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Isolation and Molecular Identification of *Campylobacter* spp. from Vaginal Swab Sample Obtained from Sheep Herds with Abort History

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Abstract

Campylobacteriosis is a contagious and zoonotic infection characterized by abortion and infertility in cattle and sheep. The objective of this study was to investigate *Campylobacter* spp. cause to abortion in sheep herds in Kars province. For this purpose, a total of 350 vaginal swab samples obtained from sheep with abortion were examined by cultural and molecular methods. Swab samples were inoculated on Preston Campylobacter Selective Agar for isolation of *Campylobacter* species. Following the incubation, suspected colonies were subjected to Gram staining, mobility, oxidase and catalase tests for identification. Multiplex PCR (m-PCR) was used for the identification of *Campylobacter* isolates at species level. *Campylobacter* spp. was isolated in 8 (2.28%) of the 350 vaginal swab samples examined. Of 5 isolates were identified as *Campylobacter jejuni* and 3 were *C. coli* by m-PCR. According to the data obtained from this study, it was revealed that *Campylobacter* species should be taken into consideration in the abortion cases of sheep in this region. Considering the risk of this infection both in terms of animal health and human health, it is thought that more attention should be given to protection and control measures.

Keywords: *Thermophilic Campylobacter spp., Sheep, Vaginal swab, PCR*

Abort Öykülü Koyun Sürülerinden Alınan Vaginal Sıvap Örneklerinden *Campylobacter* spp. İzolasyonu ve Moleküler İdentifikasyonu

Öz

Campylobacteriosis, sığır ve koyunlarda yavru atımı ve infertilite ile karakterize, bulaşıcı ve zoonotik bir infeksiyondur. Bu çalışmada, Kars ilindeki koyun sürülerinde gözlenen abort olaylarının *Campylobacter* spp. yönünden araştırılması amaçlandı. Bu amaçla, abort olgularının görüldüğü koyun sürülerinden alınan toplam 350 adet vaginal sıvap örneği kültürel ve moleküler yöntemlerle incelendi. *Campylobacter* türlerinin izolasyonu amacıyla alınan örneklerin Preston Campylobacter Selektif Agara ekimleri yapıldı. Üreme sonucu şüpheli kolonilere identifikasyon amacıyla, Gram boyama ve hareketlilik muayeneleri ile oksidaz ve katalaz testleri uygulandı. *Campylobacter* spp. olarak belirlenen şüpheli kolonilerin tür düzeyinde identifikasyonu için Multiplex PCR (m-PCR) kullanıldı. Toplamda incelenen 350 vaginal svap örneğinin 8 (%2.28)'inde *Campylobacter* spp. izolasyonu gerçekleştirildi. Multiplex PCR sonucunda 3'ü *Campylobacter jejuni* ve 5'i *C. coli* olarak tespit edildi. Bu çalışma sonucunda elde edilen verilere bakılarak yöremizdeki koyunlarda meydana gelen atık olgularında *Campylobacter* türlerinin de göz önüne alınması gerektiği ortaya konulmuştur. Bu infeksiyonun hem hayvan sağlığı hem de insan sağlığı açısından oluşturduğu risk göz önüne alınırsa koruma ve kontrol tedbirleri açısından daha fazla önem verilmesi gerektiği düşünülmektedir.

Anahtar sözcükler: *Termofilik Campylobacter spp., Koyun, Vaginal sıvap, PCR*

INTRODUCTION

Sheep breeding constitutes a significant part of the animal husbandry of Turkey. According to the data of 2017, the sheep population of Turkey is about 33 million and 450 thousand of which are farming in the Kars region [1]. One of the most important problems encountered in sheep breeding and economically damaging to the breeder is the abortion case. Bacterial, viral and protozoal infections are among the causes of abortion in animals. These

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infections are important in terms of public health as well as economically. Among the bacterial infections, brucellosis, campylobacteriosis, chlamydiosis and salmonellosis are responsible for most cases of abortion [2,3].

Campylobacteria which are pathogenic microorganisms for other animals and humans, can be found commensively in the intestinal flora of various domestic and wild animals and can cause gastrointestinal and genital infections in some cases. *Campylobacter* species cause epidemics in sheep and sporadic infections in other animals. Although healthy sheep can carry the bacteria in the intestine and gallbladder without clinical infection, some *Campylobacter* species can cause systemic infections. *Campylobacter* was first isolated from the aborted sheep fetus. Agent spreads to the environment through feces and genital secretions of infected animal and aborted fetus. When the disease first comes out, abort cases in the herd is seen 60-70%. *Campylobacter* infections are characterized by abortion, stillbirth, birth of premature and poor lambs in the 4-5th month of pregnancy and death of sheep due to metritis [4-8].

Campylobacter jejuni, *C. coli* and *C. fetus* subsp. *fetus* are species that are common in the world and cause reproductive diseases in sheep. The agent is Gram negative, motile and microaerophilic. Environmental samples such as soil, water and food can be contaminated with *Campylobacter* spp. as the result of contact with contaminants such as feces and aborted fetus. It is known that *Campylobacter* species cause cross-infection among some animal species [9]. Roug et al.[10], isolated *C. jejuni* and *C. coli* from sheep, goat, cattle and pigs in agricultural fairs in California. Results of this study are thought to show transmission *Campylobacter* species among animal species. Pao et al.[11], showed that sheep in small ruminant farms were exposed to *C. jejuni* infections at a greater risk than goats. Healthy sheep serve as reservoirs for *Campylobacter* species, especially in stressful conditions such as birth, weaning, and nutritional changes [12,13].

In this study, it was aimed to investigate the *Campylobacter* spp., which is one of the important abortion agents, from vaginal swab samples collected from sheep herds in the Kars region.

MATERIAL and METHODS

Ethical Approval

The experiment was carried out with the approval of Kafkas University Local Ethical Committee for Animal Experiments (KAÜ-HADYEK/2018-114).

Samples

Totally 350 vaginal swab samples obtained from 7 sheep herds in the Kars region were investigated for *Campylobacter* species.

Bacterial Isolation and Identification

In this study, vaginal swab samples were examined by the culture method. For pre-enrichment step, samples were inoculated in Preston Campylobacter Enrichment Broth containing 7% defibrinated horse blood and Preston Campylobacter selective supplement (SR117, OXOID) and were incubated in microaerobic conditions at 37ºC and 42ºC for 48 h. After incubation, 100 µL of the pre-enriched culture was plated on Preston Campylobacter Selective Agar plates and the plates were incubated at 37°C and 42°C for 48-72 h. The growth cultures were evaluated for the colony morphology, microscopic appearance, catalase and oxidase properties [14,15].

DNA Extraction and Multiplex PCR

The classical phenol-chloroform extraction method [16] was used for DNA extraction from the Campylobacter isolates. Then, the multiplex PCR (m-PCR) technique was carried out for thermophilic *Campylobacter* and the multiplex PCR was for *C. fetus* and *C. veneralis* [17,18]. The primer sets targeting the 23S rRNA gene of *Campylobacter* spp., the *hipO* gene of *C. jejuni*, the *glyA* gene of *C. coli*, *C. lari*, the *cstA* gene of *C. fetus*, the *virB11* gene of *C. veneralis* were used with the exception of the specific amplified products as 650, 323, 126, 251, 764 and 233 bp respectively *(Table 1)* [17,18]. Both genus and specific-specific PCR was conducted in a single reaction.

Each multiplex PCR tube for thermophilic *Campylobacter* spp. contained 200 µM dNTP (Thermo Scientific, Lithuania); 2.5 µL of 10x reaction buffer (Thermo Scientific, Lithuania), 20 mM $MgCl_2$ (Thermo Scientific, Lithuania); 0.5 µM *C. jejuni* and *C. lari* primers; 1 µM *C. coli* and *C. fetus* primers, 2 µM *C. upsaliensis* primers; 0.2 µM 23S rRNA primer *(Table 1)*; 1.25 U of *Taq* DNA polymerase (Thermo Scientific, Lithuania), and 2.5 µL of whole-cell template DNA. The volume was adjusted with sterile distilled water to give 25 µL. DNA amplification was carried out in a thermocycler (Bio-rad, U.S.A) using an initial denaturation step at 95°C for 6 min followed by 30 cycles of amplification (denaturation at 95°C for 0.5 min, annealing at 59°C for 0.5 min, and extension at 72°C for 0.5 min), and was finalized with an extension at 72°C for 7 min.

Each multiplex PCR tube for *C. fetus* subsp. *fetus* and *C. fetus* subsp. *veneralis* contained 0.5 mM of each dNTP (Thermo Scientific, Lithuania), 2 µL of 1x reaction buffer (Thermo Scientific, Lithuania), 2.5 mM $MgCl_2$ (Thermo Scientific, Lithuania), 0.625 µM MG3F/MG4R primer set, 0.375 µM nC1165g4F/nC1165g4R primer set, and 1.5 U Taq DNA polymerase (Thermo Scientific, Lithuania), 1 µL of whole-cell template DNA. The volume was adjusted with sterile distilled water to give 20 µL. For amplification, the following cycling conditions were used: initial denaturation for 3 min at 95ºC followed by 35 cycles of denaturation for 30 sec at 94ºC, annealing for 30 sec at 53ºC, and extension for 1 min at 72ºC.

Table 1. *Primer sequences used in the multiplex PCR assay and the expected sizes of the amplified products*

Primer	Sequence (5'–3')	Size	Target Gene
23SF 23SR	TATACCGGTAAGGAGTGCTGGAG ATCAATTAACCTTCGAGCACCG	650	*C. jejuni* 23S rRNA
CJF CJR	ACTTCTTTATTGCTTGCTGC GCCACAACAAGTAAAGAAGC	323	*C. jejuni* hipO
CCF CCR	GTAAAACCAAAGCTTATCGTG TCCAGCAATGTGTGCAATG	126	C. coli glyA
CLF CLR	TAGAGAGATAGCAAAAGAGA TACACATAATAATCCCACCC	251	*C. lari glyA*
MG3F MG4R	GGTAGCCGCAGCTGCTAAGAT TAGCTACAATAACGACAACT	764	*C. fetus*
nC1165g4F nC1165g4R	AGGACACAAATGGTAACTGG GATTGTATAGCGGACTTTGC	233	Cfv

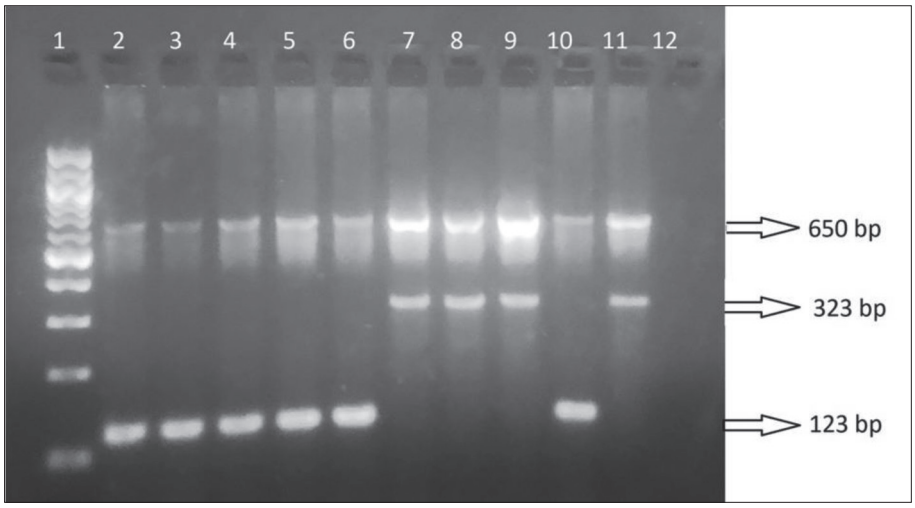

Fig 1. Gel electrophoresis image of m-PCR for thermophilic *Campylobacter* species. 1: DNA marker (Gene ruler 100 bp DNA Ladder, Fermentas); 2-9: Positive samples; 10: Positive control for *C. coli*; 11: Positive control for *C. jejuni*; 12: Negative control

The PCR reaction is accompanied by the *Campylobacter* reference strains and the amplified products were visualized by 1.5% agarose gel electrophoresis and the images were photographed under UV transilluminator (UVP, CA 91786, U.S.A.).

RESULTS

As the result of cultural examination colonies of the *Campylobacter* spp. were isolated showing microscopic characteristics such as small size, pinpoint morphology, non-hemolytic, and Gram-negative "gull-wing" shaped bacilli. Suspected isolates were subjected to biochemical tests. Thus, *Campylobacter* spp. was isolated in 8 (%2.28) of the 350 vaginal swab samples. Eight isolates, which were characterized as *Campylobacter* spp., were identified as *C. coli* (in 5 isolates) and *C. jejuni* (in 3 isolates) by using species-specific multiplex PCR *(Fig. 1)*.

DISCUSSION

Sheep farming has great importance for husbandry in Turkey. Abortions caused by infectious agents in sheep breeding are an important problem. These agents lead to significant economic losses, not only to a loss of an offspring but also to a decrease in milk yield, a decrease in the breeding value and in some cases infertility. Brucellosis (20-33.7%) was the first agent to be seen in the investigations of the infections causing abortion in sheep and this was followed by campylobacteriosis, chlamydiosis, listeriosis, and salmonellosis [3,19,20]. Campylobacteriosis is widely occurred all over the world and can be transmitted to people in contact with food, water, livestock and domestic animals, especially poultry [21,22]. Campylobacteriosis is the important cause of abortion in the sheep in many countries including Turkey [23-27]. Yardımcı et al.[28], reported that blood sera samples taken from sheep in Van region were analyzed by ELISA and detected *Campylobacter* antibody positivity in 39% of samples.

Many studies have been conducted to show *Campylobacter* spp. existence in sheep in many parts of the world. In the USA, Hansen et al.[29], reported as 5-17% risk of abortion due to *Campylobacter* species. Fallah et al.[25], have investigated 132 aborted sheep fetuses by PCR and showed 12 (9.09%) *Campylobacter fetus* subsp. *fetus* and 2 (1.51%) *Campylobacter jejuni* in Iran. Allsup [30], reported that Campylobacteriosis was the third responsible agent in

sheep abortion and increased from 6.8% in 1982 to 13.1% in 1984 in England. Species were determined according to the order of prevalence as *C. jejuni*, *C. fetus* subsp. *fetus* and *C. coli*.

Campylobacter species cause serious problems for animal and human health in our country as well as in the world and cause labor and economic losses. Karaman and Küçükkayan [31], have reported that *Campylobacter* spp. were isolated in 4 (1.3%) out of 297 aborted lambs obtained from different provinces between 1993-1997. In a similar study conducted by Küçükayan et al.[6], *Campylobacter* spp. were isolated in 6 (1.29%) out of 463 fetuses in 2003-2007 and all of them were identified as *C. fetus* subsp. *fetus*. Diker [32], had isolated *C. fetus* subsp. *fetus* from 15 (12,09%) out of 124 aborted sheep fetuses. Kenar et al.[33], reported that they isolated *Campylobacter* spp. in 20 (6,6%) of 303 aborted sheep fetuses. Kenar and Erganis [34], investigated 35 aborted sheep fetuses in Samsun and neighboring provinces during lambing season in 1991-1992 and *Campylobacter* spp. were isolated in 8 (22.9%) samples of which 5 (62.5%) were *C. fetus* subsp. *fetus*, 2 (25%) were *C. jejuni* and 1 (12.5%) was aerotolerant *Campylobacter*. Ekin et al.[24], investigated the presence of *Campylobacter* spp. in the gallbladder of healthy sheep in 2000 and 2002 years in Van region and found the *Campylobacter* spp. year-based prevalence as 27 (24.6%) and 24 (21.8%), respectively. Of the 27 *Campylobacter* strains isolated in 2000, 14 were identified as *C. jejuni*, 7 as *C. fetus*, 3 as *C. coli* and 3 as *C. lari*. Yeşilmen [12], have isolated the *Campylobacter* spp. in 10 (10%) out of 100 aborted sheep fetus in Diyarbakır province. Seven (70%) of the isolates were identified as *C. fetus* subsp. *fetus* and 3 (30%) were determined as *C. jejuni*. Büyük et al.[2], isolated *Campylobacter* spp. from 4 (10.25%) of 39 vaginal swab samples taken from sheep in Kars region. In a study conducted by Karakus [35], in Kars region, while both cattle and sheep have an important role as a source of *C. jejuni*, it was found that sheep played a more important role especially in the spread of *C. coli* to the environment.

In the present study, vaginal swab samples collected from sheep herds with abortion were examined in terms of *Campylobacter* species. *Campylobacter* spp. isolation was achieved in 8 (2.28%) vaginal swab samples. As the result of species-specific PCR analysis of isolates, 5 (62.5%) were identified as *C. coli* and 3 (37.5%) were *C. jejuni*. *Campylobacter* spp. isolation rate has varied between 1.2% and 92% in sheep in the world and in Turkey [2,26,36,37]. The results of this study were consistent with lots of researches. It is suggested that the factors cause the differences among the studies are the transport conditions of samples to laboratory, age and number of sampled animals, sampling season, isolation method and selective media used, hygiene and geographic structure [8,38].

In this study, it was revealed that *Campylobacter* infections should be taken into consideration in abortion cases occurring in sheep. It is also important since sheep can contaminate the environment and food with secreting the *C. jejuni* and *C. coli* and may play important role in human beings. Increased rate of isolation of *C. coli* from sheep will need more epidemiological investigations on this species as the *C. jejuni* is the primarily thermophilic agent in abortion cases.

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