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Short Communication

Ovine Abortion Associated with *Campylobacter fetus* subsp. *fetus* ST2 in Turkey

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Abstract

In this study, we aimed to evaluate the microbiological, molecular and pathological findings of abortus cases detected in a sheep herd consist of 200 animals. Macroscopically, irregular necrotic foci were observed in the liver in aborted fetuses. Selective and non-selective media were used for the isolation of causative agent. Phenotypic and molecular tests were performed for identification. *Campylobacter fetus* subsp. *fetus* (Cff) was isolated from organs of four foetuses aborted. While the vaginal swap samples taken from sheep that did not abort, water, feed and litter samples produced negative results for Cff, 5 of the 20 faecal samples and 5 of the vaginal swaps taken from the sheep that had aborted, yielded Cff. Multilocus Sequence Typing (MLST) was used for genotyping and all isolates were detected as Sequence Type 2 (ST2). This is the first documented report of an ovine abortion caused by Cff ST2 in Turkey. It is considered that the development and use of vaccines, containing local *Campylobacter* species, would contribute to both prophylaxis and control of abortions caused by campylobacters including *Campylobacter fetus* subsp. *fetus*.

Keywords: *Campylobacter fetus subsp. fetus, MLST, Sheep abortion*

Türkiye'de *Campylobacter fetus* subsp. *fetus* ST2 İlişkili Koyun Abortusu

Öz

Bu çalışmada, 200 başlık bir koyun sürüsünde görülen abortus olgularının mikrobiyolojik, moleküler ve patolojik bulgularının değerlendirilmesi amaçlandı. Aborte fetuslarda makroskopik olarak karaciğerde düzensiz nekrotik odaklar gözlendi. Etken izolasyonu için seçici ve seçici olmayan besiyerleri kullanıldı. İdentifikasyonda fenotipik ve moleküler testlerden yararlanıldı. Atık dört fötüsun organlarından *Campylobacter fetus* subsp. *fetus* (Cff) izole edildi. Abort yapmayan koyunlardan alınan vajinal swap, su, yem ve altlık örnekleri Cff yönünden negatif bulunurken, abort yapan 20 koyunun dışkı örneklerinin 5'i ve vajinal swap örneklerinin 5'i Cff pozitif olarak saptandı. Genotiplendirmede Multilokus Sekans Tiplendirme (MLST)'den yararlanıldı ve tüm izolatların ST2 (Sekans Tip 2) olduğu belirlendi. Bu çalışma, Türkiye'de koyunlarda abort olgularından *Campylobacter fetus* subsp. *fetus* ST2'nin bildirildiği ilk rapordur. Yurdumuzda abort olgularından izole edilen *Campylobacter* türlerini içeren aşıların geliştirilmesi ve kullanımının, *Campylobacter fetus* subsp. *fetus*'un da neden olduğu abortusların hem profilaksisine hem de kontrolüne katkıda bulunacağı düşünülmektedir.

Anahtar sözcükler: *Campylobacter fetus subsp. fetus, MLST, koyun abortus*

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INTRODUCTION

The pregnancy and delivery rates of sheep and goats are quite high during the mating season (85-95% and 75-85%, respectively). Although factors that may show negative impact on fertility are rather few in sheep compared to cattle, still major problems are encountered in the maintenance of pregnancy in ewes and the delivery of healthy lambs [1,2]. The main problem in both sheep and cattle that is faced is abortion, which also causes grave economic loss. Infectious abortions are mostly observed at herd/flock level, rather than as sporadic cases [3,4]. *Campylobacter fetus* subsp. *fetus* (Cff) colonizes mainly in the intestinal tract of cattle and sheep and may cause sporadic abortion in both species [5]. Cff is recognised as a significant causative agent of ovine abortions.

Once a flock is exposed to the Cff, it rapidly spreads within the flock via the faecal-oral route, eventually causing a high abortion rate of 50-60% throughout the lambing season, and thus, heavy economic loss occurs [1,6].

It was aimed to determine the etiology of the abortion that was seen in a flock of 200 sheep and to report *Campylobacter fetus* subsp. *fetus* ST2 isolation.

MATERIAL and METHODS

Animals

In a flock of 200 Akkaraman sheep, 20 animals aborted in the 4th month of gestation, and four of the aborted foetuses were submitted to the Faculty of Veterinary Medicine of Erciyes University (ERU). The animals were vaccinated against enterotoxemia in the 2nd month of gestation, and it was informed that the flock was fed on beet pulp silage.

Necropsy

Necropsy was performed under aseptic conditions. The liver, spleen, stomach, and different parts of the small and large intestines, as well as the pancreas, kidneys, lungs, heart, and brain were dissected. Two specimens were taken from each tissue, one for histopathological analysis and the other for microbiological analysis.

Histopathological Analysis

For histopathological analysis, the tissue samples were fixed in 10% neutral buffered formalin solution, which was changed several times. The tissue samples were dehydrated through graded concentrations of ethanol prior to automated tissue processing, and then were embedded in paraffin wax. Deparaffinised sections were stained with haematoxylin and eosin stain and a modified Brown and Brenn method [7].

Bacteriological Analysis

The foetal organ specimens collected at necropsy and 40 faecal samples and 40 vaginal swap samples taken from 20 sheep that aborted and 20 sheep that did not abort, as well as 3 feed, 3 water and 3 litter samples underwent bacteriological analysis. Gram staining was used for slides prepared from the foetal tissue (liver, abomasum content, lung) samples. For the bacterial culture method, the foetal organ samples (liver, abomasum content, lung) were inoculated onto blood agar (containing 7% sheep blood), MacConkey Agar, and Eosin Methylene Blue Agar (EMB). Furthermore, in view of the hepatic lesions showing similarity to those observed in campylobacteriosis, the foetal liver samples were also inoculated onto blood agar base No: 2 (enriched with 7% sheep blood; Skirrow Selective Supplement, Oxoid, SR0069, UK). The inoculated plates were incubated under aerobic, microaerobic (Gas generating kits, Anaerocult C, Merck, Germany) and anaerobic (Gas generating kits, Anaerocult A, Merck, Germany) conditions for 48-72 h at 37°C. For the isolation of Cff from faecal samples, vaginal swap samples, feed, water and litter, a technique combining pre-enrichment and membrane filtration was used [8]. However, in the present study, we used Brucella broth supplemented with Skirrow's supplement (Skirrow Selective Supplement, Oxoid, SR0069, UK) and a 0.65 µm pore-size cellulose acetate membrane filter.

Phenotypic tests including Gram staining, motility test, oxidase and catalase activity, growth at 25°C and 42°C tests [8] and molecular analysis [9,10] were performed for identification of the isolates.

Molecular Analysis

DNA Extraction: Template DNA was prepared from pure cultures grown on blood agar by using boiling and centrifugation method.

Identification of the Isolates at Genus, Species and Subspecies Level: Phenotypic tests including Gram staining, motility test, oxidase and catalase activity, growth at 25°C and 42°C tests [8] and molecular analysis [9,10] were performed for identification of the isolates.

We performed three different PCR for the genus, species and subspecies identification of the isolates. Genus detection PCR was performed using C412F and C1288R primers described by Linton et al.[9], while species and subspecies detection PCR were carried out according to Schulze et al.[11] by using MG3F/MG4R and VenSF/VenSR primers. In addition, identification of other *Campylobacter* species were carried out according to method of Wang et al.[10].

16S Ribosomal RNA Gene Sequencing: In order to identify the species of the isolates, 16S rRNA gene sequencing was performed using the universal primers 27F and 1492R [12]. The amplified products were purified using the QIA-quick PCR Purification Kit (Qiagen, USA), and sequence analysis was performed using the Big Dye Direct Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions. After cycle sequencing, the amplicons were

purified with Sephadex G-50 (Sigma-Aldrich, USA) by using spin columns and sequenced on the Applied Biosystems 3500 Genetic Analyser (Applied Biosystems, USA).

All sequences were analysed with the CLC Main Workbench 6 and compared with reference sequences available on the website of the National Centre for Biotechnology Information using the Basic Local Alignment Search Tool for Nucleotides (BLASTn) programme *(https://blast.ncbi.nlm. nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome)*.

Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR): The ERIC primers 1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and 2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') were used for molecular typing of the isolates [8].

Multilocus Sequence Typing (MLST): The multilocus sequence type (ST) of the *C. fetus* subsp. *fetus* isolates was determined according to a previously described MLST protocol [13]. Briefly, seven housekeeping genes of the *C. fetus* subsp. *fetus* isolates (aspA: aspartase, glnA: glutamine synthetase, gltA: citrate synthase, glyA: serine hydroxy methyl transferase, pgm: phospho glucomutase, tkt: transketolase, and uncA: ATP synthase alpha subunit) were amplified.

The PCR products were sequenced in both forward and reverse directions. Alleles, STs, and clonal complex (CC) assignments were made using the PubMLST database *(https://pubmlst.org/bigsdb?db=pubmlst_campylobacter_nonjejuni_seqdef)*

RESULTS

Necropsy Findings

Macroscopic examination showed that irregular necrotic foci with a light brown dented centre and a pale periphery were scattered throughout the hepatic lobes *(Fig. 1-A)*. No characteristic lesions were observed in the other organs examined.

Histopathological Analysis

Histologically, the liver presented with multifocal areas of coagulative necrosis, mononuclear cell infiltration and numerous large bacterial colonies surrounding the necrotic areas, sinusoidal dilatation, and haemorrhage *(Fig. 1-B,C)*. *Fig. 1-C* shows many Gram negative curved-shaped bacteria within and around an area of necrosis. Different from the liver, the other organs that were examined did not present with any characteristic histopathological finding.

Bacteriological Analysis

Numerous Gram-negative spiral shaped bacteria were observed in the stained tissue preparations. At the end of the incubation period, no specific growth was observed on the aerobic and anaerobic incubated plates, while the blood agar plates incubated microaerobically showed the growth of smooth, translucent, non-haemolytic colonies, measuring 1-2 mm in diameter. These colonies were subcultured for pure culture of the isolates. Based on the results of the phenotypic tests, the isolates were identified as *Campylobacter* spp. Thus, organs belong to four foetuses analysed were found to be positive for *Campylobacter* spp.

Molecular Analysis

All isolates tested were identified as *Campylobacter* spp. according to Linton et al.[9]. 16S rRNA sequence analysis revealed that isolates were identified as *C. fetus*. However BLAST results showed that 16S rRNA sequencing could not differentiate between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. Next, the isolates were identified as *C. fetus* subsp. *fetus* according to the PCR method *(Fig. 2)* described by Schulze et al.[11]. In addition, the biochemical 1% glycine tolerance test was used for differentiation and isolates were found to be tolerant to 1% glycine.

While the vaginal swap samples taken from sheep that did not abort, water, feed and litter samples analysed with enrichment technique produced negative results for Cff, 5 (25%) of the 20 faecal samples taken from the sheep that had aborted, yielded Cff. Similarly 5 (25%) of the 20 vaginal swap samples taken from the sheep that had aborted, were positive for Cff. *Campylobacter* isolation results of the current study are presented in *Table 1*.

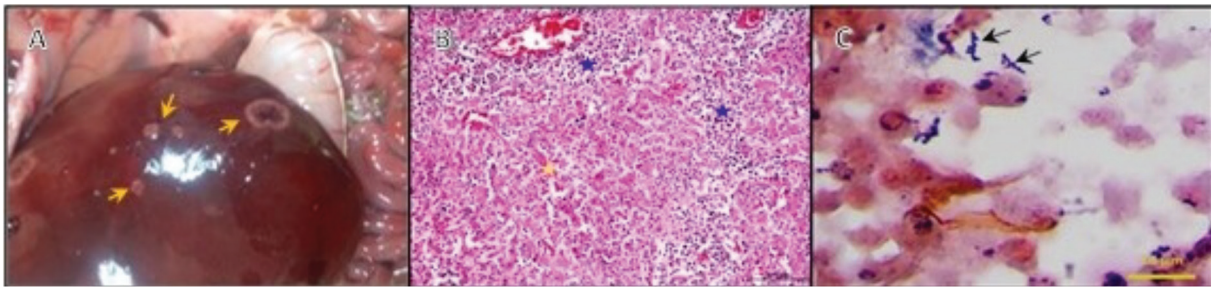

Fig 1. Pathology of the liver taken from the aborted foetus. **A)** Pale, necrotic areas scattered throughout the surface of the liver *(yellow arrows),* **B)** Multifocal moderate necrotizing hepatitis *(yellow star),* with neutrophil leukocyte and mononuclear cell infiltration *(blue stars);* Bar: 100 µm, Haematoxylin and eosin stain, **C)** Easily distinguishable clusters of spiral bacteria *(arrows)* in the hepatic lesions

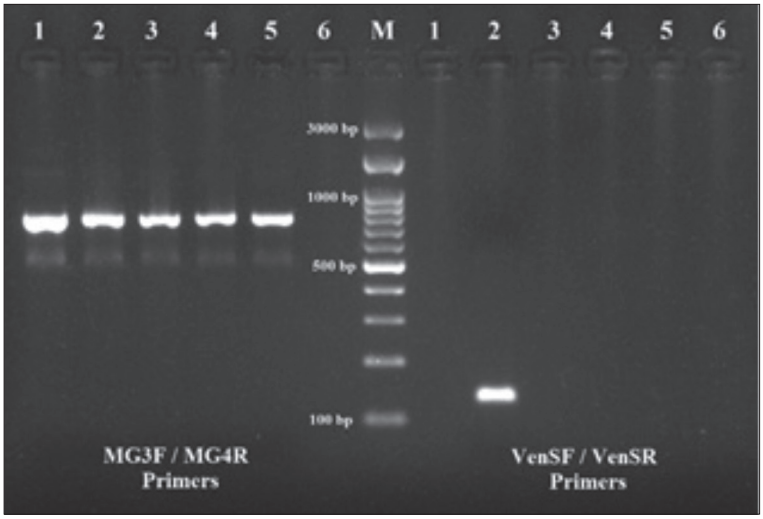

Fig 2. Agarose gel electrophoresis of PCR products obtained by using primer pairs MG3F/MG4R (750 bp) and VenSF/VenSR (142 bp); 1: *C. fetus* subsp. *fetus* control strain, 2: *C. fetus* subsp. *venerealis* control strain, 3: *C. fetus* subsp. *fetus* isolate recovered from aborted foetus, 4: *C. fetus* subsp. *fetus* isolate recovered from ovine feces, 5: *C. fetus* subsp. *fetus* isolate recovered from ovine vaginal swab, 6: Negative control, M: Marker, 100bp DNA Ladder H3 RTU, GeneDireX

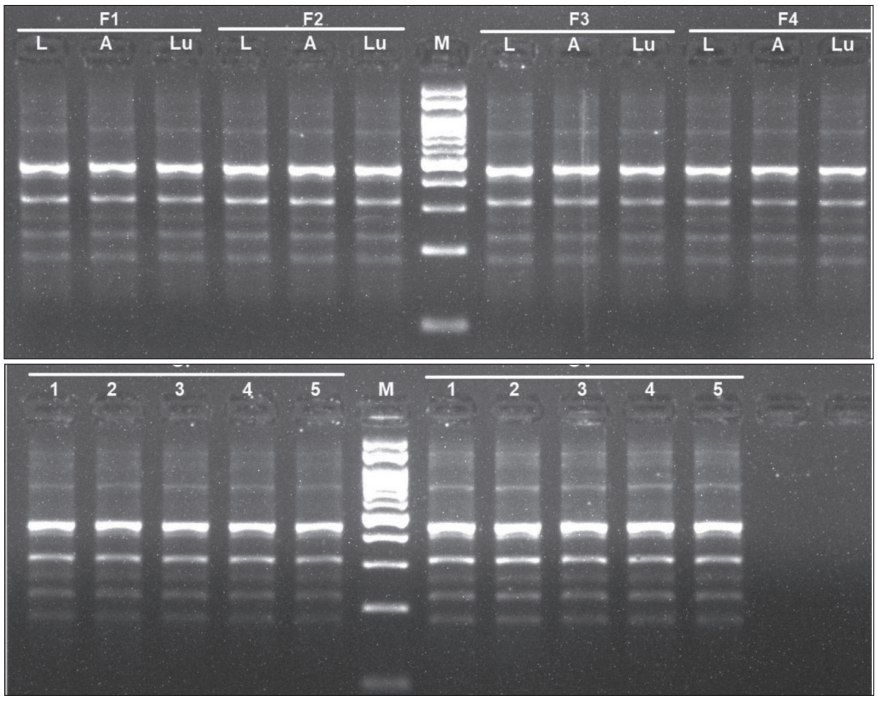

Fig 3. Agarose gel electrophoresis of ERIC-PCR products obtained by using 1R and 2 primer pairs. **F1:** Aborted foetus 1, **F2:** Aborted foetus 2, **F3:** Aborted foetus 3, **F4:** Aborted foetus 4, **L:** Liver isolate, **A:** Abomasum content isolate, **Lu:** Lung isolate, **OF:** Ovine faeces isolates (1-5), **OV:** Ovine vaginal swap isolates (1-5), **M:** Marker GeneDirex 100 bp DNA Ladder H3 RTU

In the genotyping performed by ERIC-PCR, it was determined that all of the 22 isolates *(Table 1)* obtained from aborted foetuses, sheep faecal samples, and sheep vaginal samples had the same band patterns *(Fig. 3)*; thus, a single representative Cff isolate belongs to each source was used in the MLST step of the study. The isolates which were deposited in the GenBank, were the representative Cff isolates.

16S rRNA Gene Sequencing

The 16S rRNA sequences of Cff isolates obtained from aborted foetus, ovine faeces and vaginal swaps were deposited in GenBank under accession numbers MK806573, MK818524 and MK818525 respectively.

Multilocus Sequence Typing (MLST)

In result, it was determined that the isolates obtained from the aborted foetuses, ovine faeces, and vaginal swaps were of the ST2 genotype *(Table 2)*.

DISCUSSION

Campylobacter coli [14], *C. jejuni* [15], and *C. fetus* subsp. *fetus* [16] are the main *Campylobacter* species isolated from ovine abortion cases, and several literature reports have been published on their isolation. In cases of ovine abortion associated with Cff, diagnosis is based on the results of phenotypic and molecular tests. In the ovine abortion case described in this report, the isolates obtained from the aborted foetuses and other material were identified as Cff on the basis of the results of phenotypic tests [8] and molecular analyses [9,17]. In the species identification of *C. fetus* isolates, different results have been reported for the

Table 1. *Number of samples analysed and distribution of Campylobacter species recovered*

Samples Analysed	n	*Campylobacter* Species Isolated		
		Cc	*Cff*	*Cj*
Aborted foetuses	4	-	4*	-
Faecal samples (from aborted sheep)	20	-	5	-
Faecal samples (from sheep that did not abort)	20	15	-	-
Feed	3	-	-	-
Litter	3	-	-	1
Vaginal swap samples (from aborted sheep)	20	-	5	-
Vaginal swap samples (from sheep that did not abort)	20	-	-	-
Water	3	-	-	-

Cc: *Campylobacter coli;* ***Cff:*** *Campylobacter fetus subsp. Fetus;* ***Cj:*** *Campylobacter jejuni;* ***-:*** *negative result; * number of positive samples*

Table 2. *Results from the sequence analysis of seven housekeeping genes and their allelic profiles based on MLST Database* regarding Campylobacter fetus*

Isolate Name	Housekeeping Genes Analysed and Allelic Profiles							ST
	ASP	GLN	GLT	GLY	PGM	TKT	UNC	
MK806573	1	2	2	2	1	1	2	2
MK818524	1	2	2	2	1	1	2	2
MK818525	1	2	2	2	1	1	2	2

** (https://pubmlst.org/bigsdb?db=pubmlst_campylobacter_nonjejuni_seqdef), MK806573, MK818524, and MK818525 are GenBank accesion number of the isolates whichwere recovered from aborted foetus, ovine faeces, and vaginal swaps respectively*

band size obtained with the use of MG3F/MG4R primers in previous studies. For example, while Hum et al.[17] reported to have obtained 960 bp bands, Schulz et al.[11] and Wagenaar et al.[18] reported to have obtained 750 bp bands with the use of these primers. Interestingly, the size of the bands obtained in the present case study was also 750 bp. The differences in the amplicon sizes in studies performed in different countries cannot be explained [11].

It is indicated that the reference test for the differentiation of *C. fetus* isolates at subspecies levels is the 1% glycine-tolerance test, and the results of this test are reported to be generally in agreement with PCR results [11]. Likewise, the results of these two methods were observed to be in agreement in the present study, both methods identified the isolates as Cff.

Only few literature reports are available on the MLST analysis of Cff isolates obtained from cases of ovine and bovine abortions [13]. There is no study performed on the typing of Cff with MLST in Turkey. However, in the study conducted by Van Bergen et al.[13], Cff isolates from several countries were genotyped by MLST, and of the 4 Cff isolates from Turkey that were tested, 3 were reported to have been identified as sequence type 2 (ST2), and 1 as sequence type 5 (ST5). In the current study, the isolates obtained from the aborted foetuses, vaginal swap samples and faecal samples were identified as ST2. However, there is no information on the isolation source (from abortion cases or intestinal carriage etc.) of the Turkish Cff isolates analysed in the study of Van Bergen et al.[13].

It is reported that, the macroscopic observation of multifocal necrotic areas, as if staple-punched, in the foetal liver, and the detection of coagulation necrosis at histopathological examination are specific to abortions associated with campylobacters. The macroscopic and histopathological findings detected in the foetal livers examined in the present study *(Fig. 1)* were in agreement with these specific findings reported in literature.

In conclusion, Campylobacters are frequently isolated from ovine abortions occurring in different locations in Turkey [14,15,19,20]. On the other hand, prophylactic vaccination is not periodically implemented against ovine abortions caused by campylobacters in Turkey. Therefore, it is considered that the development of inactive vaccines from local Campylobacter species isolated from the cases, and the immunisation of animals with these vaccines would contribute to both prophylaxis and disease control.

Conflict of Interest

The authors declared that there is no conflict of interest.

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