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Original article

Virulence profiling of *Campylobacter* spp., *C. jejuni* and *C. fetus subsp. fetus* abortions rise in sheep farms in Kashmir, India

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Abstract

Campylobacter spp. are the leading causes of ovine abortions leading to severe economic losses and a source of bacterial food borne illness in humans, posing a major public health concern. This study reports an increase in *Brucella* negative abortions in sheep farms in Kashmir, India in the last few years. Screening of sheep farms was carried to rule out *Campylobacter* abortion. Three Government sheep breeding farms in the Kashmir valley and some other private flocks were screened for the presence of *C. jejuni* and *C. fetus* subsp. *fetus.* A total of 217 samples comprising of 200 swabs (rectal and vaginal swabs; 100 each) from clinically healthy animals, ten vaginal swabs from cases of abortion and seven abomasal contents of the aborted fetuses were collected from sheep breeding farm Khimber (District, Srinagar), sheep breeding farm Goabal, the Mountain Research Centre for sheep and goats (MRCSG, SKUAST-K) (District, Ganderbal) and from private sheep breeders were screened. In the present study a total of 15.2% of samples were positive for different *Campylobacter spp*. by PCR. *C. jejuni* and *C. coli* were detected individually or coexisting by PCR in the rectal swabs from all farms, while *C. fetus* subsp. *fetus* was detected only in the vaginal swabs from private sheep farms and abortion samples. Additionally, *C. jejuni* was also isolated from vaginal swabs. A total of 5, 20 and 18 samples were confirmed positive by PCR for *C. fetus* subsp. *fetus, C. jejuni* and *C. coli,* respectively. The *Campylobacter* isolates obtained in the present study were screened for *fla*A*, cdt*B*, cad*F*, wla*N, *pld*A, *vir*B and *dna*J virulence determinants. However, the isolates harboured *fla*A*, cdt*B and *cad*F virulence determinants only. The recovery of virulent *Campylobacter* isolates from healthy sheep fecal swabs in the present study may have longer human health implications. The presence of abortive strains of *C. jejuni* and *C fetus* subsp. *fetus* in sheep farms has long term economic implications in the Kashmir valley. This study emphasizes the need for efforts to be taken on farms to prevent animal infections and minimizing human exposure to these pathogens through proper hygiene and production practices as suggested by World Organization for Animal Health (OIE).

Keywords: abortion, *C. jejuni*, *C. coli*, *C. fetus* subsp. *fetus*, sheep, virulence determinants

Introduction

The genus *Campylobacter* identified in 1886 by Theodore Escherich was recognized as an etiological agent of abortion in sheep nearly a century ago (McFaydean and Stockman, 1913). *Campylobacter* a gram negative, oxidase, catalase positive and non-fermentative bacterium of the family *Campylobacteraceae* requires microaerophillic atmosphere of 3-5% oxygen, 10% CO_2, and 85% nitrogen for optimal growth, with some species requiring hydrogen (Bolton and Coates, 1983). The recent years have witnessed increased human cases of *Campylobacter* gastroenteritis, making it the second most serious foodborne illness globally after Salmonellosis. C*ampylobacter jejuni* and *Campylobacter coli* are commonly found in the intestinal contents or feces of healthy sheep (Mehmut et al. 2006). Although poultry are the largest reservoir for human campylobacteriosis, research has confirmed animal faeces, mutton and milk as a source of human campylobacteriosis for their handlers.

Campylobacter spp. (*C jejuni* and *C.fetus* subsp. *fetus*) are the primary cause of ovine abortion globally, with an average occurrence rate of 23.2% within affected flocks (Sahin et al. 2008, Joens et al. 2010). *Campylobacter fetus* subsp. *fetus* has been recognized as the leading cause of sheep abortion (Sahin et al. 2017) and *C. jejuni* has also been implicated in sheep abortion (Yaeger et al. 2021).

Several virulence-associated genes play an important role in *Campylobacter* pathogenicity (Zilbauer et al. 2008). The *fla* genes (*fla*A and *fla*B) responsible for bacterial motility, encode flagellin, the ciliary protein which enables *Campylobacter spp*. cells to move and colonize. The *cad*F conserved gene, encoding the fibronectin binding protein of enterocytes, participates in the adherence necessary to induce symptoms of Campylobacteriosis, in *C. jejuni* and *C. coli* (Ziprin et al. 2001). The *vir* gene, encoded by the *Campylobacter* plasmid, encodes proteins responsible for pathogenicity (Bacon et al. 2000). The *pld*A gene having a role in cell invasion is responsible for the synthesis of an outer membrane phospholipase important for caecal colonization. The *dna*J considered to be a chaperone protein helps to cope with diverse physiological stresses (Chansiripornchai and Sasipreeyajan 2009). The *cia*B*, pld*A and *dna*J genes, recognised as heat shock protein genes are important for caecal colonisation and mutations in these genes limit the ability of the organism to colonise (Reddy et al. 2018). The cytolethal distending toxin (*cdt*) genes, *cdt*A*, cdt*B and *cdt*C, form the polycistronic *cdt* operons that are responsible for the expression of cytotoxins and are lethal for host enterocytes. The strains of *C. jejuni* may carry *wla*N which is responsible for causing Guillain Barre syndrome due to its ability to produce LOS (lipooligosaccharide) which exhibits molecular mimicry with the saccharide component of human GMI ganglioside present in peripheral nerves (Perez-Perez et al. 1996, Yuki et al. 2001, Poropatich et al. 2010).

Not much recognition was previously given to this organism as the causative agent of abortion however, it has been found to be the predominant isolate responsible for abortion (Kirkbridge 1993) in sheep in many studies. Sheep rearing is an important source of livelihood especially among migratory Gujjar Bakerwal tribes in the union territory (UT) of Jammu and Kashmir. The consumption of mutton in the Kashmir valley is high due to the severe cold climatic conditions in winter which necessitates the need for local sheep rearing and purchase from other states of the country. The losses due to *Campylobacter* abortion in sheep flocks have not been assessed previously in the UT of J&K. The past decade has witnessed a steady increase in *Brucella* negative abortions in sheep flock in the UT. Several reports of abortions from Government sheep farms in the UT with negative *Brucella* serology of the aborted animals form the basis of this study.

Materials and Methods

Sampling

A total of 200 samples (comprising 25 rectal and 25 vaginal swabs) were collected from sheep breeding farm Khimber, Mountain Research Centre for sheep and goats, sheep breeding farm Goabal, and private sheep breeding farms in the Ganderbal district. Abomasal contents (n=7) from aborted sheep foetus and vaginal swabs from ten abortion cases, which were previously confirmed as *Brucella* negative were also screened in the present study. The samples were collected in sterile vials, and transported to the laboratory on ice in Cary Blairs transport media (Hi Media, India).

Bacterial DNA Extraction and PCR assays

The samples were enriched by inoculating in thioglycollate broth (Difco) and incubated under microaerophilic conditions using a campygen gas pack (BD Gas Pak Campy Container system) initially at 30°C for 5 hours followed by incubation at 42°C for 24-48 hrs for *C jejuni/C coli* and 37°C for 24-48 hrs for *C. fetus subsp fetus.* Enriched samples were streaked on modified Cefaperazone Charcoal Agar (mCCDA) supplemented with mCCDA supplement (Hi Media, India) and plates were incubated under microaerophilic conditions using the temperature and time combination as described previously (Gharbi et al. 2018.). The identification of colonies was based on colony morphology,

Table 1. Primer sequence used for amplification of 16SrRNA *Campylobacter* genus, *Campylobacter jejuni* and *Campylobacter coli*.

S.No.	Target gene	Primer sequence (5´-3´)	Product size (bp)	Reference
1.	16SrRNA (Genus specific)	F-GGTTAAGTCCCGCAACGAGCCGC R-GGCTGATCTACGATTACTAGCGAT	283	Leite et al. (1996)
2.	*map*A (*C.jejuni*)	F-CTATTTTATTTTTGAGTGCTTGTG R-GCTTTATTTGCCATTTGTTTTATTA	589	Denis et al. (1999)
3.	*ceu*E *(C. coli)*	F-AATTGAAAATTGCTCCAACTATG R- TGATTTTATTATTTGTAGCAGCG	462	Denis et al. (1999)
4.	*CFF (C. fetus* subsp. *fetus)*	F-GCAAATATAAATGTAAGCGGAGAG R-TGCAGCGGCCCCACCTAT	435	Wang et al. (2002)

Table 2. Primer sequence for detection of virulence genes of *C. jejuni* and *C. coli.*

S.No.	Target gene	Primer sequence (5´-3´)	Product size (bp)	Reference
1.	*cdt*B	F-CAGAAAGCAAATGGAGTGTT R- AGCTAAAAGCGGTGGAGTAT	620	Hickey et al. (2000)
2.	*cad*F	F-TTGAAGGTAATTTAGATATG R-CTAATACCTAAAGTTGAAAC	400	Konkel et al. (1999)
3.	*fla*A	F-AATAAAAATGCTGATAAACAGGTG R-TACCGAACCAATGTCTGCTCTGATT	855	Konkel et al. (1999)
4.	*dna*J	F-AAGGCTTTGGCTCATC R-CTTTTTGTTCATCGTT	720	Suvamoy et al. (2003)
5.	*pld*A	F-AAGCTTATGCGTTTTT R-TATAAGGCTTTCTCCA	913	Suvamoy et al. (2003)
6.	*vir*B	F-TCTTGTGAGTTGCCTTACCCCTTTT R-CCTGCGTGTCCTGTGTTATTTACCC	494	Suvamoy *et al.* (2003)

gram staining and biochemical tests, (catalase, oxidase and hippurate hydrolysis for *C. jejuni*).

DNA extraction was performed using the phenol chloroform isoamyl (PCI) extraction method. Briefly, suspected colonies was suspended in a 1.5 ml microcentifuge tube containing 200 µl of distilled water and gently mixed by vortex. The samples were boiled for 5 min, followed by cooling on ice for 10 min followed by centrifugation at 10,000 × g in a table-top microcentrifuge (Cooling Centrifuge, Eppendorf) for 10 min. The supernatants were discarded before adding 250 µl RNase (100 µg/ml) and 250 µl of lysis buffer. A total of 550 µl of saturated phenol was added mixed thoroughly and followed by centrifugation at 8,000 × g. The supernatant was collected and extracted with phenol, chloroform and isoamyl alcohol (25:24:1) and centrifuged at 8,000 × g for 10 min. The supernatant was extracted twice using PCI as above. The aqueous phase was collected followed by cold salt precipitation with 2 M sodium acetate and 1.5 ml ethanol (100%) and kept at -20°C for 1 hr followed by centrifugation at 12,000 × g. The DNA pellet was washed with 80% ethanol, dried and suspended in 30 µl of 1X TAE buffer and used as the template (40-100 ng/µl) for PCR.

The PCR protocol as per Wang et al. (2002) was followed for detection of *C. fetus* subsp. *fetus* while the protocol of Denis et al. (1999) was followed for *Campylobacter jejuni* and *Campylobacter coli* detection. Details of the primers used in this study are provided in Table 1. The cyclic condition for the *sap*B gene amplification of *C. fetus* subsp. *fetus*, involved an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 60 seconds, and extension at 72°C for 60 seconds and a final extension step at 72°C for 5 minutes (Wang et al. 2002).

The cyclic conditions for multiplex PCR for *Campylobacter jejuni* and *Campylobacter coli* amplification included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute, and extension at 72°C for 1 minute and a final extension step at 72°C for 10 minutes (Denis et al. 1999). The *Campylobacter jejuni* and *Campylobacter coli* isolates were screened for virulence genes, *cad*F, *cdt*B, *fla*A, *dna*J, *pld*A, and *vir*B, using primers as described in Table 2.

Fig. 1. *Campylobacter jejuni* on mCCDA (Modified Charcoal Cefoperazone-deoxycholate) agar.

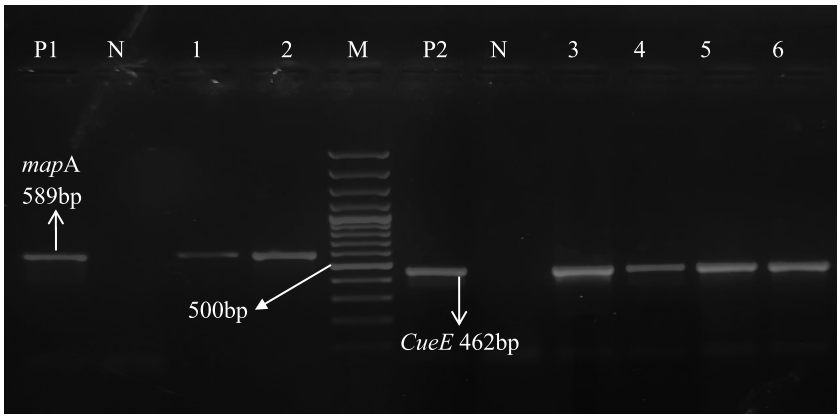

Fig. 2. Multiplex PCR for *map*A (*C. jejuni*) and *ceu*E (*C. coli*) genes.
M: 100bp ladder, P1: Positive control *C. jejuni*, P2: Positive control *C. coli.* N: Negative control, 1-2: isolates positive for *Campylobacter jejuni* 5-8: isolates positive for *C. coli*

Antimicrobial sensitivity profile

The antimicrobial sensitivity profile was determined using the standard disc diffusion method on Muller-Hinton agar containing 5% defibrinated sheep's blood. The panel of antibiotic discs included penicillin, amikacin, tetracycline, ciprofloxacin, cephalothin, nalidixic acid and enrofloxacin. The interpretation of the test was done based on M-45; Clinical and Laboratory Standards Institute (Beilei-Ge et al. 2013).

Results

In the present study, out of 33 PCR positive samples only 12 isolates of *Campylobacter spp* could be obtained. These comprised 7 isolates of *C. jejuni*, 3 isolates of *C. coli*, and 2 isolates of *C. fetus* subsp. f*etus.* Typical small, round, smooth, glistening buff coloured colonies were observed on mCCDA medium (Fig. 1) which revealed Gram negative rods with typical seagull morphology.

A total of 33 (15.2%) out of 217 samples screened from various sheep farms (both organized and unorganized) were found positive for *Campylobacter* spp by PCR. The amplified products of 283bp, 589bp, 462bp, and 435bp corresponding to the genus *Campylobacter*, *C. jejuni*, *C. coli*, and *C. fetus* subsp. *fetus* respectively, were obtained in the PCR assays (Figs. 2, 3).

From sheep breeding farm Goabal, 1 (4%) of the 25 rectal swabs, tested positive for *C. jejuni*, 1 (4%) for

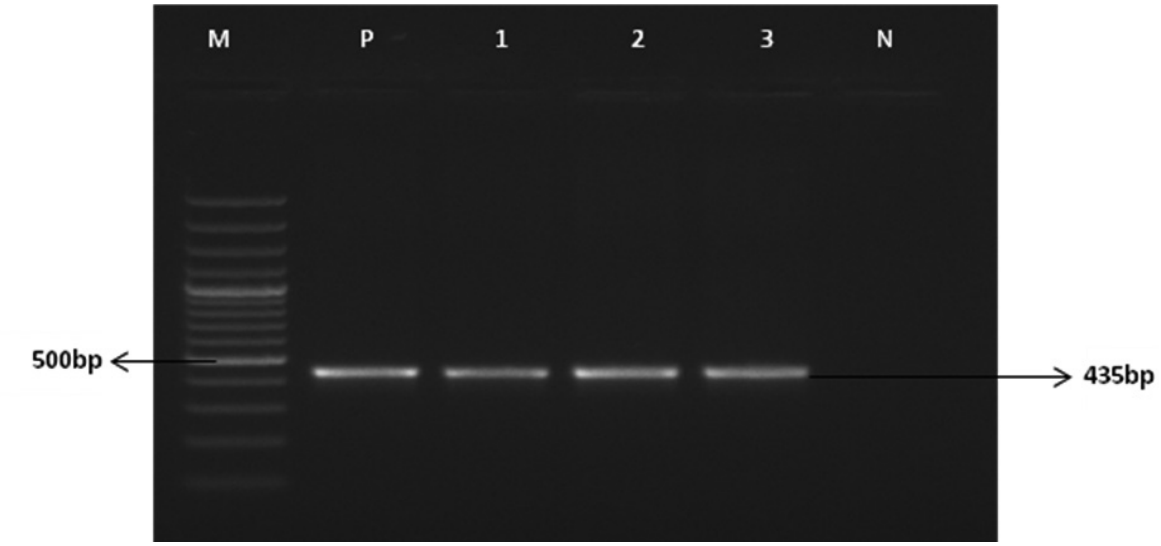

Fig. 3. Agarose gel electrophoresis showing 435bp amplicon of *Campylobacter fetus* subsp. *fetus*.
M: 100bp ladder, P: Positive control, N: Negative control, 1-3: Isolates positive for *Campylobacter fetus* subsp. *fetus*

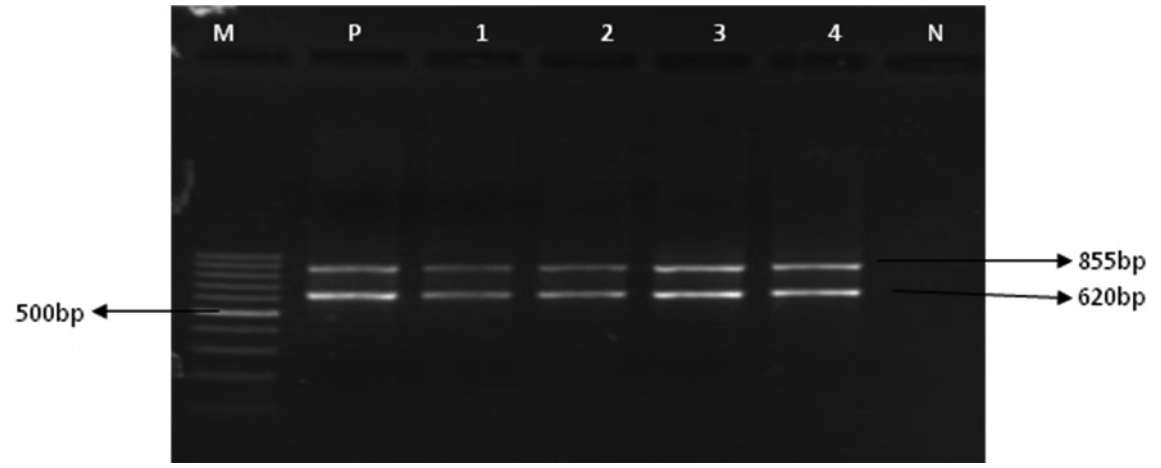

Fig. 4. Multiplex PCR for *fla* and *Cdt* gene of *Campylobacter* spp.
M: 100bp ladder, P: Positive control *Fla* (855bp) and *Cdt* (620bp) genes, 1-4: *Fla* and *Cdt* gene positive isolates, N: Negative control

C. coli, and 1(4%) sample was positive for both *C. jejuni* and *C. coli.* Of the 25 vaginal swabs collected from the same farm, 1 (4%) tested positive for *C. jejuni* by PCR. From the samples collected at sheep breeding farm Khimber, 3 d12%) of the 25 rectal swabs, were positive for *C. jejuni*, 1 (4%) for *C. coli*, and 2 (8%) has coexistent *C. jejuni* and *C. coli*. None of the 25 vaginal swabs from the farm tested positive for any of *Campylobacter spp* by PCR. In MRCG Shuhama, out of the 25 rectal swabs 3(12%) were positive for *C. jejuni*, 3(12%) for *C. coli*, and 4(16%) for both *C. jejuni* and *C. coli.* None of the 25 vaginal swabs screened from the farm revealed the presence of *Campylobacter* spp. In private sheep farms, out of the 25 rectal swabs 2(8%) tested positive for *C. jejuni*, 3 (12%) for *C. coli*, and 3 (12%) for both *C. jejuni* and *C. coli*. Out of the 25 vaginal swabs screened from these private farms 1(4%) was positive for *C. fetus* subsp. *fetus.* Out of the 7 abomasal contents from aborted sheep fetuses, 1(14.2%) tested positive for *C. fetus* subsp. *fetus*. Of the 10 vaginal swabs from *Brucella* negative aborted sheep 3 (30%) were positive for *C. fetus* subsp. *fetus.*

All the isolates of the present study revealed the presence of *cad*F, *fla*A and *cdt*B (Figs. 4, 5). However, none of the isolates harboured the *pld*A, *dna*J, *vir*B, and *wla*N gene. The distribution of *Campylobacter* spp in various organized and private farms is shown in Table 3.

The antibiotic sensitivity profile revealed that all isolates were resistant to penicillin (100%), cephalothin (100%), with resistance to tetracycline being 80%, amikacin (76%), ciprofloxacin (68%) and enrofloxacin (52%). In the present study 80% of isolates revealed sensitivity to nalidixic acid.

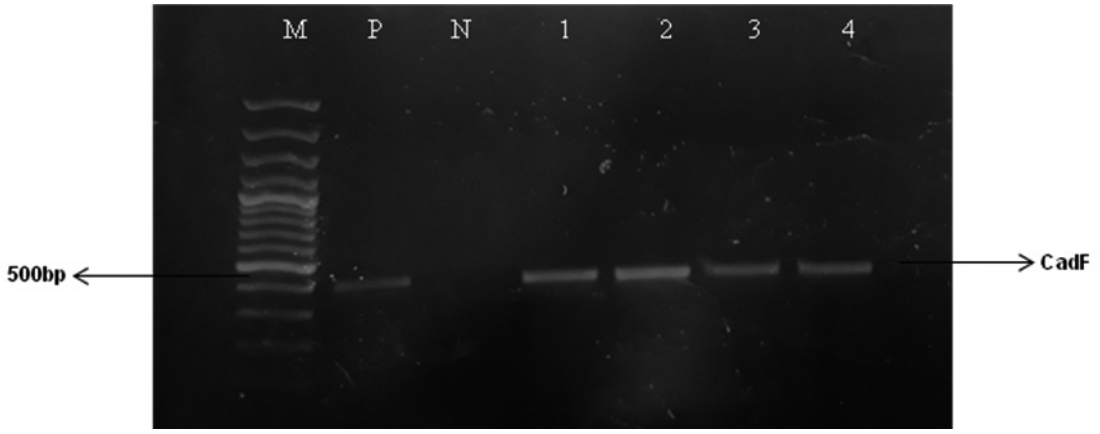

Fig. 5. Agarose gel electrophoresis showing 400bp amplicon of *Cad*F gene.
M: 100bp Ladder; P: Positive control, N: Negative control; 1-4: *Cad*F positive samples

Table 3. Distribution of *Campylobacter spp* in various farms.

S.No	Farm name & District	Sample type and no	*C. jejuni*	*C. coli*	*C jejuni* & *C. coli*	*C. fetus* subsp *fetus*	No. of Isolates		
							C.jejuni	*C. coli*	*C. fetus subsp fetus*
1.	Sheep Breeding Farm Goabal, (District Ganderbal)	Rectal swabs (n=25)	1(4%)	1(4%)	1(4%)	0	1	0	0
		Vaginal swabs (n=25)	1(4%)	0	0	0	0	0	0
2.	Sheep Breeding Farm Khimber, (District Srinagar)	Rectal swabs (n=25)	3(12%)	1(4%)	2(8%)	0	2	0	0
		Vaginal swabs (n=25)	0	0	0	0	0	0	0
3.	MRCG Shuhama (District Ganderbal)	Rectal swabs (n=25)	3(12%)	3(12%)	4(16%)	0	1	2	0
		Vaginal swabs (n=25)	0	0	0	0	0	0	0
4.	Private sheep farms	Rectal swabs (n=25)	2(8%)	3(12%)	3(12%)	0	3	1	0
		Vaginal swabs (n=25)	0	0	0	1(4%)	0	0	1
5.	Cases from veterinary hospitals	Abomasal content of the aborted sheep foetus (n=7)	0	0	0	1(14.2%)	0	0	0
6.	Cases from veterinary hospitals	Vaginal swabs from *Brucella* negative aborted sheep (n=10)	0	0	0	3(30%)	0	0	1

Discussion

Campylobacter species pose a significant threat to both animals and humans, particularly in sheep-rearing regions worldwide. *Campylobacter fetus* subsp. *fetus* and *C. jejuni* are a major cause of sheep abortion and can lead to severe economic losses in the sheep industry (Hedstrom et al. 1987, Dorsch et al. 2022). Infection occurs through ingestion of the bacteria, with most abortions occurring during the last month of pregnancy.

The genus *Campylobacter* is also recognized as an important zoonotic pathogen, with *C. jejuni* and *C. coli* being a common cause of human gastroenteritis, surpassing cases of salmonellosis in high-income countries (Cobo-Diaz et al. 2021). The disease spreads rapidly among uninfected ewes unless stringent hygiene practices are followed, as the fetus, placenta, birth fluids, vaginal discharge, and feces from the ewe are all potential sources of infection. It has been observed that if water or feeding areas become contaminated with these materials, the abortion rate can be notably high (Agerholm et al. 2006, Mearns et al. 2007).

In the present study the occurrence of 15.2% of *Campylobacter* (33/217) from both organized and private sheep farmers in the Kashmir valley was observed. A high percentage of *C. fetus* subsp. *fetus* was detected in the abortion samples (14.2% from abomasal contents of aborted sheep fetus and 30% from vaginal swabs of aborted sheep) in comparison to the vaginal swabs from healthy animals (1%). *C. jejuni* (1%) was only isolated from vaginal swabs of the healthy animals. Several studies (Hamali et al. 2014, Bisma et al. 2018) have reported the presence of *C. jejuni* and *C. fetus* subsp. *fetus* from the vaginal swabs and aborted samples. Bisma et al. (2018), reported 10% of cases positive for *Campylobacter jejuni*, and 3.33% of cases for *Campylobacter fetus* subsp. *fetus* from 150 vaginal samples of aborted ewes. Hamal et al. (2014) reported a prevalence of 9.09% of cases of *C. jejuni* and 1.51% of cases of *C. fetus* subsp. *fetus* from 132 aborted fetus and placental samples. Out of the 100 rectal swab samples screened from sheep in the same study 19% were *C. jejuni* and 18% were *C. coli.* Adesiyun et al. (1992) reported a prevalence rate of 17.92% *Campylobacter* sp from lamb rectal swabs.

A passive filtration technique using 0.45µ pore size filters post enrichment with campy thioglycollate broth was used for isolation of *Campylobacter*. Only 23.2% (10/33) of *Campylobacter sp*p could be isolated from the samples. A plausible reason for this low isolation rate is attributed to its extremely slow growth and fastidious nature (Brand et al. 2004, Chon et al. 2022). All the *C. jejuni* isolates were hippurate hydrolysis positive, and all *C. fetus* subsp. *fetus* isolates were positive in the nitrate reduction test. Harvey et al. (1980) and Steinhauserova et al. (2001) have reported hippurate hydrolysis and nitrate reduction tests as one of the important biochemical tests for identification of *C. jejuni* and *C. fetus* subsp. *fetus,* respectively.

All the isolates of the present study revealed the presence of *fla*, *cad*, *cdt* virulence genes. The pathogenesis of *Campylobacter spp.* particularly *Campylobacter jejuni* and *Campylobacter coli* has been attributed to the presence of several virulence genes viz; *fla*, *cad*, *vir*B11, *cdt*, *wla*N, *dnaJ* and *pld*A (Gilbert et al. 2000). The polar flagellum encoded by (*fla*), responsible for the characteristic darting motility of *C. jejuni* is involved in the colonization and invasion (Guerry, 1997), *cdt* which blocks the G2/M phase of eukaryotic cells prior to cell division, induces a cytoplasmic distention and ultimately causes cell death (Jeon et al. 2005), and *Campylobacter* adhesion to fibronectin protein (*cad*F) promotes intestinal epithelial cell binding (Monteville et al. 2003). These virulence determinants in *Campylobacter* strains isolated from various sources has been reported by Bang et al. (2003), Datta et al. (2003) and Muller et al. (2006) emphasizing the important role of these virulence markers in *Campylobacter* pathogenesis.

None of the isolates in the present study revealed the presence of *dna*J*, vir*B11, *pld*A and *wla*N genes. Similar findings have been reported by Datta et al. (2002) who analysed 40 *Campylobacter* isolates from different parts of Japan by PCR. All the isolates were found positive for the *fla* gene, 16 (32%) for *cad*, 17 (42.5%) for *rac*R and 7 (17.5%) for *cdt*. No samples were positive for *wlaN*. Bisma et al. (2018) also reported the presence of the virulence genes *fla*, *cdt*, and *cad* in seventeen isolates of *C. jejuni* and three isolates of *C. coli*.

Antimicrobial resistance is a major public health concern in both developed and developing countries in recent years (Padungton and Kaneene 2003). The most common antibiotics used for the treatment of *Campylobacter* associated enteritis and abortions in animals are macrolides, fluoroquinolones and tetracycline. A high degree of resistance has been reported in recent years to these antibiotics around the globe (Wieczorek and Osek 2013). The *in vitro* antibiotic sensitivity profile of *Campylobacter* isolates in the present study revealed highest sensitivity of the isolates to nalidixic acid and resistance to penicillin, cephalothin, tetracycline, amikacin, ciprofloxacin and enrofloxacin. Several researchers such as Bisma et al. (2018), Abdullah et al. (2022) and Chepkwony (2016) have reported the multidrug resistance of *Campylobacter spp* to various classes of antibiotics.

This study emphasizes the need for monitoring and controlling *Campylobacter* infections in sheep farms of the union territory of J&K, India to safeguard animal health and mitigate the risk of zoonotic transmission to humans. Proper management practices and hygienic measures are crucial in curbing the spread of this disease and ensuring the well-being of both the sheep population and humans.

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