Determination of *Campylobacter fetus* subsp. *fetus* and *Campylobacter jejuni* in Aborted Sheep Fetuses by Multiplex PCR Assay

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ABSTRACT

Campylobacteriosis is a contagious and zoonotic infection characterized by abortion and infertility in animals. Ovine campylobacteriosis is caused by *Campylobacter (C.) fetus* subsp. *fetus* or *C. jejuni*. As a result of the slow-growing bacterial agents and the high lability of *Campylobacter* species, laboratory diagnosis of these agents has always been problematic. Several publications on detection of *Campylobacter* spp. DNA from reference strains or pure culture have been presented. However, studies that have been carried out on field or clinical samples of sheep are quite limited. The purpose of the current study was to evaluate the utilization of multiplex-PCR (m-PCR) assay in the diagnosis of ovine campylobacteriosis from abomasal content samples of aborted sheep fetuses. A total of 116 aborted sheep fetuses were tested and *C. fetus* subsp. *fetus* was isolated from 8 (6.9%) samples. The m-PCR assay was conducted and amplified *C. fetus* subsp. *fetus*-specific DNA in 13 (11.2%) of the abomasum contents. Cohen's kappa coefficient revealed substantial (*κ:* 0.74) agreement between bacteriological culture and m-PCR methods. The results of this study suggest that the m-PCR assay is more sensitive and reliable than conventional bacteriological culture for detection of *Campylobacter* species.

Key words: *C. fetus* subsp. *fetus*; *C. jejuni*; Sheep; Abortion; m-PCR; Culture.

INTRODUCTION

Campylobacter species are Gram negative, motile, relatively slow growing, slender, curved or gull-shaped bacteria (1). These microaerophilic organisms grow best on enriched media in an atmosphere of increased CO_2 and decreased oxygen tension. *Campylobacter* species are found in the intestinal and genital tracts of domestic animals and are widely distributed in terms of geography (2,3,4). These microorganisms cause a variable set of disorders in a diverse set of hosts: from abortions in sheep, to gastritis and septicemia in humans mostly with compromised immune systems (5,6,7,8). Three species, namely *C. fetus* subsp. *fetus*, *C. fetus* subsp. *veneralis,* and *C. jejuni,* are recognized pathogens of veterinary significance. *C. fetus* subsp. *fetus* and *C. jejuni* result in outbreaks of abortion in sheep characterized by gross lesions in the placenta and/or fetus. Transmission of both organisms is by the fecal-oral route. During the pregnancy period, bacterial infection in the uterus of ewes may take place after bacteremia. Following, necrotic placentitis may result in abortion at later stages of pregnancy or cause lambs that are stillborn or weak. Abortion leads to substantial contamination of pasture with widespread infection in the flock. Recovered ewes are known to be immune for at least 3 years, and the flock fertility in future breeding seasons is generally not affected (4,5,6).

The diagnostic methods for campylobacteriosis include tests for isolation and identification, tests for detection and estimation of antibodies induced in response to the agent in ruminants. Serologic techniques are not definitive, as all

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infected animals do not produce measurable quantities of antibodies. For example, the vaginal mucus agglutination test detects about 50% of infected, infertile cows on a herd basis (2,3,9). The sensitivity of bacteriological culture technique is based on the usability and amounts of *Campylobacter* spp. in the specimen, and the nature of the specimen that is usually contaminated with other bacteria. Although bacteriological culture techniques are effective and commonly used in detection of the agents, they are difficult to perform and time consuming, causing disadvantages while handling large-scale samples or attempting to make a rapid diagnosis (3,10,11). In detection of weak or slow-growing bacterial agents, PCR may provide fast and reliable results. A few studies on detection of *Campylobacter* spp. DNA from reference strains or pure culture have been performed (8,11,12,13). Studies that have been carried out on field or clinical samples of sheep are also rather limited (10,14).

Campylobacteriosis is a topic of interest for the ruminant breeding all over the world as the disease causes substantial economic losses (15). *C. fetus* subsp. *fetus* and *C. jejuni* have been reported as the major causes of abortion in sheep in most countries where sheep breeding is performed extensively (16,17,18), including Turkey (19, 20). The purpose of this study was to investigate the applicability of the multiplex-PCR (m-PCR) assay as a diagnostic tool for detection of *C. fetus* subsp. *fetus* and *C. jejuni* DNA in the abomasum content samples of aborted sheep fetuses and compare the results to conventional bacteriological culture methods.

MATERIAL AND METHODS

Samples

A total of 116 aborted sheep fetuses were collected from 98 different commercial sheep flocks in the Van region of Turkey. Necropsy was carried out immediately after the abortion at Van Yüzüncü Yıl University, Faculty of Veterinary Medicine. Liver, pharynx, and lung samples as well as abomasum content (3-7 ml) were aseptically collected for bacteriological culture and m-PCR analyses. Bacteriological culture analyses were performed immediately and the samples that were planned for m-PCR were kept at -70˚C until processing.

Bacteriological Culture

Swabs taken from the abomasum content, liver, pharynx and lung samples were inoculated immediately onto duplicate plates of blood agar base no: 2 (CM0271, Oxoid, Hampshire, UK) with contents of 7% (v/v) defibrinated horse blood, *Campylobacter* growth supplement (SR084E, Oxoid, ThermoFischer, Scientic, Basingstoke, UK) and *Campylobacter* selective supplement (SR069E, Oxoid). One series of plates was incubated for 3 days at 37˚C, and another series was left for incubation at the temperature of 42˚C in a microaerophilic atmosphere, ThermoFischer, Scientic, Basingstoke, UK (5-10% O_2, 5-10% CO_2, 5-9% H_2) (CN0035A, Campygen, Oxoid, ThermoFischer, Scientic, Basingstoke, UK). Suspected colonies were examined by macroscopic and microscopic morphology, catalase and oxidase tests and non-fermentative activities (1,3,5). *Campylobacter* isolates were identified according to the capability to grow at 25˚C and 42˚C, sensitivity to nalidixic acid and cephalothin and hippurate hydrolysis. Additionally, the samples were also inoculated onto blood agar plates (1.10886, Merck, Darmstadt, Germany) containing 5% (v/v) defibrinated sheep blood. The plates were incubated at 37˚C aerobically for 1-3 days. Identification of bacterial agents was achieved by conventional bacteriological culture methods (3).

m-PCR

Extraction of DNA from Abomasum Contents

Bacterial DNA was extracted from the abomasum content samples using a commercially available DNA extraction kit (K0721, Thermo Scientific, Genejet Genomic DNA purification kit, Lithuania) following the manufacturer's recommendations. DNA concentrations were determined spectrophotometrically (GBC, Dadenong, Australia) by reading absorbance values at 260 and 280 nm. The samples were stored at -20˚C until used as templates for amplifications.

Primers, Amplification Conditions and Agarose Gel Electrophoresis

The m-PCR was carried out at a final mixture volume of 25 µl. The mixture consisted of 2 µl of template DNA, 15 µl of 2xPCR master mix (K0171, Thermo Scientific) and 1 µl 25 mM of each primer. The oligonucleotide primers utilized in the m-PCR process were specific for *Campylobacter* spp. (21), for *C. fetus* subsp. *fetus* (22) and *C. jejuni* (13) presented in Table 2. m-PCR was performed using the following protocol: after an initial denaturation at 94˚C for 3 minutes, the next 35 cycles were carried out at 94˚C for 1 minute

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Table 1: Sequences of primers, product lengths and references

Bacteria	Primers	Sequence (5'-3')	Product Length (bp)	Reference
Campylobacter spp.	*c412*-F	GGATGACACTTTTCGGAGC	816	21
	c412-R	CATTGTAGCACGTGTGTC		
C. fetus subsp. *fetus*	*cfch57*-F	GCAGTCGAACGGAGTATTA	997	22
	cfch57-R	GCAGCACCTGTCTCAACT		
C. jejuni	*hipo*-F	GACTTCGTGCAGATATGGATGCTT	344	13
	hipo-R	GACTTCGTGCAGATATGGATGCTT		

(denaturation), 54˚C for 1 minute (annealing) and 72˚C for 1 minute (extension) in a thermal cycler (Thermo, Electron Corp., Waltham, USA). The final extension was at 72˚C for 3 minutes. Sterile, DNase/RNase-free, diethylpyrocarbonate (DEPC)-treated water (Applichem, Darmstadt, Germany) without DNA template was used as the negative control. As the positive control, DNA isolated from *C. fetus* subsp. *fetus* (ATCC 33246) and *C. jejuni* (ATCC 33560) was supplied by the Department of Microbiology, Faculty of Veterinary Medicine, Van Yüzüncü Yıl University. To determine the reliability of the results and detect any external contamination, all m-PCR samples were processed in duplicates.

The products that were amplified were subjected to analysis by electrophoresis on a 1.5% (w/v) agarose gel that contained ethidium bromide (0.3 mg/L) at 80-90 volts for 1.5-2 hours. The products that were amplified were visualized by an image-analysis software system (Spectronics Co., Westburg, NY, USA). m-PCR outcomes that had a molecular size of 816 bp were accepted to be positive for *Campylobacter* spp., those with 997 bp were *C. fetus* subsp. *fetus* and those with 344 bp were *C. fetus*. A 100-bp DNA ladder (GeneRuler ThermoFisher, USA) was utilized as a size marker.

Detection of *C. fetus* subsp. *fetus* and *C. jejuni* in Abomasum Content Samples by m-PCR

C. fetus subsp. *fetus* (ATCC 33246) and *C. jejuni* (ATCC 33560) strains were grown in a microaerophilic atmosphere on blood agar base no: 2 (CM0271, Oxoid, Hampshire, UK) with contents of 7% (v/v) defibrinated horse blood at 37˚C for 3 days. The bacteria were harvested from the plates with sterile phosphate-buffered saline (pH: 7.2, P4417, Sigma-Aldrich, St. Louis, Missouri, USA), and the suspensions were adjusted spectrophotometrically (GBC) to an optical density of 0.18 (23). To detect *C. fetus* subsp. *fetus* and *C. jejuni* in the process of m-PCR, eight abomasum content samples that were negative by culture and m-PCR methods were artificially contaminated with *C. fetus* subsp. *fetus* ($1.4x10^6$ CFU/ml) and *C. jejuni* ($1.6x10^5$ CFU/ml) strains. *C. fetus* subsp. *fetus* DNA and *C. jejuni* DNA were extracted from the abomasum contents and processed by m-PCR as previously described.

Statistical Analysis

In this study, Cohen's kappa coefficient (agreement degree, κ) was used to determine the agreement between the bacteriological culture and m-PCR methods (24). The data was analyzed with MedCalc Software Program (MedCalc Version 19.7, MedCalc Software Ltd., Belgium).

RESULTS

Isolation

In the examination of 116 aborted fetuses, *C. fetus* subsp. *fetus* strains were isolated from 8 (6.9%) of abomasum content samples, 6 (5.1%) from livers, 4 (3.4%) from pharynxes and 1 (0.8%) from lungs. *C. jejuni* or any other *Campylobacter* species were not isolated in the remaining fetuses.

m-PCR

The m-PCR assay amplified *C. fetus* subsp. *fetus*-specific DNA from 13 (11.2%) of the 116 abomasum content samples (Fig 1). All *C. fetus* subsp. *fetus* positive samples were also positive for *Campylobacter* spp. (Fig 2). PCR-positive samples were obtained from 13 different sheep flocks.

Comparison of m-PCR Assay with Bacteriological Culture

Of the 116 abomasum content samples, eight (6.9%) were positive by bacteriological culture and 13 (11.2%) were also found to be positive by m-PCR assay. In comparison of the

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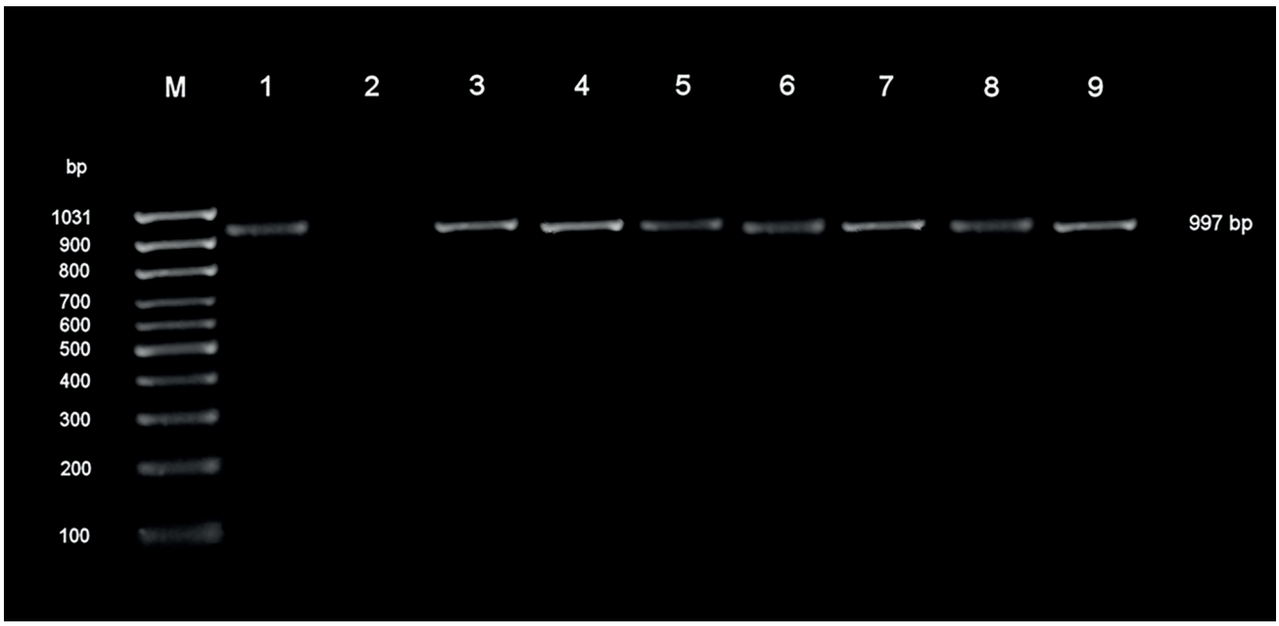

Figure 1. *C. fetus* subsp. *fetus* m-PCR products obtained from abomasum content samples of aborted sheep fetuses separated on a 1.5% (w/v) agarose gel. M: GeneRuler™ 100 bp DNA Ladder Plus (Thermo Fisher Scientific, SMD 241), Lane 1: Control positive (based on DNA from *C. fetus* subsp. *fetus* ATCC 33246 strain), Lane 2: Control negative (DNase / RNase free sterile water), Lanes 3-6: Culture positive, m-PCR positive abomasum content samples, Lanes 7-9: Culture negative, m-PCR positive abomasum content samples.

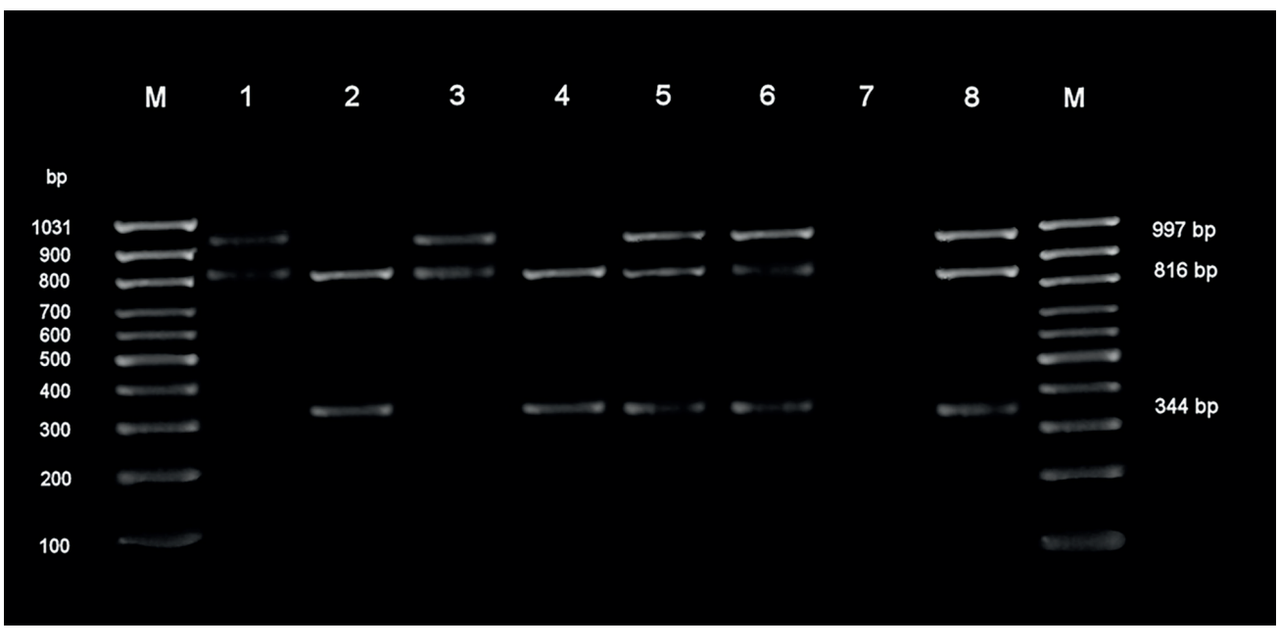

Figure 2. Optimisation of m-PCR. M: GeneRuler™ 100 bp DNA Ladder Plus, Lane 1: *C. fetus* subsp. *fetus* (997 bp) and *Campylobacter* spp. (816 bp) specific primer control (colony), Lane 2: *Campylobacter* spp. and *C. jejuni* (344 bp) specific primer control (colony), Lane 3: *C. fetus* subsp. *fetus* and *Campylobacter* spp. specific primer control (abomasum contents), Lane 4: *Campylobacter* spp. and *C. jejuni* specific primer control (abomasum contents), Lane 5: *C. fetus* subsp. *fetus*, *Campylobacter* spp. and *C. jejuni* specific primer control (colony), Lane 6: *C. fetus* subsp. *fetus*, *Campylobacter* spp. and *C. jejuni* specific primer control (abomasum contents), Lane 7: Control negative (DNase/RNase free sterile water), Lane 8: Control positive [DNA from *C. fetus* subsp. *fetus* (ATCC 33246) and *C. jejuni* (ATCC 33560)].

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results of the culture and m-PCR methods, 8 (6.9%) positive and 103 (88.8%) negative fetuses were confirmed with both methods. While all culture-positive fetuses were also positive by m-PCR, 5 (4.3%) m-PCR-positive fetuses were found to be negative by bacteriological culture. Cohen's kappa coefficient revealed substantial (κ: 0.74) agreement between bacteriological culture and m-PCR methods.

DISCUSSION

Turkey had about 49.8 million sheep in 2019 with 2.7 million of them located in the Van region. Sheep supply an important source of meat and milk for human in Turkey (25). Reproductive disorders like abortion or infertility in ruminants are major problems in the animal industry all over the world.

Abortion is a major factor resulting in decreased rates of lambing, and hence the profitability of sheep breeders. Many causes of abortions, especially bacterial agents, in animals may also bear risk for human handlers. Abortions in sheep may be caused for many reasons and can be broadly classified under infectious and non-infectious factors. Many infectious agents like bacterial, viral, protozoal, chlamydial and fungal agents are known to inflict a direct effect on the reproductive health of animals (26, 27, 28).

Several studies have been performed to determine the etiological role of *Campylobacter* species in sheep abortion cases in some countries. For example, a total of 132 aborted sheep fetuses were tested by PCR in Iran to detect the cause of abortion. In that study 12 (9%) and 2 (1.5%) samples were found as positive for *C. fetus* subsp. *fetus* and *C. jejuni*, respectively (14). In another report from New Zealand, *C. fetus* subsp. *fetus* was isolated from 4 (40%) of 7 aborted sheep fetuses and 3 placentae (29). Erganiş *et al*. (30) identified *C. fetus* subsp. *fetus* in 13 (22.4%) cases out of 58 aborted sheep fetuses collected from different flocks in the Central Anatolia Region of Turkey. Büyük *et al.* (31) also studied 39 milk samples and 39 vaginal mucus samples from 35 sheep and 4 goats by bacteriological culture and PCR methods in Kars region of Turkey. *C. coli* was isolated from 4 (10.2%) samples were in that investigation.

In the current study, *C. fetus* subsp. *fetus* was isolated from 8 (6.8%) of the aborted sheep fetuses, and positive m-PCR products with a molecular size of 997 bp were recorded in 13 (11.2%) samples. Our results clearly demonstrate that campylobacteriosis is an important cause of abortion in sheep in the Van region of Turkey, and *C. fetus* subsp. *fetus* which may play a major role in the abortion of ewes and be responsible for 11.2% of sheep abortions in the Van region of Turkey. These results are in accordance with those obtained by Fenwick *et al.* (29), Erganiş *et al.* (30) and Fallah *et al.* (14) that identified *C. fetus* subsp. *fetus* as the causal agent of sheep abortion samples.

Identification of the role of *Campylobacter* species in abortion cases in sheep may provide great advantage where sheep breeding is performed on an intensive basis. Different samples have been examined for isolation of *Campylobacter* species from aborted sheep fetuses (14,30,31). In the current study, abomasum content, lung, liver and pharynx samples were used for isolation of *Campylobacter* species. In the examination of 116 aborted fetuses, *C. fetus* subsp. *fetus* strains were isolated from 6.9% of abomasum content samples, 5.1% from livers, 3.4% from pharynxes and 0.8% from lungs. These results indicate that abomasum content analysis may be a better and more reliable choice to isolate *C. fetus* subsp. *fetus* than other organs of fetal material.

Although there are many studies on thermophilic campylobacter species in sheep in Turkey, there are only a few studies on *C. fetus* subsp. *fetus*. Previous studies (32,33,34) revealed that the prevalence of *Campylobacter* spp. in the gallbladder of slaughtered sheep varied from 23.1% to 66% in some regions of Turkey. In a study performed on 25 aborted fetuses in late stages of pregnancy, pure cultures of *C. jejuni* were obtained from fetal liver, lung and abomasum contents, while no other agent was detected as a cause of abortion, in Central Anatolia of Turkey (19). In another study, a total of 220 gallbladder specimens of healthy slaughtered sheep were examined bacteriologically, and the 51 (23.2%) samples were evaluated as positive for campylobacter (34). Among the 51 *Campylobacter* strains that were isolated, 28 (54.9%) were identified as *C. jejuni*, 14 (27.4%) were identified as *C. fetus*, 6 (11.7%) were identified as *C. coli,* and 3 (5.8%) were identified as *C. lari*. It was stated by the authors that *C. jejuni* is the most frequently encountered campylobacter species isolated from the gallbladders of sheep in the Van region of Turkey. Sahin *et al.* (4) reported that *C. jejuni* has recently undergone a shift in prevalence in the etiology of *Campylobacter* abortions, and now *C. fetus* subsp. *fetus* is the main primary cause of sheep abortion in the United States. Although, *C. jejuni* is responsible as the primary agent of sheep abortion cases

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(4,19), this agent was not isolated from any of the aborted sheep fetuses in the current study. This may be attributed to the adaptation of *C. jejuni* to the gallbladders of sheep or that *Campylobacter* species are frequently associated with epidemiologically distinct reproductive diseases in ruminants (35).

A few PCR based techniques have been proposed for detection of *Campylobacter* species in pure cultures or reference strains (8,11,18). However, there is limited information available on direct detection of *Campylobacter* species from clinical samples. In a previous study, 132 aborted sheep fetuses were tested by the PCR method; 9% and 1.5% of them were found to be positive for *C. fetus* subsp. *fetus* and *C. jejuni*, respectively (14). In another study, *C. coli* were identified from 10.2% of 35 sheep and 4 goat samples by the bacteriological culture and PCR methods (31). In the current study, *C. fetus* subsp. *fetus* was isolated from 6.9%, and m-PCR assay amplified *C. fetus* subsp. *fetus*-specific DNA from 11.2% of the abomasum content samples from aborted sheep fetuses (Fig. 1). These results indicate that campylobacter prevalence is similar to that of reported by Büyük *et al.* (31) and Fallah *et al.* (14). On the other hand, m-PCR provided higher number positive results than the bacteriological culture method in this study. Lack of isolation or the stage of the infection may explain the superiority of the PCR assay compared to isolation methods. Hum *et al.* (12) identified *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* isolates by m-PCR method, and reported that it was useful for direct detection of *C. fetus* subsp. *fetus* in abomasal samples from aborted fetuses. Similarly, *C. fetus* subsp. *fetus* and *C. jejuni* were directly determined by the m-PCR method in artificially infected abomasum content samples of aborted sheep fetuses in the current research (Fig. 2). This finding is important for determination of the epidemiology of sheep abortion cases caused by *C. fetus* subsp. *fetus* and *C. jejuni.*

C. fetus subsp. *fetus*, *C. jejuni* and *C. coli* have been commonly implicated in sheep abortion (3,14,18). *C. fetus* subsp. *fetus* was solely identified as an etiological agent in abortions in the current study. This findings implicates that *C. fetus* subsp. *fetus* may be taken into consideration as the most common agent in aborted sheep fetuses due to campylobacteriosis.

PCR based assays have turned out to be a good alternative for reaching fast and highly specific bacterial diagnoses. The research on naturally infected sheep materials to compare the diagnostic performance of PCR assays to bacteriological culture methods has been limited so far. In this study, 8 (6.9%) and 13 (11.2%) abomasum content samples of aborted sheep fetuses were found to be positive by bacteriological culture and m-PCR assay, respectively. All positives cases by culture were also positive by m-PCR. Five samples were negative by culture, but positive by m-PCR. The data from this study suggests that, due to its speed and high sensitivity, the m-PCR protocol could be used as an alternative to conventional bacteriological culture methods for identifying *C. fetus* subsp. *fetus* in the abomasum content specimens of aborted sheep fetuses.

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