples taken from broilers and layer hens, and sequencing of S1 gene and phylogenetic analyses revealed

high similarity with IS-Var2. Although 4/91-like viruses were recently detected from layer flocks at almost the same proportions with the IS-Var2 IBVs, the latter was still considered as the major cause of IB infections in broiler flocks in Turkey. In the present study, IS-Var2 was found to be responsible for IB infection in the layer chickens, and the findings indicated that it was not possible to make the genotype prediction without sequencing the isolated strain, due to the fact that nephropathogenicity and egg production problems could be caused by any of the IBV genotypes like 4/91, IS/1494/06 or M41-like viruses circulating in the country.

Detection and genotyping are crucial steps to control IB infections in chicken flocks by vaccination. This study employed virus isolation on embryonated SPF eggs and a commercial real time RT-PCR for detection of the virus, and S1 gene sequencing for genotyping of the isolate. When the sequencing results were considered, it was observed that commercial real-time RT-PCR test yielded false result by identifying our isolate as 793B (4/91) genotype. Whereas, nucleotide sequencing which is regarded as gold standard for genotyping revealed that our isolate was actually an IS-Var2-like IBV. The results of this report and our previous experience indicated that although commercial RT-PCR tests used for genotype detection can be regarded as quick and cheaper, they may fail in detecting the correct genotype in the samples.

We therefore suggest that nucleotide sequencing should be employed to correctly determine IBV isolates originated from diseased chickens in the field. Otherwise, mistyping results might lead to improper selection of vaccine strains in the field in order to protect the flocks which will consequently result in great economical losses.

Heterogeneity between vaccine strains and field strains is a significant problem in the field as observed in this report. Keeping in mind that no complete cross-protection is available among the IBV serotypes, novel vaccines containing variant strains are needed to combat this disease. We therefore recommend that continuous monitoring of IBV genotypes by virus detection and S1 gene sequencing, followed by selecting genotype or protectotype-match vaccine strains for immunizing is the most plausible way to control IB infection in chicken flocks.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Short Communication / *Kısa Bilimsel Çalışma*

Determination of the reproductive characteristics of Saanen goats using estrus synchronization and the growth performances of kids

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Abstract: This study aimed to determine the reproductive characteristics of Saanen goats of Australian origin using an estrus synchronization program with hormones (MPA and PMSG) in two different mating periods (Period I and Period II) and to investigate the survival rates and growth performances of kids. According to the results of the research, it was determined that pregnancy rate and litter size were higher in the mating period I (87% and 2.16) compared to mating period II (80.5% and 2.03), and it is hypothesized that this situation is related to the length of dry period of the goats. Although the mortality rate ($P<0.001$), and body weight ($P<0.05$) at birth were affected by kidding season, the survival rate and their body weights of the Saanen-kids at days 30, 60, 90, and 120 were not affected by the kidding season ($P>0.05$). These results indicate that the kidding season should be an environmental factor to be taken into account when planning the production. In addition to this, it is important to optimize the care and management conditions, especially during the kidding season, due to the high multiple birth types for the estrus synchronization program in Saanen goats.
Keywords: Estrus synchronization, kidding season, mortality rate, Saanen goats.

Östrus senkronizasyonu uygulanan Saanen keçilerinde üreme özellikleri ve oğlaklarda büyüme özelliklerinin belirlenmesi

Özet: Bu araştırmanın amacı iki farklı çiftleştirme döneminde (Dönem I ve Dönem II) östrus senkronizasyon programı (MPA and PMSG) uygulanan Avusturalya orjinli Saanen keçilerinde üreme özellikleri, oğlaklarında yaşama gücü ve büyümenin belirlenmesidir. Araştırma sonuçlarına göre çiftleştirme dönemi I 'de elde edilen gebelik oranları ve bir doğuma oğlak sayısı (% 87 ve 2,16) çiftleştirme dönemi II 'ye (80,5% ve 2,03) göre daha yüksek olduğu tespit edilmiştir. Bu durumun keçilerin kuruda kalma süresi ile ilişkili olabileceği düşünülmektedir. Doğumdaki ölüm oranı ($P<0,001$) ve canlı ağırlık değerleri ($P<0,05$) oğlaklama döneminden etkilenmesine rağmen, oğlakların 30, 60, 90 ve 120. günlerdeki yaşama güçleri ve bu dönemlerdeki canlı ağırlıkları oğlaklama döneminden etkilenmemiştir ($P>0,05$). Bu sonuçlar, üretim planlaması yapılırken oğlaklama döneminin dikkate alınması gereken bir çevresel faktör olduğunu göstermektedir. Buna ek olarak, Saanen keçilerinde östrus senkronizasyon programı için çoklu doğum türlerinin yüksek olması nedeniyle özellikle çocukluk mevsiminde bakım ve yönetim koşullarının optimize edilmesi önemlidir.
Anahtar sözcükler: Oğlaklama dönemi, ölüm oranı, östrus senkronizasyonu, Saanen keçisi.

There are fewer goats than other farm animals in Turkey; however, the increasing demand for high-yield goat breeds has led to an increase in the number of goats in recent years (16, 18). Estrus synchronization with hormone applications in goat breedings a popular practice to ensure continuity in production, to increase the number of offspring, to reduce production costs such as care, feeding, and labor, and meeting the market demand for goat products at the appropriate time (2). In recent years, the Saanen goat has taken its place among the most preferred breeds in our country due to its high milk and fertility characteristics. For profitability and permanency in animal production, reproductive performance, survivability of the offspring and their growth characteristics should be evaluated together. No studies are evaluating the reproductive performance and offspring

yields of exotic goat breeds. This study aimed to determine the reproductive characteristics of Saanen goats of Australian origin using an estrus synchronization program with hormones in two different mating seasons and to investigate the survival rates and growth performances of kids. The Saanen goats had Australian origins and were certified as high-class dairy goats in this study. The study was carried out in a private enterprise located in the Bolvadin district of Afyonkarahisar province with a semi-humid steppe climate during the period from 2011 to 2013. In the research, the same animals were used in both mating seasons (mating period I and mating period II). The first mating period consisted of 100 randomly selected female goats, the youngest one was 18 months old. They are defined as the mating period I (mating in August 2011). While 34 of these animals were lactating, the remaining 66 animals were recorded to be dry for at least one month (39±9 days). After the synchronization, these 87 goats gave birth and were dried off on about 150 days of lactation. These goats were defined as the mating period group II (mating in June 2012). The kidding season of mating period I and mating period II occurred in January 2012 (kidding season I) and November 2012 (kidding season II), respectively. The ages of dams were classified into three groups: 18 to 23 months of age (dam age group I), 24 to 35 months of age (dam age group II), and 36 months of age and older (dam age group III). A hormonal synchronization program was applied during the anestrus period of the animals in both periods for sexual stimulation. For this purpose, sponges containing 60 mg of medroxyprogesterone acetate (Esponjavet, Hipra, Turkey) were applied intravaginally and kept in the vagina for 10 days. On day 10 of the protocol, injection with 500 IU pregnant mare serum gonadotropin (PMSG; Gonas, Hipra, Turkey) was performed, then vaginal sponges were removed at 12 days. Hand mating was carried out as a buck for 30 goats. The goats used in the study were fed with two different rations. Each goat was fed 700 grams of roughage (7% crude protein, 1800 kcal ME/kg) and 350 grams of concentrated feed (20% crude protein, 2800 kcal ME/kg) per day until the third month of pregnancy. Then, advanced pregnancy rations 1.3 kg/goat/day roughage (9% crude protein, 1900 kcal ME/kg) and 700 g/goat/day concentrated feed (24% crude protein, 2900 kcal ME/kg) were applied. In addition to these feeds, the goats were taken to natural pasture for grazing between 06:00 and 10:00 a.m., 3:00 and 8:00 p.m. each day. Newborn kids were kept with their mothers for 10 days after birth. After this period, the kids were separated from the doe. The feeds of the kids consisted of meadow grass *ad libitum* and a concentrated mixture (100 g/goat/day) containing 16% crude protein and 2500 kcal ME/kg. After their mothers had been milked, the kids were allowed to suck only twice a day (morning and evening) for one hour each, until the weaning period (90 days). The kids' growth was determined using a digital scale sensitive to 50 grams. Fertility characteristics were calculated according to formulas specified by Akçapınar (2). Statistical analyses were performed using the software package SPSS 17 for Windows Significances of differences in reproductive characteristics of the goats were determined using the Chi-square test. The growth of the kids at different periods were calculated using general linear model (GLM). Yijkl = μ + Ci + Dj + Ek + Fl + eijkl Where; Y = the dependent variable, μ = the overall mean, Ci = the fixed effect of sex (i= female or male), Dj = the fixed effect of birth type (j= single, twin, triplet, quadruplet), Ek = the fixed effect of maternal age (k=I, II, III), Fl= The effect of kidding season (l=I, II), Eijklm = the random error. The Tukey's multiple-range test was used for multiple comparisons of groups statistical significance was taken at $P \leq 0.05$ (11, 13).

The data of reproductive characteristics of Saanen goats were presented in Table 1. In the present study, litter size was similar to the results of other studies using Saanen goat breeds of Australian origin in Turkey (9, 20). This value is higher than the data of other studies (4, 6, 8, 12, 14, 16, 17, 19) conducted with goats that were stated to be Saanen goats but which origin is unknown. The average pregnancy rate of Saanen goats in this study was found to be higher than in some studies (4, 6, 16, 19) and lower than in others (9, 14, 17). It is thought that the origin or genotype differences of goats may have affected litter size and pregnancy rate. Also, the difference in the climatic conditions of goats (6, 14, 17), hormone application to goats for the estrus synchronization or use of different synchronization methods (8, 12, 16), and different

Table 1. Reproductive characteristics (n; %) of Saanen goats using estrus synchronization by mating period.

Group	TNG	PN	PR	Single Birth		Twinning Birth		Triplet Birth		Quadriplet Birth		Litter Size
Mating period	n	n	%	n	%	n	%	n	%	n	%	
I	100	87	87	24	27.6	33	37.9	22	25.3	8	9.2	2.16
II	87	70	80.5	21	30.00	30	42.9	15	21.4	4	5.7	2.03
Total	187	157	83.95	45	28.66	63	40.1	37	23.6	12	7.64	2.10
P			**ns**		**ns**		**ns**		**ns**		**ns**	**ns**

ns: P>0.05. TNG: Total Number of Goats, PN: Number of goats pregnant, *Pregnancy rate: PN.*

Table 2. Survival rate (%) according to kidding period, dam age, sex and birth type in Saanen-kids.

Factors	n				Survival Rate (%)			
	Total number of born kids	Number of dead kids at birth	Kids born alive	Mortality rate at birth%	30. day	60. day	90. day	120. day
Kidding season				***	ns	ns	ns	ns
I	188	67	121	35.6	91.7	89.3	86.8	83.5
II	142	25	117	17.6	95.7	92.3	91.5	89.7
Sex				ns	ns	*	**	**
Male	148	37	111	25.0	92.8	86.5	82.9	80.2
Female	182	55	127	30.2	94.5	94.5	94.5	92.1
Dam age				ns	*	*	*	*
I	35	8	27	22.9	81.5^{a}	74.1^{a}	74.1^{a}	70.4^{a}
II	186	59	127	31.7	92.9^{a}	91.3^{b}	89.0^{ab}	85.0^{a}
III	109	25	84	22.9	98.8^{b}	95.2^{b}	94.0^{b}	94.0^{b}
Birth type				*	*	*	*	*
Single	58	12	46	20.7	97.8^{a}	93.5^{a}	93.5^{a}	89.1^{a}
Twinning	111	17	94	15.3	98.9^{a}	98.0^{a}	93.6^{a}	91.5^{a}
Triplet	97	28	69	28.9	95.7^{a}	89.9^{a}	89.9^{a}	88.4^{a}
Quadruplet	64	35	29	54.7	65.5^{b}	65.5^{b}	65.5^{b}	62.1^{b}
Total	330	92	238	24.80	96.75	91.70	91.70	88.75

*P>0.05,ns, *: P<0.05,* **: P < 0.01,***: P<0.001. $^{a, b:}$Means within a column with different letters are significantly different.

Table 3. Effects of kidding season, sex, dam age and birth type on body weight in Saanen-kids (kg) (X±Sx̄).

Factors	n	Birth		d 30		d 60		d 90		d 120
Kidding season		*		ns		ns		ns		ns
I	121	2.85±0.06	111	6.10±0.17	108	10.25±0.27	105	14.04±0.35	101	18.93±0.49
II	117	3.05±0.06	112	6.54±0.18	108	10.87±0.33	107	14.36±0.38	105	18.94±0.32
Sex		***		***		***		***		ns
Male	111	3.19±0.07	103	6.82±0.29	96	11.96±0.35	92	15.52±0.41	89	20.28±0.51
Female	127	2.74±0.06	120	5.86±0.14	120	9.45±0.21	120	13.19±0.31	117	17.91±0.38
Dam age		***		ns		ns		ns		ns
I	27	2,06±0.14^{a}	22	5.44±0.36	20	9.56±0.54	20	13.04±0.65	19	17.19±0.80
II	127	3.03±0.05^{b}	118	6.38±0.18	116	10.61±0.28	113	14.16±0.35	108	19.03±0.65
III	84	3.12±0.07^{b}	83	6.47±0.20	80	10.75±0.48	79	14.57±0.46	79	19.23±0.74
Birth type		***		**		***		*		ns
Single	46	3.21±0.11^{a}	45	6.73±0.35^{a}	43	11.34±0.59^{a}	43	15.00±0.65^{a}	41	19.75±0.66
Twinning	94	3.15±0.07^{a}	93	6.57±0.18^{a}	92	11.18±0.35^{a}	88	14.80±0.39^{a}	86	19.35±0.57
Triplet	69	2.75±0.08^{b}	66	5.96±0.18^{ab}	62	9.76±0.29^{ab}	62	13.47±0.43^{ab}	61	18.32±0.66
Quadruplet	29	2.38±0.11^{c}	19	5.33±0.31^{b}	19	8.44±0.54^{b}	19	12.03±0.79^{b}	18	17.17±1.22
Total	238	2.95±0.04	223	6.32±0.12	216	10.56±0.22	212	14.20±0.26	206	18.94±0.32

P>0.05,ns; *: P<0.05; **: P<0.01; ***: P<0.001. $^{a-c}$: means within a column with different letters are significantly different *(P<0.05).*

management strategies (4, 14) in the investigated studies may explain the differences. In the current study, the litter size and pregnancy rate obtained in the mating period I was higher than that of mating period II. However, the pregnancy rate of the 27 animals in the mating period I that was still lactating and dried off for only a month was similar (pregnancy rate 79.41, litter size 2.00) to the results obtained in mating period II. According to these results, we hypothesize that the differences observed in the litter size and pregnancy rate may be due to the short length of the dry period, as uterine involution may not have been fully realized in this short amount of time (5, 10). The data of survival rates and growth characteristics of Saanen-kids up to 120 days were presented in Tables 2 and 3. The birth weights of the offspring in this study were lower than those in similar studies conducted on Saanen goats in Turkey (1, 3, 6, 8, 14, 17, 19). Birth weight differences may be due to higher rates of triplet and quadruplet births in this study, together with genetic traits of the dams and environmental effects compared to other studies (2, 15). The mortality rate of the offspring in this study was higher than in various studies (6, 9, 14, 16, 17). These differences

may be due to the low birth weights of offspring observed in the present study (7, 15). The survival rate and body weight values obtained in the study were found to be lower than the values reported in some studies on unsynchronized Saanen goats (3, 9). This may be related to the lower birth weight of the offspring obtained in the current study compared to other studies. In addition, in this study, triplet and quadruplet birth rates were higher than in other studies. In this case, because the offspring were not able to get sufficient colostrum, they may have been at higher risk of developing various diseases (11, 15). Although the bodyweight of male kids were significantly higher than those of female kids during the growth periods at 30, 60, and 90 days of the growth period ($P<0.001$), it was found that female kids had a higher survival rate than those of males kids, especially at 60, 90 and 120 days of the growth period ($P\leq0.05$). This situation may be related to the sex of kids or it may be associated with the fact that the enterprise carries more attention to female kids for future breeding. In this preliminary study, it was noted that in Australian-origin Saanen goats raised in semi-humid steppe climatic conditions (Afyonkarahisar) with which synchronization protocols were applied during the anestrus period, the pregnancy rates and litter size obtained in the mating period I were higher compared to mating period II. Moreover, the survival and body weight values were similar in kidding season I and kidding season II, except for birth. Although the mortality rate ($P<0.001$), and body weight ($P<0.05$) at birth were affected by the kidding period, the survival rate and their body weights of the Saanen-kids at days 30, 60, 90, and 120 were not affected by the kidding season ($P>0.05$). According to the results, we hypothesize that the length of the dry period may be an important factor in reproductive efficiency. However, further studies are needed to confirm this theory. These results indicate that the kidding season should be an environmental factor to be taken into account when planning the production. Also, if the estrus synchronization program will be implemented in Saanen goats, it is important to optimize the care and management conditions, especially during the kidding season, due to the high multiple birth types.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Case Report / *Olgu Sunumu*

Occurrence of *Neophilopterus tricolor* (Burmeister, 1838) on a black stork (*Ciconia nigra*) in the Kızılırmak Delta, Turkey

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Abstract: A dead black stork (*Ciconia nigra*) was found by an a birdwatcher in the Kızılırmak Delta near Samsun, Turkey and brought to the Parasitology Laboratory of the Faculty of Veterinary Medicine at Ondokuz Mayis University for ectoparasitological examination in December 2019. During the examination, 45 specimens of chewing lice (21 males, 18 females and 6 nymphs) were collected. All of the specimens were identified as *Neophilopterus tricolor* (Burmeister, 1838) (Ischnocera: Philopteridae). *Neophilopterus tricolor* is reported from the black stork (C. nigra) for the first time in Turkey.
Keywords: Black stork**,** *Ciconia nigra*, *Neophilopterus tricolor*, Philopteridae, Phthiraptera.

Türkiye'de Kızılırmak Deltası'nda, bir kara leylekte (*Ciconia nigra*) *Neophilopterus tricolor* (Burmeister, 1838) olgusu

Özet: Samsun'da Aralık 2019'da, Kızılırmak Deltası'nda, bir kuş gözlemcisi tarafından bir adet ölü kara leylek (*Ciconia nigra*) bulunmuş ve ekto-parazitolojik incelemesi yapılmak üzere Ondokuz Mayıs Üniversitesi Parazitoloji Anabilim Dalı Laboratuvarı'na getirilmiştir. Ektoparazitik muayenede 45 örnek (21 erkek, 18 dişi ve 6 nimf) incelenmiş ve tümü *Neophilopterus tricolor* (Burmeister, 1838) (Ischnocera: Philopteridae) olarak tanımlanmıştır. *Neophilopterus tricolor* Türkiye'de ilk kez bir kara leylekte (*C. nigra*) rapor edilmiştir.
Anahtar sözcükler: Kara leylek, *Ciconia nigra*, *Neophilopterus tricolor*, Philopteridae, Phthiraptera.

In studies conducted on domestic and wild birds, over 4000 Mallophaga (Amblycera, Ischnocera) species have been identified worldwide (1, 3, 18). Phthiraptera species are obligatory ectoparasites that spend their entire life cycle on the host; they are obliged to leave their host within a short time after its death and find another host or they cannot survive for more than a short period (2). About 100 species of lice have been reported from domesticated and wild birds in Turkey (7, 12).

There are three species in the bird family Ciconiidae (Order Ciconiiformes), namely the black stork (*Ciconia nigra*), white stork (*Ciconia ciconia*) and yellow-billed stork (*Mycteria ibis*); only the chewing lice fauna of the white stork (*C. ciconia*) have been studied in Turkey (6, 11, 13). The ectoparasitic genera, *Ardeicola*, *Ciconiphilus*, *Colpocephalum*, *Laemobothrion* and, *Neophilopterus*, have been recorded on storks (18). Usually, chewing lice infestations are seen on all birds in the same nest (4, 16).

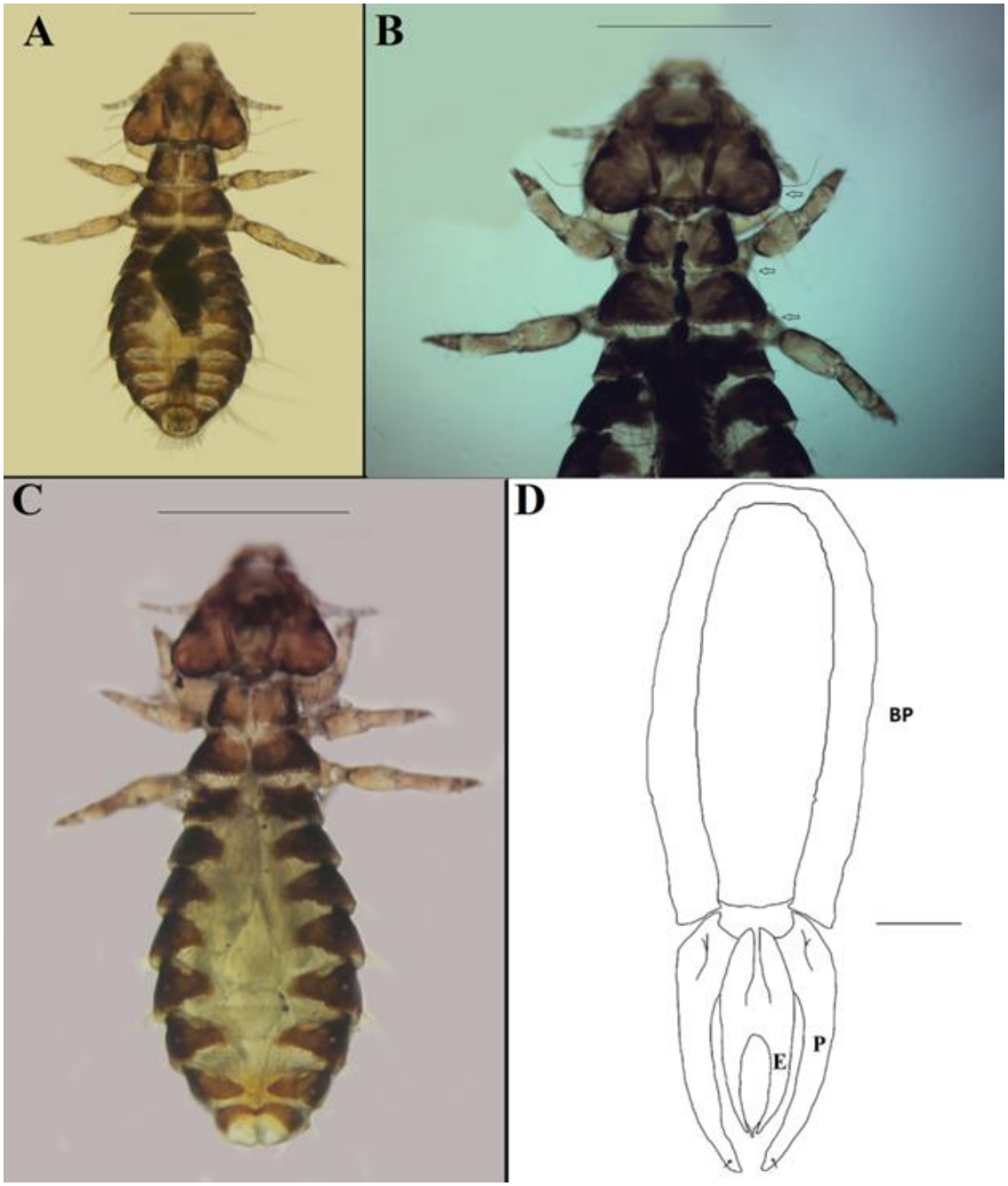
The aim of the present study was to identify the species of chewing lice removed from a black stork (*C. nigra*) found dead in the Kızılırmak Delta in Samsun Province, Turkey.

A one-year old, black stork found dead in the Kızılırmak Delta by an a birdwatcher was brought to the Parasitology Laboratory of the Veterinary Faculty at Ondokuz Mayis University in Samsun, Turkey for examination in December 2019. After the ectoparasitological examination, the collected material, namely chewing lice, was preserved in 70% ethyl alcohol before processing. For identification purposes, the

specimens were mounted on slides, according to the Canada Balsam technique (17). After mounting, the specimens were examined under a stereo-microscope (Nicon SMZ 1500) and then diagnosed according to the keys of Cummings (5) and Lanzarot et al. (14). The male genital organ was drawn with the aid of InkScape (Version 0.92).

In this study, a total of 45 chewing lice specimens (21 males, 18 females and 6 nymphs) were collected from a dead black stork. All of the specimens were *Neophilopterus tricolor* (Burmeister, 1838) (Philopteridae).

The head of *N. tricolor* (Burmeister, 1838) was large and triangular, the front edge was slightly concave, the width of the head was greater than its length. The preantennal region was shorter than the postantennal region. There were 6 marginal temporal setae, 4 long and 2 short (Figure 1A, B). Thoracic width was greater than the length. The prothorax and pterothorax were clearly separated in females and males (Figure 1A, B, C).



A: Male, B: Female (Setae of head and thorax, arrows), C: Female (Scale bar: 1mm); D: Male genitalia (BP: Basal plate, E: Endomeral plate, P: Paramere) (Scale bar: 50 µm).

Figure 1. *Neophilopterus tricolor*.

The posterolateral corners of the prothorax were rounded. On both sides of the prothorax, there were 2 + 2 setae, one long and one short. There were 24 setae, 12 + 12, on the posterior edge of the pterothorax (Figure 1B). There were two rows of tergal setae on each segment, with 10-15 setae distally and 8-10 setae in the central area.

The basal plate was convex and longer than the parameres. The area of the basal plate was groove-shaped. The parameres were wide, rounded, slightly curved inward and tapering towards the distal end. At the end of the endomeral plates, there was a membrane-like penis located between the basal endomeral region (Figure 1D).

There have been a number of studies on the chewing lice species of white storks in Turkey (6, 8, 9, 10, 11). Dik and Uslu (6), Dik et al. (8) and İnci et al. (11) reported the infestation of white storks with four different chewing lice species, *Ardeicola ciconiae*, *Ciconiphilus quadripustulatus*, *Colpocephalum zebra* and *N. incompletus*, in Turkey. In the present study, 45 chewing lice specimens were removed from a black stork found dead in the Kızılırmak Delta; all of the specimens were from the same species, *N. tricolor*.

Neophilopterus tricolor (Burmeister, 1838) is morphologically similar to *N. incompletus* (Denny, 1842), which has been reported to infest *C. nigra* (14). These species are host specific to *C. ciconia* and *C. nigra*, respectively (14). However, the morphological characters of the two species are slightly different; they can be easily distinguished via the differences in the genital area and mesosoma (5). In addition, the marginal setae on the temples were compared by Zlotorzycka (19) who reported six temporal marginal setae (four long and two short) on *N. tricolor* and five temporal marginal setae of almost the same length on *N. incompletus*. Another difference is the positions of the tergal setae in the abdominal segments: there are two rows of abdominal tergal setae, one apical and the other on the midline of the segment, in *N. tricolor*, and two rows, one apical and one basal, in *N. incompletus* (14, 15).

In the present study, *Neophilopterus tricolor* is reported for the first time from the black stork in the Kızılırmak Delta in Turkey.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Case Report / *Olgu Sunumu*

Acute fipronil intoxication in Squirrel Monkey *(Saimiri sciureus)*

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Abstract: A biocidal product in gel form containing 0.05% fipronil active substance was used for pest control in the habitat of thirteen Squirrel Monkey (*Saimiri sciureus*) in a zoo. Following the application, sudden death was observed in three primates. Routine bacteriological, histopathological and toxicological analyses on one cadaver and tissue samples from the cadaver did not produce any pathogenic agent, pesticide and any pathognomonic findings. In an attempt to investigate the presence of known fipronil intoxication biomarkers, fipronil and fipronil sulfone, we applied modified QuEChERS extraction processes on tissue samples and determined the fipronil and fipronil sulfone levels as 27.1-3.5 µg/kg in cutaneous tissue and 13.8-47.2 µg/kg in brain tissue, respectively, by using UHPLC Q-Orbitrap Mass Spectrometer. In the light of the anamnesis, necropsy, microbiology and histopathology results, and after evaluating the presence of fipronil-fipronil sulfone in tissues, we considered that the deaths could have resulted from acute fipronil toxicity due to species-specific active substance susceptibility.
Keywords: Biocidal, fipronil, fipronil sulfone, intoxication, primate.

Sincap Maymununda (*Saimiri sciureus*) akut fipronil toksikasyonu

Özet: Bir hayvanat bahçesinde barındırılan on üç Sincap maymununun (*Saimiri sciureus*) yaşam alanında haşere mücadelesi amaçlı %0,05 fipronil etkin maddesi içeren jel formunda biyosidal ürün kullanılmıştır. Uygulamanın ardından üç primatta ani ölüm şekillenmiştir. Ölen primatlardan bir tanesinin kadavrasında yapılan nekropsi ve akabinde alınan doku numunelerinde rutin bakteriyolojik, histopatolojik, toksikolojik analizler sonucu patojen etken, patognomik bulgu ve pestisite rastlanılmamıştır. Fipronil toksikasyonu biyobelirteçleri olarak bilinen fipronil ve fipronil sülfon maddelerinin varlığının araştırılması için alınan doku numunelerine modifiye QuEChERS ekstraksiyon işlemleri uygulanmış ve UHPLC Q-Orbitrap Kütle Spektrometresi ile fipronil ve fipronil sülfon sırasıyla deri dokusunda 27,1-3,5 µg/kg ve beyin dokusunda 13,8-47,2 µg/kg düzeyinde tespit edilmiştir. Anamnez, nekropsi, mikrobiyoloji ve histopatoloji bulguları ışığında, doku fipronil-fipronil sülfon varlığı değerlendirildiğinde türe özgü etkin madde hassasiyeti olabileceği düşünülerek akut fipronil toksikasyonu sonucu ölüm şekillendiği kanaatine varılmıştır.
Anahtar sözcükler: Biyosidal, fipronil, fipronil sülfon, toksikasyon, primat.

Fipronil is a phenylpyrazole derivative broad-spectrum insecticide, which has been used in pets, households, and farming areas as an active substance for the control of target pests since 1993 (15). Upon considering the exposure of other non-target organisms, it was determined that intoxication occurred in humans generally through splash and spills during the use of fipronil products for their pets, or during their stay in houses with insufficient ventilation after fipronil use (14). It was reported that environmental contamination levels caused by fipronil use have reached concentration levels that can pose extensive negative biological and ecological effects on such non-target species as harmless terrestrial and aquatic invertebrates (e.g. bees and worms), which play fundamental roles in ensuring the functioning of the ecosystem (18). Taking into consideration the common use and the environmental life cycle of the drug, investigating its non-target toxicity effects on ecosystems and food chain (apart from the targeted efficacy on pests) is a remarkable current topic for the one health concept.

A day after the application of a gel biocidal product containing 0.05% fipronil for pest control, three animals

were found dead in a cage containing 13 Squirrel Monkey (*Saimiri sciureus*) animals, homeland of which was South America. Furthermore, one animal had a number of symptoms of depression, inappetence, lethargy and body weight loss, which have reduced and disappeared over time, and the animal recovered in time. To investigate the cause of death, the cadavers of five elderly male animals were brought to Pendik Veterinary Control Institute. The cadavers were examined by using the primate necropsy techniques (4), samples were collected in line with the routine protocols followed in sudden death cases, and cultured in Blood Agar and MacConkey Agar for isolation and identification under the scope of microbiological controls. No growth was observed at the end of the incubation period. For histopathology examination, tissue samples were stained with Hematoxylin-Eosin (HE) and examined under a light microscope. Histopathology examination revealed parenchymatous degeneration in heart muscle, emphysema, edema, thrombosis, and fibrin matrix in lungs, and hemorrhage and necrosis areas in spaces of Disse in the liver. Extraction was performed with Quechers extraction kits (Agilent Tech., cat. no. QP6150S) for toxicological examination, and no detectable levels of pesticides were determined in the cadaver brain, liver and skin tissue samples by Gas chromatography - Mass Spectrometry (GC-MS) in terms of the pesticide groups of organic phosphorus, organochlorides, carbamates and pyrethroids. Since no other macroscopic pathological results were observed in the cadaver apart from the generalized cachexia that was in line with fipronil intoxication results in the literature, it was decided to perform chemical examination on tissues for fipronil and its metabolites/degradants (mainly fipronil sulfone), which are known to be residual biomarkers of fipronil exposure, in order to confirm fipronil intoxication (8). The presence of fipronil - fipronil sulfone was investigated in cadaver brain, liver and skin tissue samples against the skin, brain and liver tissue samples taken from a six-month-old male Balb/c mouse, which was known to be blank (as per the permission by Pendik Veterinary Control Institute Animal Experiments Local Ethics Committee 10/2019-220). The samples were analyzed with some changes on the method (5) in the literature and by using the Q Exactive High Performance Quadrupole Orbitrap Mass Spectrometer.

Fipronil reference standard (120068-37-3 cas no, Dr. Ehrenstorfer) and fipronil sulfone reference standard (120068-36-2 cas no, Sigma-Aldrich) were prepared for working standards at concentration of 5 µg / mL with acetonitrile (Sigma, LC MS grade). 2.5 grams brain, liver and skin tissue samples weighed for the blank and cadaver. Concentration of 5 µg/mL fipronil and fipronil sulfone working standard were loaded in blank mouse skin, brain, liver tissue with 50, 100 and 150 µL spikes. Then, 5 ml of pure water was added to all blank and cadaver samples. After mixing for 45 minutes at low speed, it was kept in an ultrasonic bath for 45 minutes. After the ultrasonic bath, 10 ml of acetonitrile was added to the sample, and vortexing was performed for 5 minutes. Then, 3 grams of $MgSO_4$, 1 gram of NaCl and 2 grams of sodium acetate were added to the sample, and vortexing was performed for 5 minutes. After vortexing, centrifugation was performed at 4500 rpm for 22 minutes at 22 degrees Celsius, the lower phase was taken with a sterile injector and passed through a 0.45 µm filter to the vial.

The prepared samples were delivered to the Q Exactive High-performance Quadrupole Orbitrap Mass Spectrometer. Thermo Scientific Accucore Phenyl Hexyl 100 x 2.1 column was used for analysis. For the flow to be used in the analysis, Ultra-Pure Water containing 0.1% formic acid-2 mM Ammonium Format on line A and Methanol solution containing 0.1% formic acid- 2 mM Ammonium Format on line B were used. The created instrumental method was determined as 15 minutes. The flow gradient was set at 90% from line A, 50% at 3 min, from 100% B line at 5 min to 9 min, and at 0.3 ml / min from 90% A line at 9 min to 15 min. Fipronil and fipronil sulfones were determined in the UHPLC Q-Orbitrap system by making 5 µL injection from the sample that was extracted into the vial prepared for analysis.

Fipronil and fipronil sulfone was determined to be 27.1-3.5 µg/kg in monkey skin tissue and 13.8-47.2 µg/kg in monkey brain tissue, respectively. No detectable levels of fipronil and fipronil sulfone were found in the cadaveric liver tissues.

In light of medical history, necropsy, microbiology, and histopathology results and after considering the presence of fipronil - fipronil sulfone in tissues, it was concluded that the reason of death was acute fipronil intoxication due to possible species-specific active substance susceptibility.

Preparations of fipronil are commonly used in veterinary treatment for ectoparasitary infestations of cats and dogs such as lice, flea, and ticks in spot-on and spray form for external use. Since it is not approved in other animal species such as primates, there are dose and administration recommendations on the literature for its extra label use upon need (9). Fipronil prevents the flow of chloride ions in chloride channels bound to the receptors of gamma-aminobutyric acid (GABA). It leads to excessive nerve activation, and hyperexcitation and death in target species with its GABAergic antagonistic effect formed through nervous system's primary inhibitor neurotransmitter, GABA. The main metabolite in mammals is fipronil sulfone in liver, adipose tissue and urine, and this metabolite is formed by cytochrome P450 in humans (15). Fipronil degrades in the environment through reduction, hydrolysis, oxidation and photolysis

reactions and rapidly forms five main active metabolites (fipronil-sulphate, fipronil-amide, fipronil sulfone, fipronil-desulfinyl, fipronil sulfonic acid) (7). According to the route of administration, technical fipronil (97% purity) is classified in toxicity category II (moderately toxic) or toxicity category III (slightly toxic) (15). Due to the differences in the affinity of main compound fipronil against receptors, while it was reported to be relatively safer in mammals since it showed 500-fold selective toxicity compared to insects (8), it has been reported that the primary biological metabolite of fipronil, fipronil sulfone is 20 times more active in mammalian chloride channels compared to chloride channels of insects, and that primary environmental metabolite fipronil desulfinyl was 9-10 times more active in mammalian chloride channels compared to the main compound (3). Upon examining the species that had been exposed to fipronil, its metabolites and environmental effects, it was reported that no gender difference was determined in the toxicokinetics or metabolism despite significant interspecies differences (2). Although fipronil is reported to be highly toxic for a number of bird species (e.g. chicken), it is not toxic against others (e.g. duck) (12), and it was also reported upon examining fipronil and fipronil-sulfone distribution and bioaccumulation in susceptible birds exposed to fipronil that it passed the blood-brain barrier, sulfone formed significantly high tissue concentrations in brain, liver and adipose tissue, and that the elevation in brain sulfone levels being simultaneous with specific intoxication effects provided insight for possible toxicity mechanisms in susceptible species (11). Placental passage or passage through milk to offspring was reported in mother rats exposed to fipronil and it resulted in neurotoxic effects in central nervous system including modified memory behaviour (16), and it was also reported that fipronil and its active metabolite sulfone caused reduction in motor coordination (17). Results on humans have determined that fipronil sulfone in maternal serum passed through from placenta to the fetus and formed negative side effects for infants (10). A series of adverse reaction reports of anorexia, lethargy, contractions, and death have been reported after the off-label use of fipronil in young and juvenile rabbits (19). Moreover, very small doses of fipronil applied to control grasshoppers have been reported to show negative effects on non-target species such as Coleoptera, Hymenoptera and Diptera, which are important for agriculture (7, 18). A pretty large part of pesticides in the market is used in agricultural areas. Particularly in vertebrate populations exposed to metabolites of environmental pesticide cycle, negative effects may be observed directly through intoxication or indirectly through the effects on growth, development, and reproduction due to the decrease in food sources (6, 13). The homeland and living area of Squirrel Monkey that was addressed in this case report is largely limited to Amazon River Delta within the borders of Brazil (1). It has been reported that Brazil is among the largest producers and exporters of agricultural products, and accordingly one of the largest pesticide consumers around the world, and that 1.068.60 tons of fipronil was used in Brazil for agricultural purposes in 2012 (15). It is known that fipronil metabolites form a significant effect on mammalian receptors, and through exposure to these metabolites, it is known that selectivity among receptors of non-target species such as insects and humans is decreased compared to the main compound. This case was shared particularly to emphasize that these metabolites in the environmental pesticide cycle may lead to larger problems for non-target species in the ecosystem such as the Squirrel Monkey, and to form an important step in the investigation of the environmental pesticide exposure. In the future, there is a need for detailed studies to be performed on susceptible species and their living areas in order to reveal fipronil exposure status and possible toxicity mechanisms.

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Ethical Statement

This study was approved by the Pendik Veterinary Control Institute Animal Experiments Local Ethics Committee (10/2019-220).

Conflict of Interest

The authors declared that there is no conflict of interest.

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Review / *Derleme*

The role of bio-detection dogs in the prevention and diagnosis of infectious diseases: A systematic review

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Abstract: Infectious diseases have been lately considered as one of the most important global risks, which negatively impact not only the health but also the socioeconomic conditions of countries. Globalization influences the spread of infectious diseases as a result of increased travelling and interaction in humans. Thus, it is highly important to prevent and diagnose new infectious diseases by using accurate and quick diagnostic methods.

Bio-detection dogs have a great potential to accurately diagnose infectious disease as they have a great ability to sense disease-specific volatile organic compounds (VOCs) originate from infectious agents and/or pathophysiological processes in the human body. The use of these dogs to detect infectious diseases has come to focus in particular after the recent global health crisis due to the SARS-CoV-2 infection.

This review discusses the potential use of bio-detection dogs in the prevention and diagnosing of infectious diseases. Moreover, factors affecting the scent of the disease, e.g. VOCs, are tried to be highlighted.

Keywords: Bio-detection dogs, diagnosis, infectious disease, volatile organic compound (VOC).

Enfeksiyöz hastalıkların önlenmesi ve teşhisinde biyo-dedektör köpeklerin rolü: Sistematik bir derleme

Özet: Bulaşıcı hastalıklar, son zamanlarda sadece ülkelerin sağlığını değil, ekonomisini de olumsuz yönde etkileyen en önemli küresel risklerden biri olarak kabul edilmektedir. Küreselleşme, yeni insan seyahat modellerinin ve artan insan etkileşiminin bir sonucu olarak bulaşıcı hastalıkların yayılmasını etkilemektedir. Dolayısıyla yeni bulaşıcı hastalıkların doğru ve hızlı teşhis yöntemleri kullanılarak önlenmesi ve teşhis edilmesi büyük önem taşımaktadır.

Biyodedektör köpekler, insan vücudundaki (pato)fizyolojik süreçler sırasında oluşan hastalığa özgü uçucu organik bileşikleri (VOC'ler) ayırt etme konusunda büyük bir yeteneğe sahip oldukları için bulaşıcı hastalıkları doğru bir şekilde teşhis etmede önemli bir potansiyele sahiptir. Bu köpeklerin enfeksiyöz hastalıkları teşhis etmek için kullanılması özellikle SARS-CoV-2 enfeksiyonu nedeniyle yakın zamanda yaşanan küresel sağlık krizinden sonra araştırmaların odak noktası haline gelmiştir.

Bu derleme, bulaşıcı hastalıkların önlenmesi ve teşhisinde biyodedektör köpeklerinin potansiyel kullanımını tartışmaktadır. Ayrıca hastalık kokusunu etkileyen faktörler yani VOC'ler de aydınlatılmaya çalışılmıştır.

Anahtar sözcükler: Biyodedektör köpek, enfeksiyöz hastalık, teşhis, uçucu organic bileşikler.

Introduction

A virus called the Novel Coronavirus (2019-nCoV) or Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) originating from the City of Wuhan in Hubei Province, China, has spread globally and affected more than 100 countries within a few weeks in 2020. The

World Health Organization (WHO) declared the SARS-CoV-2 a global pandemic after the epidemic criteria have been reached in a short time (12, 45, 53). Since the beginning of the recent SARS-CoV-2 outbreak, a great priority has been placed on the necessity of preparing health systems against the spread of this virus worldwide (12). Given high contagiousness of the virus and the significant role of asymptomatic individuals on the spread of the virus, the development of new strategies to detect the virus and to prevent the spread of the disease is particularly important (13, 48). Currently, real-time screening technologies are being used as the most common methods to control infectious disease in all over the world. These methods require several pre-analytical steps such as collection, appropriate storage and transportation of samples to a laboratory. Moreover, due to high cost, routine application of these methods brings a serious economic burden to the countries in case of any disease that is considered pandemic. Thus, it is of great importance to develop diagnostic methods that are sensitive, fast, accurate, economical and easily applicable to the field for public health as well as for border security management (3).

As seen in SARS-CoV-2 outbreak, new infectious diseases account for significant morbidity and mortality in humans. These diseases have been emerging in the global world due to several factors such as overpopulation in cities, increased international travel of people, and increased contact of humans with disease vectors and reservoirs in nature (63). According to the World Health Organization Report in 2007, spreading and emerging of infectious diseases are significantly faster since the 1970's. Experts also state that viral infections such as Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), H1N1 influenza, Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) which all of them are life threatening globally (13) have emerged in last decades. Therefore, emerging infectious diseases are considered as serious threats to global public health and economy. One of the main aims of the global public health security is to take proactive and reactive measures to decrease the risks, which negatively affect the human health (64).

Bio-detection dogs have come to the fore lately to detect "asymptomatic" individuals having an infectious disease in many countries because of their high ability to diagnose the disease and, further of their ease of use in the field (22, 29). These dogs are specifically trained dogs using their sense of smell to diagnose infection-specific VOCs. Domestic dog (*Canis familiaris*) has a high developed olfactory system which can detect VOCs related to metabolic and infectious diseases in body fluids and breath. Although these specific VOCs can be identified by methods such as gas chromatography-mass spectrometry (GC-MS), potential use of this method as a diagnostic tool is limited because it is time consuming, expensive and it requires qualified operators (42). It has been reported that dogs' exceptional sniffing performance is comparable to the current mass spectrometry-based laboratory applications and is capable of detecting compounds close to the femtomolar level (33, 62).

The focus of this review is to provide an overview of the current literature and future perspectives related to the use of bio-detection dogs in diagnosing infectious disease including SARS-CoV-2 infection.

Olfactory System of Dogs

A dog's sense of smell is 10,000 to 100,000 times more accurate than a human's smell because of their highly developed olfactory system which is specialized to detect messenger chemicals, e.g. pheromones for communication and evaluation of the environment (9, 62). The olfaction is involved in a complex chemical network such as perception of an odor which is mediated by olfactory receptors (OR) in the nasal cavity and the identification process through which the chemical signal is converted into an electric signal and transmitted to the brain. The olfactory system of dogs comprises of two systems which are the main olfactory system and the accessory system. The main olfactory system includes the olfactory mucosa, which contains the respiratory epithelium and the olfactory epithelium with olfactory receptors. These two epithelia cover the nasal turbinates within the nostrils. Surface of the mucosa is increased by three turbinates located in each nostril (44). The major cells located in the olfactory epithelium are olfactory bipolar neurons (4, 87). The dentrites of these neurons end in cilia, which help increasing interaction between odorant molecules and olfactory receptors (ORs) located on the cilial membrane (87). The olfactory nerve including the axon groups of the olfactory neuron transmits the signal to the olfactory bulb. The olfactory bulb is a relay station, e.g. synaptic area between primary and secondary olfactory neurons within the glomeruli. The perception of odors depends on the glomeruli map as each odor may activate a different glomerular pattern (19).

The accessory system comprises of the vomeronasal organ (VNO) and the accessory olfactory bulb. It was previously believed that the main olfactory system and accessory system are anatomically and functionally different from each other. The accessory system was tought to be responsible for numerous neuroendocrine and behavioral responses by responding to pheromones, while the main olfactory epithelium was responsible for conscious scenting by responding the volatile chemicals (38, 67). However, it is now known that both systems can respond volatile compounds simultaneously with different selectivity (67).

Many studies show that dogs have high packing density of neurons (44), and an increased number of functional genes against pseudogenes in the olfactory receptor gene family (49). Compared to humans, dogs have ~ 3 times more genes encoding olfactory receptors, a 30 times larger extension of the olfactory epithelium, approximately 50 times more olfactory receptors, and 3 times larger bulbus olfactorius (37). Thus, VOC biomarkers are within the detection range of the canine olfactory system. In addition to all these features, the nasal cavity in dogs contains hundreds of millions of sensory neurons in the nasal epithelium. Although the acuity of smell in dogs is associated with the large sensory organ size and receptor gene repertoire, the fluid dynamics of the transmission during sniffing are also of great importance in perception of smell (15).

History of Bio-detection Dogs

Detection dogs have long been used for hunting purposes, solving criminal cases, finding landmines and searching for victims of natural disasters successfully. Recently, a new field for detection dogs has emerged, in which they are trained to identify medical diseases. These dogs are called bio-detection dogs and trained to use their sense of smell to detect a wide variety of substances, mostly volatile organic compounds (VOCs), which are exceptionally or specially produced in people with metabolic or infectious disease.

The first publication regarding the detection dog in the medical field was a case report published in 1989 by Williams (65). According to this report, a Border Collie and Doberman Pinscher cross dog constantly sniffed a spot on the owner's leg for a long time. It was later found out that the spot where the dog was pointing was melanoma. The first studies on the use of trained dogs in cancer diagnosis were published in earlier 2000s. Willis et al. (66) reported that dogs were able to diagnose bladder cancers. Pickel et al. (43) later confirmed that dogs were successful in diagnosing melanoma. More importantly, in that study it was claimed that the further examination of a patient with negative initial pathology revealed melanoma after the dogs indicated this patient as positive. Another study conducted by McCulloch et al. (36) demonstrated that detection dogs showed high sensitivity and specificity in the diagnosis of lung and breast cancers from patients' breathing air. Horvarth et al. (25) further reported that dogs were successful not only in distinguishing between cancerous and normal tissue, but also in distinguishing non-cancerous pathological tissue (diseases that cause inflammation, necrosis or the emergence of metabolic products) from cancerous tissue. In another study conducted on ovarian cancers, it has been shown that dogs showed high success in the diagnosis of cancer from blood with 100% sensitivity and 95% specificity values (26). Dogs were found to be able to diagnose early stages of colorectal cancers and can detect this type of cancer from respiratory air with 92% sensitivity and 99% specificity (55). Similarly, Malinois breed detections dogs responded correctly in 30 of 33 cases in the diagnosis of prostate cancer from dog urine with 91% sensitivity and specificity (14). Ehmann et al. (17) reported that detection dogs were able to differentiate lung cancers from chronic obstructive pulmonary disease (COPD) by sniffing the breathing air. Furthermore, they also showed that smoking and nutrition did not negatively affect the diagnosis when using detection dogs. Today, cancer detection dogs are routinely trained and used to diagnose cancer in many countries. Nowadays, many centers of foundations have been established to train and use cancer detection dogs (32).

What do bio-detection dogs smell?

Hundreds of volatile organic compounds are released from the human body. As they reflect the metabolic state of the individual, body odor of individuals with any metabolic or infectious disease changes due to VOCs (52). Numerous studies have shown that VOCs can be specific to a specific pathogen or infection (8, 50). For instance, different VOC expression patterns were detected in primary human tracheobronchial cells infected or not infected with human rhinovirus (50). Similarly, VOCs produced by B lymphoblastoid cells following infection with three different viable influenza virus subtypes have been reported to be unique for each virus subtype (2). In a different study, VOCs associated with bacterial and viral growth were detected and it was revealed that VOCs can be used in the differentiation of these infections (1). Furthermore, it has been demonstrated that VOCs in the respiratory air change after the H1N1 vaccine (34). These studies show that unique VOC profiles can be associated with viral pathogens, which can be detected in patients.

Today, physical, biochemical and molecular biological methods are accepted as common routine methods used for medical monitoring and clinical diagnosis. In these analyzes, blood and urine are mostly used as samples. Diagnoses based on breath analysis are much less common. Compared to research on human fluids such as urine, feces, and blood sampling, analysis of breath is preferred as it is considered a non-invasive approach. The sample quantity is unlimited. The measurement and detection of volatile compounds in a gaseous matrix is much simpler than in a more complex biological matrix such as blood. Although analyzing and characterizing the breath sample and defining the diagnostic VOC model with statistical data allows to obtain strategic information for clinical diagnosis, it has not yet widely been used in clinical practice.

Breath analysis for VOC detection is now routinely performed for certain reasons as follows:

- Diagnosis of *Helicobacter pylori* infection by monitoring the carbon dioxide level (21),

- Diagnosis of airway inflammatory conditions by monitoring the fractional exhaled nitric oxide (FeNO) level (39, 47),

- Ethanol and acetaldehyde concentrations in the blood alcohol test (11),

- Detection of acute and/or chronic diseases (54, 56).

Is the VOC in breath useful for bio-detection dog training? Changes in VOC profiles may arise from pathogens themselves, host-pathogen interactions, and host immune responses (16). Numerous experiments have been performed over recent years using breath gas analysis for the non-invasive identification of different diseases. (5). VOCs are assumed to be transferred from various organs to the lungs via blood and subsequently excreted by diffusion via the alveolar pulmonary membrane and exhalation by air (61). The majority of published reports have centered on VOC analysis from exhaled breath for establishing the etiologic diagnosis of respiratory infections (31, 40).

By sampling breath, feces, urine, blood, and tissue, detection dogs are capable of detecting cancer. In certain cases, in exhaled breath, which includes the lowest known volatile VOC levels, dogs were able to detect disease states (51). Besides the several advantages to use breath, there are still some limitations such as sample collection. Sonoda et al. (55) trained a dog using samples of exhaled breath to test patients with colon cancer. Breath samples were collected into a breath-sampling bag, sealed and stored in a refrigerator until dog training. The sensitivity and specificity of this dog in breath samples for cancer detection was 0.91 and 0.99, respectively (55). A cylindrical polypropylene organic vapor testing tube was used in another study and showed that the cumulative sensitivity of canine scent detection of lung cancer using exhaled breath samples was 0.99, with a precision of 0.99 (36).

Regardless of the sampling method, it can be said that the ability of dogs to recognize the smell of breath is quite high and unique. In view of the risks associated with the collection of breath samples in infectious diseases, the correct method should be chosen and these samples should be used in dog training. The selection of methods and materials to be used is very important for both biodetector dogs and public health.

Detection of infectious disease by bio-detection dogs

History showed that various pathogens such as bacteria, viruses and parasites can cause outbreaks, which may become a threat to human population. These pathogens include bacteria such as *Bacillus anthracis*, *Francisella tularensis, Yersinia pestis*, viruses such as Variolavirus, filoviruses (Ebola), arenoviruses (Lassa), and influenza viruses as well as parasites such as *Plasmodium falciparum* (Malaria). Authorities outlined the critical importance of affordable, fast and reliable diagnostic methods to prevent spread of dangerous infectious agents as the outbreaks affect not only the health issues and economy but also they result in social disruption and panic in the human population (27).

Lately, bio-detection dogs have come to the focus of intense attention as these dogs have been reported to detect various kinds of infectious agents with a high sensitivity and specificity from body fluids, extracts and respiratory air of humans. These promising results revealed high potential for using bio-detection dogs in the diagnosis of infectious agents in populations and further in prevention of spread of infectious diseases. In this part, we will discuss the studies about the use of dogs in detection of various infectious diseases.

Bacterial Diseases: For the detection of bacterial diseases, dogs are expected to identify volatile metabolites, which are bacteria-specific fingerprints and produced during microbial replication (33). Two different training approaches have been conducted in the studies on the detection of bacterial infections by dogs. Accordingly, dogs were either trained to point the highest concentration or source of odor in an ambient air or they are trained to detect the compounds in the steam accumulating from the samples presented to the dog (58). After the training, detection dogs can perform rapid diagnosis of disease-causing pathogens in ex-vivo clinical specimens of patients or in-vitro bacterial cultures.

Clostridium difficile Infections: *Clostridium difficile* is a Gram-positive bacterium that causes symptoms such as formation of gas in the digestive system, diarrhea and abdominal pain. If this situation is not noticed in the early period, more severe cases such as colitis and megacolon can be observed in the affected individuals and the rapid and easy transmission of the agent may also cause important hospital infections.

The first study showing that dog's developed olfactory system can be used in the diagnosis of an infectious disease agent was conducted by Bomers et. al. (6). In this study, researchers reported that a well-trained Beagle dog detected *C. difficile* in stool samples with 100% specificity and sensitivity. The dog also detected *C. difficile*-infected patients with a high predictive sensitivity (83%) and specificity (98%). The encouraging results obtained from the previous study motivated the researchers to test the diagnostic efficacy of the same detection dog during a *C. difficile* outbreak in a hospital in the Netherlands (7). In the following study, the same dog performed 651 scans of 371 patients during 9 hospital visits and was able to detect 12 of 14 cases with 86% sensitivity and 97% specificity. In this study, it has been

shown that the detection dog can detect not only the current infection but also the clinical cases that are likely to occur in the near future (7).

Another group of researchers investigated whether a detection dog can detect environmental *C. difficile* reservoirs by smell. A dog was trained to detect odors released from pure culture and fecal samples found positive for *C. difficile*. The dog was successful in detecting agent-specific odors with high sensitivity (100%) and specificity (97%). As a result of this study, it was reported that since trained dogs can successfully detect *C. difficile* scent in environmental sources, this feature can be used to take necessary cleaning measures in the relevant health facility and they can be used successfully in infection control programs as well (10).

In a different study using detection dogs, it was reported that dogs were able to detect *C. difficile* strains containing the toxin gene in stool samples, but the inter-rater reliability was not high enough, suggesting that dogs have limited practical value in the point-of-care diagnosis of *C. difficile* infection and will never reach the efficacy of molecular tests that provide diagnosis with extremely high accuracy (57).

Methicilline Resistant Staphylococcus aureus (MRSA) Infections: Detection dogs were successfully used for identification of bacterial infections in medicine. By analyzing the differences in volatile organic compound (VOC) profiles, even antibiotic-resistant and susceptible strains of the same bacterial species can be distinguished from each other. In a study based on this feature, detection dogs were successful in distinguishing methicillin-resistant *Staphylococcus aureus* strains, one of the most important hospital infection agents, not only from other *Staphylococcus* species, but also from methicillin-susceptible strains of the same species (30).

Urinary Tract Infections: Urinary tract infections (UTI) are among the most common hospital infections in hospitalized patients. Urinary system infections are caused by infectious bacteria such as *Escherichia coli* (responsible for 80% of UTIs), *Staphylococcus aureus*, *Proteus* spp., *Enterococcus* spp., *Pseudomonas* spp., *Enterobacter* spp., and *Klebsiella* spp. as well as fungal infections such as *Candida* spp.

In a study conducted by Maurer et al. (35), dogs were trained to distinguish culture positive urine samples from culture negative urine samples in cases of bacteriuria. Dogs have successfully detected the agent with a sensitivity of 99.6% and specificity of 91.5% in 250 samples containing $1x10^5$ *E. coli* colonies per milliliter. Dilution of samples at 1% and 0.1% concentration did not alter the diagnostic accuracy. Moreover, diagnostic accuracy was achieved with 100% sensitivity and 93.9% specificity in 50 samples containing *Enterococcus* spp., 100% sensitivity and 95.1% specificity in 50 samples containing *Klebsiella* spp., and 100% sensitivity and 96.3% specificity in 50 samples containing *S. aureus*. These results revealed that dogs can be trained and used successfully for early and accurate diagnosis of bacteria that cause UTI.

Detection Dogs in Veterinary Medicine: Detection dogs have been used in Veterinary Medicine to diagnose cows with mastitis. Dogs were trained using specific bacterial cultures, artificially contaminated raw milk samples and field milk samples collected from cows with mastitis to detect *Staphylococcus aureus* that is a common pathogen isolated from clinical and subclinical mastitis samples. Their respective sensitivity and specificity were 91.3% and 97.9% in bacterial cultures, 83.8% and 98% in raw milk and 59% and 93.2% in samples obtained from cows with mastitis (18).

Parasitic Diseases: The studies on detection dogs to identify pathogens are not limited to the bacterial infections. In a recent study, dogs were reported to detect malaria from nylon socks of asymptomatic children with a greater sensitivity than the routine diagnostic methods. The results in asymptomatic malaria-infected and non-infected individuals were broadly in line with the WHO's criteria for the provision of rapid diagnostic tests, by which the test should be able to detect at least 75% of the parasite agent of *Plasmodium falciparum* samples (23). This study was one of the pioneer studies showing that dogs are able to detect infectious disease even in asymptomatic patients.

Viral Diseases: Viruses, like bacteria, are found in all living organisms, including humans, animals and plants. Since viruses rely on host cellular functions to propagate and replicate, they cause metabolic reprogramming in the cells (60). It was claimed that metabolic changes occurred in host cells because viral agents mirror metabolic changes seen in cancer cells (41, 60). These metabolic changes include upregulation of nutrient consumption, nucleotide and fatty acid synthesis as well as exhibiting the Warburg effect, e.g. increased glycolytic metabolism to support viral replication and rapid cell proliferation (41). Recent studies have reported various metabolic changes in the host cells induced by different viral agents such as adenovirus (59), human cytomegalovirus (68), influenza A Virus (46) and herpesvirus (20). Routinely used methods for identifying viral infections are limited and expensive or resource-demanding conditions. Given dogs have the ability to detect VOCs caused by cancer and bacteria, they are considered as potential detectors which can detect metabolic changes caused by viruses in human body.

Dogs' ability to detect metabolic changes due to viral infections was demonstrated by different studies. In a study, dogs' ability to distinguish BVDV, BHV1 and BPIV3 infected kidney cell cultures were investigated. In

this study, dogs detected bovine viral diarrhea virus (BVDV) with high diagnostic specificity (95%) by smelling the infected cell cultures which suggest that dogs can be used as a realistic real-time mobile pathogen detection technology in diagnosing viral pathogens in cultured cell groups (3).

Recent studies on bio-detection dogs have mostly focused on SARS-Cov-2 infection. For example, Grandjean et al. (22) showed that dogs can diagnose SARS-CoV-2 by the odor of sweat released from axillary glands. Four out of eight dogs used in this study succeeded in detecting positive samples with a 100% accuracy. The success rates of other dogs were reported as 83%, 84%, 90% and 94%, respectively. Similarly, Jendrny et al. (28) reported that dogs were successful in the diagnosis of SARS-CoV-2 with 82.63% sensitivity and 96.35% specificity by sniffing saliva and trancho-bronchial secretions.

Conclusion and Future Direction

Studies on bio-detection dogs emphasized several advantages of using bio-detection dogs in detecting infectious disease. As these dogs are mobile, they are suitable for searching infectious both indoor and outdoor environments. They can be an important real-time and rapid diagnostic tool in areas such as airport, farm, slaughterhouse, etc. where diagnosis is critical. They can easily be trained for new scents and, thus for diagnosing new infections. Furthermore, the ability of dogs to recognize infectious diseases by smell may be evidence that special volatile compounds are produced in these infectious diseases, which allows the development of new laboratory diagnostic methods.

However, there are some limitations needed to be discussed in the use of bio-detection dogs. In addition to the risk of possible infection with the targeted pathogens, if biosecurity rules are violated, they may play a role in the transmission of the agent to the environment and people. Therefore, they should be tested regularly for possible infection with the investigated pathogen. It has been demonstrated that some drugs used in the treatment of dogs affect their sense of smell negatively. Thus, dogs on any medical treatment should exclude from detection work.

Detection dogs also have an important potential in the diagnosis of infectious diseases in veterinary medicine. Primarily, they can be used as a pre-diagnosis tool in herd screening for bacterial endometritis and mastitis infections seen in large animals. They can be used in the investigation of chronic and subclinical diseases such as paratuberculosis, which are difficult to diagnose in the early period. They can further be trained to investigate the presence of bacterial pathogens and mycotoxins in food and feed samples. Another issue that needs to be investigated is whether these dogs have the potential to recognize more than one factor at the same time (multiple diagnoses) for a particular clinical case.

As a conclusion, detection dogs are promising and rapid diagnostic tools in diagnosing infectious diseases in both human and veterinary medicine. However, for success in olfactory-based diagnosis, attention should be paid to factors such as using the correct dog, proficient trainer, correct sampling method, biosafety rules and appropriate training method.

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Review / *Derleme*

Precision livestock farming technologies: Novel direction of information flow

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Abstract: Precision livestock farming (PLF) is a digital management system that continuously measures the production, reproduction, health and welfare of animals and environmental impacts of the herd by using information and communication technologies (ICT) and controls all stages of the production process. In conventional livestock management, decisions are mostly based on the appraisal, judgment, and experience of the farmer, veterinarian, and workers. The increasing demand for production and the number of animals makes it difficult for humans to keep track of animals. It is clear that a person is not able to continuously watch the animals 24 hours a day to receive reliable audio-visual data for management. Recent technologies already changed the information flow from animal to human, which helps people to collect reliable information and transform it into an operational decision-making process (eg reproduction management or calving surveillance). Today, livestock farming must combine requirements for a transparent food supply chain, animal welfare, health, and ethics as a traceable-sustainable model by obtaining and processing reliable data using novel technologies. This review provides preliminary information on the advances in ICT for livestock management.
Keywords: Internet of things, livestock data management monitoring tools, precision livestock farming, health, welfare.

Hassas hayvancılık teknolojileri: Bilgi akışının yeni yönü

Özet: Hassas hayvancılık (PLF), bilgi ve iletişim teknolojilerini (ICT) kullanarak hayvanların üretimini, üremesini, sağlığını ve refahını ve sürünün çevresel etkilerini sürekli olarak ölçen ve üretim sürecinin tüm aşamalarını kontrol eden dijital bir yönetim sistemidir. Geleneksel hayvancılık yönetiminde kararlar çoğunlukla çiftçinin, veterinerin ve işçilerin değerlendirmesine, muhakemesine ve deneyimine dayanmaktadır. Üretime yönelik artan talep ve hayvan sayısı, insanların hayvanları takip etmesini giderek zorlaştırmaktadır. Bir kişinin, yönetim için güvenilir görsel-işitsel veriler almak için günde 24 saat sürekli olarak hayvanları izleyemeyeceği ise açıktır. Son teknolojilerle bu bilgi akışı hayvandan insana olarak değişmiş ve bu da toplanılan güvenilir bilgilerin, operasyonel ve efektif olarak bir karar alma sürecine dönüştürmesine (örn. Üreme yönetimi veya buzağılama takibi) yardımcı olmuştur. Günümüzde hayvancılık, yeni teknolojileri kullanarak güvenilir verileri elde ederek ve işleyerek izlenebilir ve sürdürülebilir bir model olarak şeffaf bir gıda tedarik zinciri, hayvan refahı, sağlık ve etik gerekliliklerini birleştirmelidir. Bu yayında, hayvancılık veri yönetiminde kullanılan bilgi ve iletişim teknolojileri alanındaki gelişmeler hakkında güncel bilgiler derlenmiştir.
Anahtar sözcükler: Hassas hayvancılık, hayvancılık veri yönetimi, izleme araçları, nesnelerin interneti.

Introduction

"Man goes to nature to learn what nature is, but, in so doing, he introduces possibilities of distortion through his own presence." –

T.C. Schneirla (154)

Before the industrial revolution 4.0, livestock management decisions were mostly based on the observation, judgment and experience of a human. The last decade has seen a great metamorphosis and brought a novel concept named "Precision livestock farming (PLF)", which is a digital management system that periodically or continuously measures production, reproduction, health and welfare of animals and environmental impacts of the herd through a "per animal" approach by using monitoring tools, mainly the internet of things (IoT), and controls all stages of the production process (11, 15). Thus, the use of automated measurement methods to monitor animal behavior has become increasingly widespread, and a number of models have been introduced that can distinguish reasonably accurate traits of daily physiological routines.

Different behavioral monitoring studies implemented on activity (50), eating (30), and milking (6) have created the fundamental infrastructure of PRL. The pioneering study was conducted by Farris, 1954 (50), for the detection of estrus by monitoring the mounting activity and counting the number of steps during estrus. For eating, the initial methodology was investigated in cattle (30) and soon after in sheep (127) by classifying the jaw movements to distinguish bites from chews. These pioneer studies have led to the idea of recording biting and chewing sounds with a wireless microphone attached to the forehead of the animals (103) and thus revealed a simple approach on intake and time per bite, for researchers to focus on classifying jaw movements (biting sounds were louder than chewing with differences in spectral composition) of the grazing process. However, PLF tools are still under adaptation and the evolution of digital technologies varies greatly between different sensor systems and application areas. Therefore, the development of PLF was driven by a number of variables in the care of livestock such as the growth of herd size, and the resulting inability of farmers to care for individual animals, the economic efficiency of farming, and increasingly environmental factors such as many other developments in the fields of agriculture, computing, and engineering. As these variables create more considerable complexity in farmers' work, it has become necessary for farmers to be able to monitor variables related to basic livestock production processes (11). The elementary principles for the emergence of PLF tools is to provide accurate and relevant information to take decision to a farmer (60). These incentives resulted from challenges for farmers and provide opportunities for farmers, veterinarians and engineers. PLF is possible due to technological advances, but challenges for local farmers such as animal identification in larger herds, productivity demands, and more recently, sustainability and welfare have offered unique opportunities for innovative technologies to be tested and applied. Therefore, the definition of the PLF technology was that the combination of computer and ICT use to make the production chain more efficient due to the increased control it affords resulting in improvement in animal welfare and benefits the best in using the resources resulting in decreased environmental pollution (13). The introduction of process control procedures has resulted in significant improvements in other industries (181).

PLF is a management system, which use sophisticated intelligent software’s and systems to combine variety of data from different sources of hardware’s for monitoring. This data driven system enables improved health, welfare and production along with minimized undesirable environmental impact through complex monitoring mechanisms such as tele-surveillance.

Overview of the Existing Tools

Overview of tools for precision livestock farming are considered at the levels of collection and management of data gathered by monitoring using different technologies.

Monitoring devices: Many researches were conducted to discover the potential implementation and validation of monitoring systems, while these systems are constantly developing. Behavioral and physiological monitoring of animal variables can be complicated as the method used to collect the data may change and there will always be interindividual variabilities. In order to monitor animal variables several technologies were adopted including image and sound analysis using cameras, sensors or other devices including water/feed consumption, scales etc. For image analysis, as the devices are not required to be placed on the animal no extra stress is produced; while in the prediction stage, it is difficult to retain good precision as the developed software for target tracking and extraction of animal foreground depends on various complicated image factors. Image analyses are used for weight and body condition score estimation, water/feed intake, assessing the gait and lameness along with detection of marked animals in estrus behavior monitoring. At the current state, mainly electronic wearables such as active smart ear tags which receive data from individual animals such as temperature and activity/ingestion patterns; neck and leg collars for rumination and activity loggers and other sensors used for prediction of diseases are widely used alone or in combination with robotic milkers, automatic feeders and inline milk sensors. In some farming, both image and sensing monitoring devices are combined to receive maximal efficiency and data (121). Selected biosensor based monitoring devices according to the system used, feed follow-up, monitoring of behaviour, biological parameters and applied area are listed in Figure 1.

With the increasing number of available sensing tools, a vast amount of generated data is expected to be processed and analyzed where the internet of things (IoT) is the major system for monitoring and data collection. A list of current commercial PLF tools to monitor and support cow health and performance, that includes over 100 tools is accessible by farmers and it is being updated regularly (4D4F Technology Warehouse).

The Internet of Things

Data obtained by the monitoring tools are connected through various technologies including Machine-to-Machine (M2M) communications, Cyber-Physical-Systems (CPS) Web-of-Things (WoT) and Internet-of-things (IoT). As a part of IoT, communication between machines and devices are mainly attributed as M2M where cloud computing infrastructures are available using telecommunication services (4G, 4.5 G, 5G, satellite). On the other hand, IoT comprises a broader scope of interactions between devices/things/people. CPS systems under IoT comprise physical sensing devices such as biosensors to the digital world. WoT enables the resources using mainstream applications such as HTML, Java, PHP etc. Therefore, IoT allows connecting the data gathered by all the monitoring tools to the internet for improvement of valuing of all livestock related operations (4).

The use of IoT in PLF meets five possible indications classified as surveillance, drug tracking, localization, feed follow-up, monitoring of behavior and/or biological parameters.

Surveillance: Animal protein deficit is thought to be the cause of global epidemics. This raises great concerns about disease transmission from animals to humans, making animal health a high priority (41). On the other hand, it is predicted that the demand for meat worldwide will increase by at least 40% in the next 15 years (123), this figure will reach 498 tons in 2050, and the number of animals per farm will increase in response to the demand (43). Consequently, consumer demands such as animal health, welfare and the reduction of antimicrobials use put pressure on veterinarians and farmers.

It is necessary to solve many problems such as monitoring animal health and welfare in the livestock sector, reducing the environmental impact (15% of global CO2 emission, 1/3 of the arable land and 8% of fresh water) and ensuring the efficiency of the process (43). Today, livestock farming must combine requirements for a transparent food supply chain, animal welfare and health, and an enhanced, traceable and sustainable model. The concept of epidemiologic surveillance system gathers high-quality information in animal health and food safety to make proper decisions and implement actions regarding the prevention of zoonotic disease for public health (world health organizations), animal services (public veterinarians), and private organizations (54).

Practice for the surveillance of animals involves identification and measuring components connected to the animals at least with one tool (For example, ear tags, transponders, accelerometers) wirelessly connected to measure the individual characteristics for specific groups of animals by modern information networks. Analysis for animal disease monitoring and surveillance are usually conducted by epidemiologists; which is crucially important for management of health related issues and risk analysis. Health surveillance is regarded as a tool to monitor the trends in diseases that are of significant economic, trade and security of food importance. Animal disease surveillance includes observing a group of animals strictly to evaluate and focus on a specific condition or disease individually/the whole population with ascertain variations in prevalence and define the frequency and route of epidemic spread. There are also the use of syndromic surveillance systems to detect the vector borne diseases like “Blue Tongue” through the use of pregnancy length and milk yield (108). The above mentioned statement turned into an abbreviation called MOSS (monitoring and surveillance systems) that measures disease and surveillance of animal population (150).

The Moss system includes systematic acquisition, research, analysis, and up-to-date information on health/production / reproductive data for both animal and public health. Public and animal health, as well as monitoring and identification of pandemic diseases of exotic origin such as corona, are among the purposes of use of surveillance systems. These programs provide guidance in determining effective prevention and control strategies. It also serves to monitor the progress and completion of response programs and to indicate the non-infectious and non-hazardous status of animals and animal-derived products in the animal health field. Ensuring that surveillance plans are on target is ultimately superior (48).

Drug tracking: Improved management with PLF allows an increase in the efficacy of drugs used in food-producing animals; as a medication is only used as an adjunct to a good management system bearing responsibilities for public health in livestock. Early detection of individual changes in the health parameters relating to diseases has great importance in early diagnostic interventions as well as successful chemotherapeutic treatment (101). Global misuse of veterinary antimicrobial agents led to an emerging increment in bacterial resistance at an alarming level leading to both human and animal clinical treatment failure. In order to achieve the rational dose regimen of antibiotics for prevention and treatment of diseases along with minimizing the resistance, risk relies on the optimization of pharmacokinetics through assessment and characterization of interindividual variability of drug intake (106). These novel systems therefore not only allow early detection of diseases leading to early drug interventions but also allow the evaluation of the amount of feed and water intake to calculate the exact amount of drug and associate with certain health effects along with transparency/traceability, folow-up of recovering and for refined phenotypes. The future of pharmacokinetic/ pharmacodynamics studies are expected to be guided by these smart systems merging the changes in health, welfare, and productive status (65).

Localization systems: Localization has crucial importance since the accuracy of the localization affects the cost and the limitations of the system; (33, 78). Indoor positioning systems (IPS) combines sensing and communicating technologies to determine the location of objects/animal in indoor environments (20). Various types of localization techniques are adopted such as Global Navigation Satellite Systems (GNSS), Inertial Navigation Systems (INS), wireless localization and environmental signals (magnetic, air pressure, light, sound) with database matching, dead-reckoning (magnetometer, odometer, inertial sensor based motion sensor), vision sensors (camera, light detection and ranging-LiDAR). These techniques all have advantages and limitations over each other and usually it is difficult to evaluate cost effective high performance localization system; where farm specific solutions are expected to be adopted (102).

The GPS (Global Positioning System) tools are intelligent design to track or find the animals remotely with the valuable aid of GPS tracking collars. This tool determines the exact satellite position by using the global positioning system and updates this information in given intervals. Detected positions are updated regularly or

could be downloaded remotely. The initial technology only allows assessing the obtained data when the detachable collar was accessed directly on-site. However, nowadays innovative technology allows us the acquisition and assessment of reliable data remotely. The collars generally use GSM (The Global System for Mobile Communications) operator signals to receive and typically transmit the specific location. GPS collars use "Geostationary Satellites" to promptly send the precise positioning along with other valuable information to the tracking server or standard PC (35). The above mentioned technology led the dairy farmers to monitor/control their herds effectively at extensive grazing systems in large areas with outdoor positioning systems (8). Tracking animals to find sick or missing individual or drive away predators is time and labor consuming. This day, innovative GPS devices are convenient to track the real-time location of outdoor cattle (166). Feasibly the most significant benefit of GPS trackers obtains the peace of mind they offer dairy farmers (10). On the other hand, indoor positioning systems created different possibilities for the development of tools such as determining the exact position of the cow in heat in large cow herds (148), body condition score (149) determining hot and cold areas in the barn that could adversely affect welfare (131), virtual fence (170), dynamic and smart grazing rotary systems (62).

Feed follow-up systems: Monitoring "cattle's feed intake is considered an excellent tool to form an opinion on their general well-being. Sick cattle will spend time eating less food due to loss of appetite. Thus, rumination time is becoming a key indicator for health monitoring in that animal regurgitates a bolus of food into her mouth and masticates thereafter. Hereby, when cattle become ill, it eats and ruminates less which allows us to create a rumination chart individually for animal health status. Also, rumination is an important part of the digestive process, and a healthy cow ruminates for 400 to 600 minutes a day, average daily grazing is around 6–10 h per day (179), lactating cows spend around 4.5 h/d eating (range: 2.4–8.5 h/d) and 7 h/d ruminating (range: 2.5–10.5 h/d), with a maximum total chewing time of 16 h/d. The ruminant activity also helps to keep the rumen pH at a level suitable for microbial activity. In the beginning, studies were more focused on pressure sensors mounted on the jaw with a halter (Figure 1) to detect the rumination pattern (30, 88, 185). Then, Burfeind (22) gathered the data and turned it into a monitoring system that can evaluate the data to differentiate the eating and rumination through a computer acquisition system. Today, with rumination activity patterns, the prediction of the feed intake, health status, and environmental impacts is possible. Thus, the difference between healthy and unhealthy conditions like metabolic disorders (ketosis, acidosis, dysplasia, etc.) or stress-caused circumstances (heat stress, estrus, social interactions, etc.) can be monitored. Different types of rumination detections are present (129); such as the movement of neck muscles by ear tag, bolus sensors (66), and/or as wearable sensors (183) for grazing activity, indoor positioning (126), and feed intake with video recording (133) Figure 1.

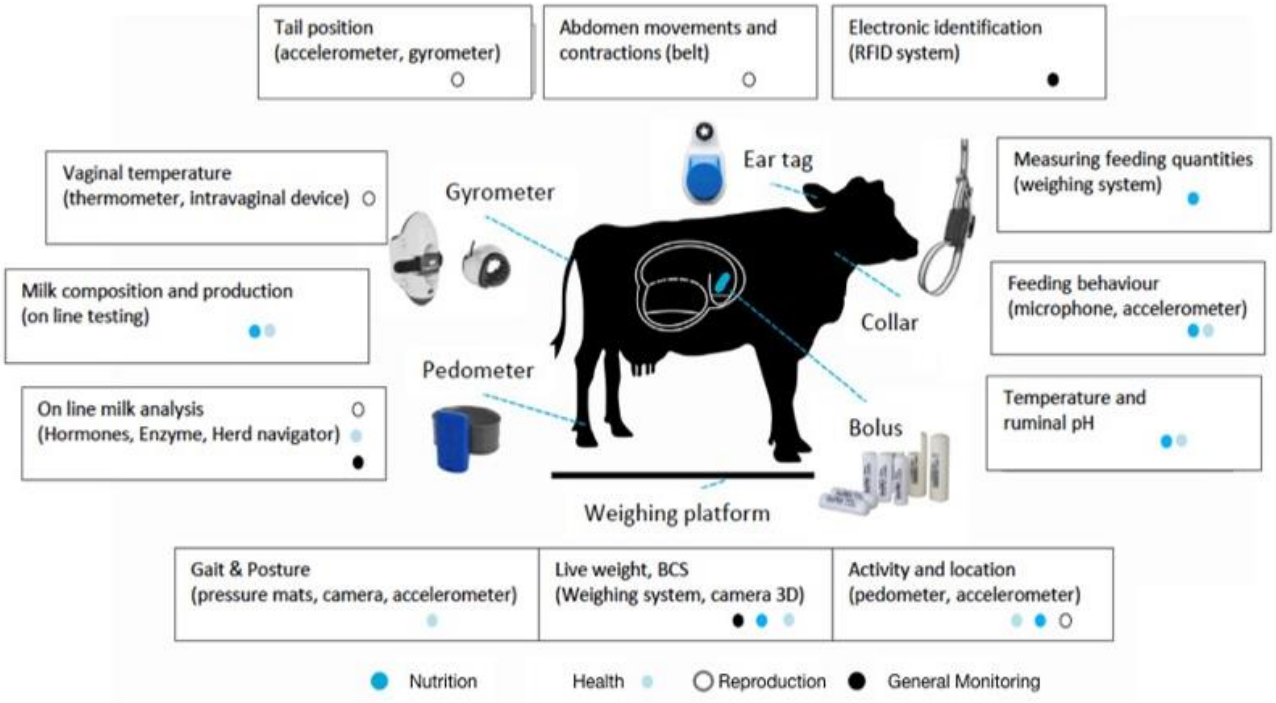

Figure 1. High-Tech Cow (64).

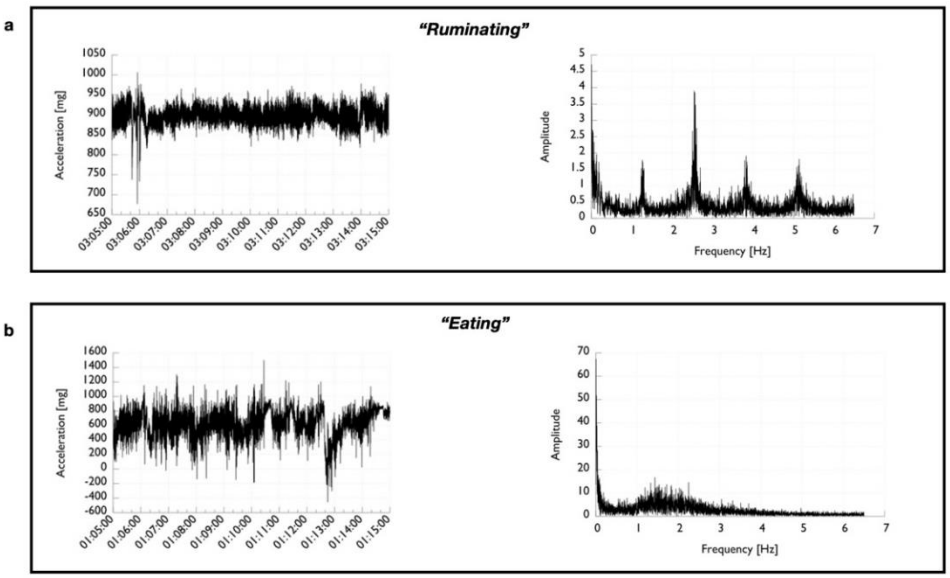

Figure 2. Time series representation (left) and corresponding frequency spectrum (right) a) "ruminating" behaviour b) "Eating" behaviour (65).

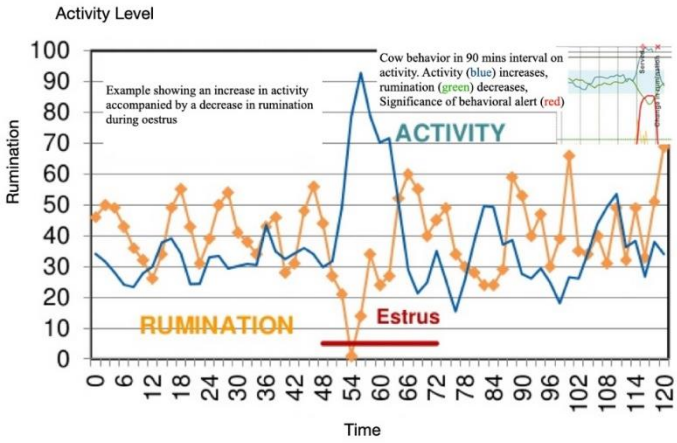

Figure 3. Example showing an increase in activity accompanied by a decrease in rumination during oestrus" (65).

It is necessary to understand the underlying mechanism of rumination tools for reliable PLF technology. For example, during eating, the cow must tear/pick up (eg grass) from the ground, chew it partly, and then swallow for rumination. As a result, the muscular movements observed in the neck are quite large and can be observed with the frequency differences of these acceleration measurements (5). This behavior is described using just a simple measurement of the acceleration at its height. With the difference between these two frequencies, the accelerometer processed map of rumination behavior is obtained (Figure 2a).

While the cow eating, the jaw movements of the cow indicates much more about rumination. Because in the course of eating, jaw indicates a wider movement pattern than the head. The frequency of behavior showing less rhythmic activity than rumination during eating can be seen in Figure 2b. The high level of variance produced during eating with lower frequency movements but lacks identifiable frequency peaks compared to the ruminant map (Figure 2b).

Figure 2 shows the regularly updated activity change of a cow during ninety minutes . When "estrus" behavior occurs in cows, "anxiety" levels increase, thus, this diversity can be observed in the behavioral model. The red line shown in the figure is considered a measure of how much the cow differs from its normal behavior and sends a warning message to the breeder for insemination. The other two lines strengthen the estimation for the insemination time. The green line shows the level of rumination decrease of the cow compared to the previous week (5). It provides an additional criterion to reinforce the diagnosis as it is known that rumination should decrease as estrus signs become evident in the cow. It also shows that the duration of feed intake during this period increased as a "dark blue" trace, compared to the average feeding time of the last week. This is actually the failure of the classification process to distinguish certain types of behavior. In fact, the cows do not eat more, they just spend more time rubbing other cows with their heads before the heat. Meanwhile, the difference observed is the map of secondary estrus behavior pattern (5) (Figure 3).

In addition to using eating patterns, there are 3D automated camera systems that can automatically measure the body condition score which is highly essential for maintaining the longevity, productivity, thus the animal welfare (67). Another interesting approach is monitoring the water volume, drinking frequency and the total water intake. These predictions are being used to detect the heat stress and the reducing the morbidity rates in dairy cattle (26), and growing rates in beef cattle (3).

With the help of smart animal feeding systems, feed consumption can be measured individually and at the same time, it can be ensured that the right ration needed by each individual is taken regularly. Apart from that, the regular weight gain and development of the animal is enabled to be more efficient with the use of these smart feeding models. While this increases the economic efficiency of the enterprise, it also helps to significantly reduce the workforce and early diagnosis of the health condition (stress, metabolic disease, etc.) and reduce the use of antibiotics for treatment (63).

Monitoring systems of behavior and/or biological parameters: The development of behavioral monitoring enabled the operational management interventions in large-scale dairy farms with a collar (transponder) or accelerometer that collects individual activity data from animals. Remote or wearable sensors can be uniquely combined with smart algorithms to continuously monitor a broad range of animal responses intimately linked to stress, health status, and welfare. The concept behind this technology is to create an accurate measurement that ensures reliable basic operational decisions for heat/respiratory stress (69), health condition like sick/healthy (63) and, welfare status social/conformations (167, 176). In order to gather health data for specific expectations (such as calving, mastitis, estrus, diseases) several methods are be combined or used alone including; body temperature (83) (vaginal, udder, ear, rectal and reticulo-rumen), indoor positioning for daily routine (milking and feeding), surveillance cameras, metabolic status (lameness, rumen temperature and pH boluses, rumen bolus, pH), external sensors (neck collar or ankle ribbon). We are able to read the estrus indicators of a cow through monitoring the position inside the barn, rumination behavior, stand-up time, lying time and inactive time with smart herd tracking systems. Based on these parameters, we can predict that the time of delivery with the abrupt cessation of rumination and eating behavior before calving (Table-1).

Table 1. Daily time budget of a dairy cow.

Lying/resting	12-14 hours
Ruminating	7-10 hours
Eating	3-5 hours 9-14 meals a day
Social interactions	2-3 hours
Milking	2-3 hours
Drinking	0,5 hours

Mastitis, as the main treat in dairy cows, is the focus for the development of various types of sensors in dairy industry as early warning and management systems would provide vast economic profits. Within the development of this sensor technology in nineties, various types of sensors are developed in advanced laboratories and introduced to markets. Nevertheless, routine detection of abnormal milk using visual observations during milking and availability of cow site tests limited application of these sensors in a large scale (72). It was the introduction of automatic (robotic) milking systems that boosted the need for sensors to detect clinical mastitis and abnormal milk due to the reduction in inspection time needed to identify mastitic cows. A variety of milk monitoring or sensing equipment to detect electrical conductivity, somatic cell count, milk colour, lactate dehydrogenase activity, milk yield, milk flow rate, incomplete milking have been incorporated and algorithms that use and integrate data captured during the milking process have been developed (79, 117).

Inline sensors are capable of monitoring and recording changes continuously as milk flows through the line or in automatically-collected milk samples. Inline sensors are adapted to be incorporated in conventional and automatic milking systems for mastitis detection (77). Inline sensors allow monitoring of subtle changes in milk non-invasively with remote accessibility to data for multiple diseases, and the ability to store the data. Unfortunately, a high number of false alerts makes individual changes in a single milk-associated parameter inconclusive for mastitis indication. Inadequate sensitivity and specificity by single-sensor methods is largely explained by the influence of other factors, such as milk temperature, milking interval, milk composition variations during the milking process (146).

Mastitis is associated with multiple changes in milk and udder of cow's udder and combining data from different sensors is helpful to obtain a much clearer picture and greater predictive ability. Hence, utilising multi-sensor information is the most recent approach to improve mastitis detection performance (92). Many multiple sensor-based approaches (6, 74, 85, 162) have been suggested to improve mastitis detection performance. In addition to mastitis detection, sensor systems progress toward identification of causative organisms, improvement of treatment and other management decisions at quarter, cow and herd level (128). The disease is multifactorial that affects both animal's physiological and behavioural responses. As sensors such as collarmounted accelerometers and heat detectors are becoming readily available to monitor behavioural changes automatically, such data might be of use for further enhancement of automatic detection of mastitis. Behavioural changes associated with mastitis include alteration in feeding time, lying time, standing time, self-grooming, rumination, head turning frequency, kicking, isolation character, preference for lying on one side, and increase of restless behaviour (39, 55, 89, 112, 135). Such sensor-derived data can increase the accuracy of mastitis detection if combined with milking related data (91).

Precise detection of estrus in cows is essential to maintain reproductive performance, especially in dairy herds using AI. Standing to be mounted is the primary and most characteristic external sign for determining when a cow is in estrus and considered sexually receptive for artificial insemination (137). Signs of estrus are often more intense in evening and night hours. Traditionally, estrus in cows is detected by visual observation (46). However, estrus detection by visual observation is highly labor intensive. In addition, increasing farm sizes and workloads limit the time available for observation of individual animals, resulting in unobserved estrus and remarkable economic losses (37). Furthermore, intensive genetic selection for high milk production has resulted in decreasing durations and weaker signs of estrus (140).

Precision monitoring technologies that continuously monitor and measure behavioral and physiological changes in the cow are commonly used to supplement or replace visual estrus detection. The development of automated estrus detection systems began in the 1980s and several types of automated heat detection devices for dairy cattle were marketed over the years (118). At present, a great number of fully automated technologies including pressure sensing systems that monitor mounting activity, activity meters, temperature measurements, video cameras, impedance or conductivity measurements, and hormone analyses are available (147). Parameters with potential to be used in automated estrus detection systems include but not limited to mounting events, activity level, lying time, rumination events, blood or milk progesterone levels, feeding time, body temperature (47, 59, 147, 156).

In general, automated estrus detection technologies detect estrus in cows mainly through secondary signs of estrus behaviour (59); mainly through multi behaviour patterns (82, 137, 152). To date, most technologies for identifying cows in estrus are based on automated activity measurement (47, 109). Pedometers or accelerometers attached to the leg or neck are able to detect estrus, with a predictable association with the timing of ovulation (9, 71, 119, 139). Automated activity monitoring systems are profitable for most dairy farms and producer satisfaction with their performance is generally high (146). Investment in automated activity monitoring technologies contributes to farm profitability in many scenarios (2, 114). Automated activity monitors use software specific algorithms to compare the activity of each animal with that of an individual specific previous reference period or with the average activity of the herd aggregated over time to create an estrus alert when a set threshold is exceeded. However, many environmental and metabolic effects as the type of housing, the herd management practices, animal health problems and heat stress have negative effects on the performance of automated activity monitoring to identify cows in estrus (2, 130, 148, 156). Other systems including video-software, body temperature measurements and biosensors integrated with in-line milking systems are expected to be combined with existing tools for multivariate estrous detection in near future (37, 118, 148).

Prediction of parturition is central to good calving management affecting animal health, animal welfare and farm economics (113). Supervision during the calving period enabling timely calving assistance is likely to reduce the risk of dystocia associated with increased calf mortality and morbidity, increased health problems in the dams, and the economic impacts that arise from increased treatment costs, reduced calf performance, and reduced reproductive efficiency (155). Historically, a combination of breeding records and visual symptoms has been used to estimate calving time; however, these efforts are hampered by the need for 7/24 monitoring and inconsistency between cows in visual behavioral and physiological changes related to calving (18).

Interpretation of behavioral and physiological changes related to calving, provide the opportunity to develop an automated system for the prediction of parturition, while no large-scale systematic research has provided insight into possible practically implementable solutions (147). Maternal body-temperature monitoring has been the first line application of precision technologies in calving detection, however, reticulorumen, skin, rectal and vaginal temperature monitors are not validated for prediction of parturition (23, 36). Recently, potential use of a calving prediction model based on continuous measurement of ventral tail base skin temperature with supervised machine learning (70) along with intravaginally inserted temperature and telemetry was reported (125, 163). Calving is related to many behavioral changes including lying bout, number of step and eating; while these parameters can be easily monitored using available tools mentioned earlier (124, 144). Meanwhile, it is still contradictory to attribute the changes in the mentioned behaviors to calving only for accurate prediction; as animals might exert other behavioral changes in noncommercial environment (151). Tail raising is another calving related behaviour. Tail-raising events dramatically change prior to calving (81, 115). In a recent study, a tail-mounted inclinometer sensor was used at 5 different intervals (i.e., 1, 2, 4, 12, and 24 h until calving) to calculate sensitivity and specificity. Depending on the interval preceding the onset of parturition, sensitivity varied from 19 to 75% and specificity from 63 to 96%. (178). As a distinct predictor, tail raising monitoring is considered as the best behavioral change to estimate the time for calving using this smart systems; while this prediction accuracy can be increased by combination of eating and rumination behaviors (116).

These systems continuously records individual animal and measured data are processed with sophisticated software, and the data is downloaded wirelessly to a computer each time the animal enters the receiving area of a base station. Alerts showing the animal's status are displayed on a local computer or in the cloud. Each leash learns normal behavioral patterns and the owner warns only when intervention is necessary and allows the farmer to plan a corrective action. Significant differences in the variance of the measured raw data allow the derivation of various behaviors such as rumination and feeding (51, 153). The energy consumed by the animal during its daily routine is individually mapped and defined.

The most important reason for the immediate adoption of animal tracking technologies has been the need to optimize offspring yield in dairy cattle. In cattle, it caused an increase in fertility, selective breeding practices, other welfare factors such as reproductive diseases (eg metritis, ovarian cyst, foot diseases), and consequently a decrease in fertility (58). Additionally, lack of management practices, malnutrition, and inadequate estrus detection contribute significantly to low pregnancy rates. The cost of bovine infertility arises from the loss of income from milk production, artificial insemination, labor cost, and late calving (58). This situation causes an estimated loss of approximately $ 2,333 per cow (98) and according to de Viries (2007), it is $ 555. Although the fertility of the herd depends on many factors, estrus detection is predicted as the most important factor. Detection of estrus in cattle was carried out by a skilled observer or farmer looking for visual signs of estrus.

The Economical Aspects of PLF

In principle, the tools related to precision farming serve a purpose in the management of input allocation to the farmers to decrease the expense of production and increase the outputs by aiming to improve health status, management and production efficiency as well as reducing the labor (14, 146). However, these technologies are available at a considerable price (Table 2).

Table 2. Price range of various precision livestock tools.

Item	Location	Targeted Measurement	Price	Notes
Identification				
Ear tag (RFID)	Ear	Identification/ accelerometer (X, Y, Z axis), Body temperature	1-5 € $1,425.00 for reader	Standard price with little variations
Collar-Transponder	Neck	Identification/ accelerometer (X, Y, Z axis),		Varies according to size and manufacturer
Ruminal bolus	Rumen	Identification Rumen pH, core body temperature	5–450€	Varies according to size and manufacturer
EID Injectable	Subcutaneous	Identification, body temperature	5 €	Only available for dogs with temperature measurement
Injectable EID reader	Subcutaneous	Laboratory Animals	150-500 €	Varies according to manufacturer
Wearable sensors				
Accelerometer	Ear, leg	Activity tracker	$55	
GPS systems	Neck, leg	Geo-satellite positioning system		
Precise Farm Management Tools				
Cow Scale	Stationary	Weight	5 500–7 280 €	Price difference based on size and complexity
Feeding computer (Spider)	Stationary	Automatised feeding	€ 225.75 per unit	Controls up to sixteen feed dispensers within a ten-metre range.
PipeFeeder	Stationary	In-parlour feeding	950-440 €	Prices are per milking point and based on a 2x8 milking parlour. Mounting hardware included.
Feed Station walk-through	Stationary	Walkthrough forwards	4000-6000 €	One/two type of feed
Walk over weight	Stationary	Weight	12 000–15 000 €	Product available for cattle
Automatic Dispensing Liquids	Connected to feed station, milking robot or milking parlour	Liquid intake	Float set (€ 48.08 per unit) not included / 500-2100	Mounting hardware included.
Pasture management tools				
Virtual fencing	Neck, nose	Sound and electrical vibration	$5000 to set up $60-90 each collar	Ongoing maintenance

Milking parlour technology				
Smart milking parlour	Stationary		$40 000–80 000	Variable price according to outfitting, 12–24 stations
Robotic milker	Dynamic/rotational		$150,000-200,000	for 50 to 70 head of cattle
Software				
DairyLive	On PC		$179.00	Dairy management for up to 50 animals
Automated health-monitoring system	On PC		$150-$175 USD per animal (collars + data system)	
CowView	On PC/Smart Phone	Standing time, frequency and time spent in cubicles, time walking, how far and how fast, frequency of visits and time spent at the feed table.	£150	
Let's nurture	On PC	Male and Female Wire Gps device Acid sensor Lithium battery Heart beat sensor	$8000-9000	
Let's nurture	On PC	QR scanning to connect with device Alert for treatment Show all Cattle list Medicine dates Animal Doctor list Hospital list Gps tracking Report of whole day	Cost for application development $7,500 – 10,000 (Include in android and IOS application) and $5000 for web application.	
Smart phones for cows				
Collar	Smart Phone, Virtual Glass	Temperature calving time Estrus detection	The GSM radio costs £2,500 collar £70-£80.	

Table 3. The risk of false positive for health disorders.

	Maximizing Sp	**Maximizing Se**
Alert	**Rumination or activity**	**Rumination or activity**
Sp (%)	97	51
Se (%) (n=404)	21	77
False Positive Rate (Detection/d/100 cows)	2	19

The economic value of the PLF naturally depends on various key factors, including the herd size, characteristics of the farm, accuracy of the reliable data, the value of obtained information that could prevent expenses, number of workers, as well as the social impact. Livestock farms have remarkably variety in terms of their size, housing, nutritional practices, labor, genetics, keeping the records, reproductive management, herd health and welfare, overall substitution strategies, and personal goals, so when there are PLF systems, the concept of "one size fits all" are not valid for all. Even if the critical action could be the same, the ROI (Return on Investment) may vary based on the application used to enforce the action (27, 142). The farm based examples have been illustrated and assessed in a limited number of studies although in general, the returns to investment are still not clear and in terms of yield and economic performance, the output of these precise technologies have not been well-demonstrated yet (7, 90).

In an example, one of the most commonly preferred systems, the implementation of an automated heat detection into a labor and capital intensive dairy farm provided an estimated of € + 7,362 profit while the

economic benefit reaches up to € 3,815 in a labor intensive capital extensive dairy farms (86).

Apart from that, welfare monitoring of the animals is frequently related with production and profit. For example, owing to the use of PLF technologies metabolic disorders such as subclinical ketosis can be prevented. A quick detection of subclinical signs of ketosis could be achieved with an in-line milk test for ketone bodies (160), that could prevent the further economic lost due to decreased milk yield and veterinary treatment cost as € 709 per animal for clinical ketosis. Moreover, if the welfare is emphisased in the desicion model, significant value will remain gained via targeted treatment due to the information provided by these technologies as observed in Subacutre ruminal acidosis (141, 142).

Even though few studies demonstrated the negative effects of automated milking systems on economic performance, currently it is presumably the most widespread PLF technology implemented worldwide due to the reduction in extensive labor and maximizing the time efficiency (73, 146, 161). Similarly, a great economic difference could be encountered between the farms which inseminate once owing to the knowledge of estrus timing provided by PLF technologies and inseminate two-three times a day (99).

The accuracy of predicted ROI depends on the level of monitoring. For example, pig groups ready-slaughter or significant disruption in pig growth can be determined efficiently with multi-level monitoring body weight (164, 165), although, since there are no individual warnings, it is impracticable for a farmer to identify and treat a specific pig. The economic benefit of early treatment of a pig will affect the value of individual identification. Similarly, a dynamic monitoring system was developed for litter size at the herd to estimate future production (17). This idea was adapted to automated milking system data in dairy herds as changing the feeding strategies for selected cows by the overall response measured in milk production (164). In this sense, PLF technologies can provide different and useful decisions for farmers, but elaborates the determination of ROI since more information could be profitable for particular types of farms (141, 174).

The intermittent use of PLF system aids to the detection of the problems a few hours early that could provide the time to act on critical decisions in particular situations to prevent further economic loss such as tail biting on a pen (172).

The low or even negative profitability of some PLF tools (16, 61, 141, 174) may not initially rationalize the large investments of purchasing these systems. However, the use of the framework to assess economic ROI also reveals that most cases of how to use supplementary information are related to operational decisions since the more precise information can provide strategically superior decisions as well as long-term implications. The effects of modifying strategic decisions can also be tangible, making it difficult to define (86, 96, 161) while making them visible will increase the transparency in critical evaluation of the ROI.

As a summary, economic investments (costs) arising from the purchase of sensors and vehicles, would compensate the profits from avoided production problems, along with associated with avoided/reduced losses.

Concerns related to PLF systems

Accuracy of the tools: Although the PLF-tools provide objective measurements, several factors can affect the sensitivity and specificity of the collected data/information and its interpretation. For example, in a broiler farm, the average weight of the flock is assessed by manual measurements or automatically by random sampling a certain number of birds to reveal the growth trend of the flock. Nowadays, “step-on scales” are developed to automatically collect the average weight of the birds in the flock. However, factors determinant of the accuracy of automated weighing relies on the ability of the birds to visit the scale, and in such conditions that impair the mobility of the birds as aging, having excess weight, sickness and lameness, the system may fail to represent the growth trend of the whole flock. In order to encounter these limitations, various methods have been introduced. The bodyweight of broilers on average with a relative error of about 11% from image surface area by introducing a computer-assisted image analysis was estimated (42). Since PLF systems are being improved and updated continuously, more reliable technologies replace the old ones as in this situation, due to the discovery of a relationship between weight and vocalization frequency, it was proven that a reasonably accurate growth trend could be obtained at the farm level (56). In another research, an automated method has been implemented to report the malfunctioning in a broiler farm by using cameras and image analysis software and resulted in a 95.24% accuracy of events (20 out of 21) in real-time. The PLF system used an algorithm that compared the measured distribution of animals with a predicted value to give an alarm to farmers when a 25% more difference was found in the measured distribution from the predicted value (87). When compared with a different algorithm utilizing water use of the birds (132), true positive cases were found 33.3% more while false positives were reduced from 28.6% to 0% with the distribution modeling. Another camera-based technology equipped with automated image processing and transfer function modeling has been utilized to estimate the water use of pigs and resulted in 92% accuracy in the estimation of half-hourly water use (52).

Based on the findings of (71) and (84) the sensitivity of the activity meter to detect the cows which were about the ovulate was 80% while the specificity was 95%.

In a recent attempt to validate the tracking ability of a PLF system, two rounds of video recording were analyzed and resulted in an overall accuracy of over 90% due to the performed optimizations in system configuration after the first recording. Further research has been conducted to determine the variation of the measured values in different tools. Although the lying duration of the dairy cows was significantly correlated between two systems ($r=0.94$; $P<0.001$) the correlation for the number

of steps was found lower (r=0.74; P<0.001) presumably due to the difference in the measuring steps between the two systems (53). In addition, a study on the detection of different health disorders with cumulative summary of rumination and activity data, 40% of the health disorders detected by farm staff with the sensitivity around 28% for mild lameness to 85% for severe mastitis. Roundly, half of the health disorders were detected one day earlier than the farm personnel.

User Friendly Interfaces: The use of information systems does not always lead to increased business efficiency. As the information environment becomes increasingly saturated, users may start finding the data search process confusing (40). In addition, the order of information within a system can be complex, overmuch data appears on the screen, leading to information overload for the user (184) When information overload occurs, the users' decision-making performance decreases (34).

In addition, systems currently used without considering user friendliness (111) but expected to improve decision-making performance (169). A well-designed user interface can positively affect the decision-making performance of users (182). A study was conducted to overcome this issue and developed a decision-making performance and cognitive load for potential users interested in livestock, animal biotechnology and veterinary science and farming.

The importance of friendly user interfaces increase due to the integration of these technologies into the daily life routine (25). However, when the different potential users have considered the user interface becoming a key element for the system (31, 68). Nowadays systems are can be classified into basic 3 categories as software, computer, other devices that operate codes and utilize a visual graphics can be viewed remotely. Thus, technology can communicate with human beings through graphical elements, messages and early warning alerts. Furthermore, the system could learn the system elements with machine learning in time and carry on working without the need for human touch (52) and information can be understood differently by users depending on their cognitive styles (117).

Information and communication technologies revolutionized the traditional farming system and became popular these days. The most triggering factors are the educational level between farmers and difficulty to implement new technologies (29), limited information and communication infrastructure in rural areas (157) low interest of understanding and use (186).

Another issue is the human factor which needs to be improved by designing the friendly user interfaces (168). Most of the previous work on agricultural information systems focused on system elements other than interfaces to improve system utilization. However, poor user interfaces are frequently cited as a problem in agricultural information systems (44, 110).

The user interface serves as a complex programming language and a communication tool between users thus the interface is a key essential. Therefore improving the interface design makes it easier to understand the log of farm data and make correct decisions (158). There are several examples of the commercialization of PLF techniques in livestock production. The models that are being used for commercial adaptations are; the use of robotics, egg counting, bird weighing, environmental control, precise feeding systems, climate control, automatic disease detection, and growth measurement (64). Overall, there was limited evidence of commercial PLF products used on farms. As expected, farmers in techno-friendly countries are more likely to embrace technology to reduce their dependence on hard-to-find (and expensive) workers and to make their lives a little more comfortable (87).

PLF technologies are mostly developed by researchers from the beginning and that have received support from the private sector only in the last decade. This researcher-private sector collaboration is a critically essential step in the development of friendly user interfaces for the use of the ministry officials and breeders (88). Artificial intelligence systems provide suggestions by the breeder about which animal is sick, which is in heat, and which will be deliver. That's why, many PLF systems set normal range of parameters for the infrastructure of the enterprise and the routine behavior of the animal and alert the breeder when any deviation from the normal range take place. This provides the basis of reliable information and correct decision-making in the routine learning process of artificial intelligence that requires mastery (134).

Security Issues: Precision livestock technologies are gaining more attention due to the future possibility to comply with consumer demands and the global food supply chain. There are numerous precision farming use cases (12, 32, 38, 105) that indicate the impact of this new farming practice paradigm globally. In India, farm data have been used to predict and prevent crop diseases that reduce the risk associated with crop production failure (171). Smart agriculture employs not only at the production stage where health related issues are on great focus, but also the entire food supply chain. This enables a whole new revolutionary model, where big data from the entire agriculture business structure is processed to provide critical insight for on-time operational decision making (5). Intelligent agriculture increases traditional agriculture practices by offering precision tools in the field. That tools and sensors work synergistically to deliver improved crop yields as well as productive farming experiences. In spite of the fact that advantageous for industry efficiency, the utilize of diverse, IOT tools has uncovered potential cyberattacks and vulnerabilities within the agriculture industry. These assaults offer the capacity to remotely oversee and utilize sensors and independent vehicles (drones, smart tools and etc.) within the field. Potential agricultural attacks can provide a risky and inefficient farming environment. Different examples of a cyberattack have been generated, and some of these examples are overwhelming. Such gigantic facilitated assaults moreover alluded to as agro-terrorism (94), in expansion, illustrate the potential of disturbing the

economy of an agriculture-dependent country. There are such report exists on the potential risk of cyber-attack scenarios in smart farming practices and highlights the critical control points for researchers (122). A sophisticated farm-terrorism could impair the millions of consumers' health globally. Along with that fact, such a threat on farming systems can decrease the reliability of consumers' preferences and may impair the trustworthiness of the exporter countries. In a report published by the United States, it was emphasized that cybersecurity is extremely important in the agriculture and livestock sector and is one of the critical control points for national security. While 11 cyber-attacks were reported in 2016, he emphasized that the threat of cyber-attacks targeting the IoT infrastructures used by farm systems may increase. Compared to other sectors (banking, finance defense industry, etc.), the awareness about agriculture and animal industry is still weak." This offers an enormous gap between using smart farm technology and ensuring it is accurate and permanent. If not constantly monitored, cyber-attacks against smart farming technologies can have profound effects on various stakeholders in the ecosystem. These groups include farmers, end consumers, food processing industries, agricultural cooperatives, animal husbandry, government agencies, and countries that are critically dependent on agriculture (104).

Confidentiality of the data collected via PLF tools has great importance both for farmers and technology companies. Due to misuse or loss of the data, farmers may face financial or emotional impacts whereas companies may face a reputational loss. Possible scenarios might include the leakage or theft of the data, use of confidential information either to gain profit or to damage a company and/or foreign access to unmanned aerial systems. Similarly, the integrity of the data could be compromised by tampering with the data to interfere with the livestock sector, introducing rogue data into the network of a sensor to damage a herd as well as the inadequately vetted machine learning. Apart from that, specific threats to equipment availability may have an origin of either natural disasters or cyber-related issues such as disruptions to space or ground-based positioning navigation and timing systems as well as to the communication networks (127).

Animal-Farmer Relationships: The daily activity of the farmer is started to change by the adaptation with smart farming technologies directly or indirectly by the need for less contact comparing to the traditional farming managements. This new adaptation to the PLF concept may lead to the extent of the distance between human-animal relationships (57).

Meanwhile, precision livestock farming can damage an animal-farmer relationship. The time that a farmer employs to spend with the animals will decrease in time. The habit that farmers will gain through the automatized system might reduce the beneficial opportunities and recognition abilities in between. In the traditional animal farming system, there are many common practices that require direct human-animal interactions like; dehorning, injections, milking, treatments, etc. The less time that farmers spend with modern technologies, the more animal will become tempered. The ratio of positive and negative interactions can be altered equally. Regular opportunities for beneficial interaction, such as feeding times, may decrease with the bonds they create (175).

Again, the beneficial effect of PLF on the farmers daily routine could be denoted such as the decrease at the workloads like moving the animals to the dispenser pen or the milking push. Current milking technology under the smart robot control made it possible to milk the animal when they need or that are cow are not self-milking. Another advantageous condition is that when an animal received a stressful practice the rumination behavior is altered so consequently the production capacity and welfare. This condition might turn into something that, farmers never to have to spend time to bond with the animal (76, 95).

The complicated task needs to require some technical knowledge for farmers to sort the big data, visualize the data as graphics and finally take a decision with the right judgment. This might create another risk about the losing computational skills by the owner. On the other hand, the PLF provides the understanding of individual animal identity and can totally change the perspective of point in animal husbandry (17).

The adaptation of animals to new systems are relatively quick compared to humans. A study showed that the robotics milking system gives more freedom to the animal and interestingly when it is given free of choice as well (49), given that the animal has to do first, it is still a restricted system on which movement circuits are imposed. If he wants to rest or feed, go over the robot. The PLF technologies not only providing freedom to the animal but also created more time to spend by the farmer and take the focus on animal welfare and positive interactive habits (175).

The most recent technology may decrease the distance between humans and animals by collecting more reliable data with PLF, thus more individual information at the herd level. Nevertheless, the real scenario behind this is hidden underneath that "numbers farming" with providing more-in-depth information about their needs. There are opposing views about the discussion on factory based agriculture system. Some animal rights advocates claim that the technology actually triggers the growth of a factory-based agricultural industry. This claim must take into account seriously and the truth behind it must be understood and use as an encouragement for better animal care and welfare. This gives farmers the power to make better choices based not on profit alone, but rather on the actual needs of the animals and their care at all times. Another important point is the intensification of animal production systems with PLF technologies. There is a risk of abuse the animal via production climax thus altering animal welfare. Apart from that, the reliance to these technologies may lead to a point where it is possible for the owner to fail to notice the signs of important diseases due to the decreased time of animal-farmer contact (76).

In addition, new developments in the animal behavior field and updated biological parameters could change how farmers perceive animals and how animals

perceive humans. On the other hand, with the help of artificial intelligence and computers, it can affect the development of decision-making mechanisms and new job descriptions, how the new generation farmers experience today's profession and dreams, and their job satisfaction or dissatisfaction. Finally, existing technologies may not always create a significant distance between livestock and farmers. Technologies today may perhaps enable the development of new relationships with the animals of millennials. (75).

A study conducted on the human-animal relationship as a survey; where farmers were asked what they believe about the human-animal relationship. Most of them tried the avoid the question for two reasons. The first is the definition of the new term and the other reason was the personal emotional distance. For this reason, four farmers thought it was unrelated to their profession. It was more straightforward for farmers to discuss their views on an exact human-animal relationship. Frequently they spoke of the welfare of the animal, and some spoke of the animal's lack of fear of humans, even of a mutual trust between the farmer and the animals. For some farmers, good production levels reflected a satisfying human-animal relationship. For the majority, a good human-animal relationship makes it easier to work with animals, regardless of the breed. They also talk about the well-being of the farmers and good livestock farming with equipment. Interestingly, a certain number of farmers that they surveyed are reported in some studies claiming the human-animal relationship going worse (94). On the other hand, we all agree that PLF technologies must be reinforced to build up a better relationship with the animals and humans not to worsen them, but to improve and transfer to the next generations.

The ownership of the data: There is an increasing debate on the ethical issues related to precision livestock farming. One controversial discussion is related to the collection of data from animals, as if they are an instrument to improve business process control. While, animal welfare improvement relies on accurate data and real-time knowledge to be collected from these devices, strict considerations to minimize the stress for capturing and handling to fit the collar or any tracking device should be considered; as no animal “likes” to be tagged. This should be in accordance with animal rights, as animals are sentient beings that have moral status and preference autonomy that they have vital interests humans must not override” (1).

Another issue is related to the possibility of mechanization of the breeding systems as it is expected to disturb human-animal relationships, turning animals to objects of data. Objectification perspectives within “treating animals as objects' ' and “turning animals into as objects” and instrumentalization perspective on the frame of PLC, requires novel intuitions of the ethics of care (19).

As the PLF systems become more widespread tools in livestock industries, a considerable amount of data is being collected in the meantime, arising discussions on data distribution and ownership (28, 143). Apart from a number of exceptions, the data produced is not yet distributed fully among the food chain actors and there is a lack of compatibility between data sets that may favor the quality assurance of the supply chains (103). It is still in a debate who has the potential ownership of the collected data, between the two parties, the farmer, as the owner of the animals and the software manufacturer as the data processors.

Rapid improvements on the current PLF technologies rely mostly on continuous monitoring of the animals as well as deducing the relevant information by analyzing the raw data. In fact, the information as actionable insights transferred from the monitoring, in the form of charts and reports are more valuable to the farmer than the raw data. However, the companies that develop PLF technologies can benefit from this data, more particularly when it is combined with the data from other companies to form big data. Furthermore, companies can produce income directly by selling it as reference data. Besides, a great potential of value lays in for other stakeholders as third parties in the circulation, such as veterinarians, feed, breeding pharma and technology companies, slaughterhouses, retailers, the consumer, processors, certifiers, citizens, governments and researchers (93). Therefore, it might not be fair for the farmers to be the only one that pays for the technology while other stakeholders depend on the data generated by the farmer to both to and contribute to the PLF platform. The determination of the data ownership must be properly regulated with legal frameworks in order to establish collaborations and to build a future market for agricultural data (21).

Ethical questions related to the IoT

- IoT used for medical purposes on animals would have the status of medical devices if used in humans. For humans, medical devices have specific regulations to ensure the health and safety of the user. As surprising as it may seem, there is no regulatory framework in France concerning connected objects in terms of both expected efficacy and safety. The implementation of a harmonized methodology for evaluating these tools and a material vigilance system would undoubtedly be necessary.

- These tools that continuously produce data (and sometimes alerts in case of deviation of observed data from expected data) can be stressful for their users, especially when the tools lack specificity (alerts generated on non-diseased animals). Faced with this incessant flow of data and alerts, the risk is that the owner loses confidence in the tool (and stops looking at the data at the risk of missing sick animals) or, on the contrary, that he decides to do something at the first alert without discernment. The owner's better knowledge of the animals via these IoT and the optimization of the care provided thus relies largely on the performance of the tools and their operating conditions.

- The absence of a specific regulatory framework for IoT on animals often means that the only choice to equip the animal is often made by the owner. While it is obvious

that there can be no question of obtaining the direct consent of the animal, it is legitimate to question the circumstances in which one can freely decide whether or not to equip it and whether one can simply freely decide without control to equip animals, particularly when the objects may be invasive (number of tools, nature of the tools). The question of an opinion, in the absence of validation, by a specialist in animal health or behavior arises in particular to validate the interest of the equipment and, if necessary, the choice of technical solution. It is also essential that users be properly trained in all the potentialities of the tool.

- Finally, connected objects require the use of natural resources (metal, electronic circuits and rare materials that are sometimes difficult to recycle) and also energy for their proper functioning and the storage of associated data. Some connected objects can offset these environmental impacts if they make a greater positive contribution, for example by reducing the quantities of inputs or water consumption in crops. In any case, their overall impact (interest for the animal, the farmer and the environment) should be considered.

- The issue of the digital divide and white zones can also lead to a lack of equity between farmers and between zones.

Ethical questions related to the impact of connected objects on the human-animal relationship

- The diversity of the available IoTs makes it possible to access very fine data at the animal level that can modify or influence the perception that the breeder or owner has of his animal. Thus a farmer can now go beyond the knowledge of his animals by their simple performance (as allowed by the first tools developed) and have access to their movement, their behavior (feeding, sleeping) and their location. This can allow a better understanding of the animal and allows a more personalized approach to be envisaged.

- Nevertheless, while this use of technology can provide real working comfort in a context of a constantly decreasing workforce and increasingly large herds, there is a risk of a form of distancing between the farmer and his animals. As an example, a study carried out among cattle breeders with heat detection devices underlined the positive impact perceived in terms of working comfort (including safety at work due to less handling) but highlighted a fear of the farmer of loss of animal competence. It is important to consider these tools not as substitutes for the farmer's eye, but as a complement;

- The massive and continuous collection of data of interest (milk production, growth rate, behaviors, disease resistance for example) opens the way to what is known as high-throughput phenotyping, i.e. the characterization of all the apparent characteristics of an individual, continuously and almost in real time using connected sensors and tools. This fine phenotyping is the key to then carry out genomic studies allowing the selection of animals carrying the characteristics deemed to be of interest (such as disease resistance, a phenotype that is very difficult to characterize classically). To do this, the construction of the tools is fundamental in order to associate different people from different backgrounds from the outset to develop the tools, while not forgetting to associate the end user in particular. Connected tools could make it possible to bring out the individual in the group and thus give visibility for the breeder to isolated individuals, especially in large numbers. However, one could fear the opposite effect, i.e. an extreme standardization/standardization of the animals leading to genetic impoverishment or loss of the individual by eliminating individuals that go beyond the hoped or expected standards.

Ethical questions related to the status and use of data from connected objects

- Potentially, the data collected through the different IoT can serve several purposes and several people. Also, a provider is transparent if all purposes are exposed to the user. In addition, as a data collector, it must demonstrate data governance that ensures that there is no data leakage to a third party. Similarly, the question of data valuation beyond the farm arises. Indeed, for example, the high throughput phenotyping type data allowed by these tools must be able to benefit the "breeder" without the breeder paying twice for it (by first equipping himself and then paying more for the data of interest that he has helped to produce).

- Continuous and possibly remote access by the veterinarian to the data generated by the IoT embedded in the animals opens up interesting perspectives in terms of teleconsultation or tele-expertise capable of optimizing the health and well-being of the animals, particularly in areas of medical deserts that are also used on a daily basis in non-deserted areas. However, the help that these IoT could bring cannot hide the need to address the issue of land use planning and permanent health monitoring.

- The mass of data generated may make it possible to rethink the client-veterinary relationship, opening up the field of telemonitoring and an "increased" clinical examination for the veterinarian, who would thus have access to measures not otherwise available or continuously, whereas they are currently only accessible to him at the animal's bedside. Conversely, the breeder should not be overwhelmed with information and contact the veterinarian as soon as the first data is received or contact him only in a dematerialized and frenetic way. It is indeed the complementarity of the approaches that should benefit the animal. The challenge is then to explain to customers what attitude to adopt when faced with these tools, which cannot entirely replace bedside care. The use of these new technologies will also require veterinarians and owners (breeders, pet owners) to be adequately trained in the use of these tools and the data and alerts they generate.

Thus, beyond the undeniable technical advances made possible by the connected tools, the fact remains that

they raise ethical questions that, if not resolved, deserve to be debated.

Conclusion

The advance of novel technologies and informatics has increased the worldwide demand for integration of PLF systems to local farms. While monitoring tools for physiological, behavioral and environmental parameters and analyzing software are evolving around the Internet of Things; PLF provides cost-effective production with prudent/less drug use and is relatively more environmentally friendly. As IoT technologies for PLF are still in the development stage and information is more valuable in this era of the big data world, legislations and regulations, unfortunately, follow behind, in terms of safety and ownership of the data. Deanimalization and commodification are the main ethical issues discussed around the PLF topic. The increase of the efficiency and sustainability of farming and livestock production is inevitable by properly applied PLF; where the welfare of the animals would reflect the animal health. This would enable the traceability of the food chain and food safety.

Conflict of Interest

The authors declared that there is no conflict of interest.

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