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Conventional and molecular diagnosis of Campylobacteriosis associated with bovine abortion

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Abstract

Campylobacteriosis is responsible for genital tract infections of beef and dairy herds, causing a significant economic loss in livestock sector. Campylobacter foetus species is one of the important pathogens because of its potential impact in Veterinary and Human health. This study was designed to determine the regional incidence of C. foetus infection in Kerala, India by isolation, detection of C. foetus in clinical samples by Polymerase Chain Reaction (PCR), real time PCR (qPCR), and Enzyme Linked Immunosorbent Assay (ELISA) for the detection of C. foetus antibodies in sera of bovines with the history of abortion/infertility. Clinical samples (aborted materials (50), serum (50), Cervico-Vaginal Mucus (CVM) (30) and semen samples (30)) from a total of 160 cattle and buffaloes with the history of abortion and infertility were collected. Aborted materials including placenta, foetal membranes, liver, lungs and stomach contents of the aborted foetus, semen and CVM samples were processed and subjected to isolation and identification of Campylobacter foetus subsp. foetus (Cff) and Campylobacter foetus subsp. venerealis (Cfv) and molecular confirmation by PCR and qPCR respectively. Serum samples from aborted dams were tested using indirect ELISA. All the suspected clinical samples were found negative for Cff and Cfv on both culturing and PCR. All the serum samples tested were negative by ELISA as well. Conclusively the study indicated the infection of C. foetus spp. responsible for abortion in bovine are rare in the location where the study was conducted, which might be due to insignificant endemic levels. As per the breeding policy, only artificial insemination is practiced in Kerala in bovines, which is often considered as a simple control method for Bovine Genital Campylobacteriosis (BGC) and might be one of the factors that prevented extensive spread of C. foetus spp. infection.

Keywords: *Campylobacteriosis, Campylobacter foetus, Bovine abortion, Polymerase Chain Reaction, real time PCR, and Enzyme Linked Immunosorbent Assay.*

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Reproductive performance is one of the most important factors influencing profitability in the dairy and beef industries. Though the dairy industry is very important in its contribution to the economics of both developed and the developing countries of the world, it is vulnerable to various factors which adversely affect the cattle production *viz.,* physical, chemical, biological and environmental factors. Infectious biological agents are notable causes of reproductive failures and are being given high priority in the bovine industry (BonDurant, 2007). Infections with these agents are mainly manifested as abortion which is one of the important factors reducing calving rate and consequently causing a significant economic loss to the dairy industry (Mittal *et al*., 2018).

Campylobacteriosis is a widespread bacterial disease responsible for contagious genital infections and abortion in cattle around the world (Osunla and Okoh, 2017). Bovine Genital Campylobacteriosis caused by *Cfv,* causes significant economic impact in farming industries and requires a careful and thorough diagnosis and treatment for its control. BGC is a notifiable trade disease with countries requiring certification of disease free status of both cattle and semen for import. *Campylobacter foetus* subsp. *foetus* is associated with sporadic cases of abortion in bovines (OIE, 2018).

Bacteriological analyses of these pathogens primarily rely on cultural isolation and phenotypic characterization, which is reported to be the gold standard and confirmatory test for diagnosis of *C. foetus* infection (Brooks *et al.*, 2004). However, isolation of *C. foetus* is labor intensive, accompanied with a high risk for potential and rapid overgrowth of more robust contaminants (Clark and Dufty, 1978). On the other hand, serological tests like ELISA was recognized as an effective screening test for detection of C. *foetus* (Devenish *et al*., 2005). However, cross- reactive antigens especially within the species pose problems for serological diagnosis (Repiso *et al*., 2002; More *et al*., 2017). Hence, to overcome these problems, nucleic acid amplification has been tremendously exploited for the routine and rapid confirmation of these pathogens (Islam *et al*., 2020). Various methods like PCR and qPCR have been used for the diagnosis of Campylobacteriosis in human and domestic animals, as the tests are reliable and accurate for the species and subspecies identification of *C*. *foetus* (Hum *et al*., 1997; Chaban *et al*., 2012). A subspecies-specific PCR and qPCR assay for *Cfv* targeting *ISCfe-1* gene, a highly conserved, new insertion element which is present exclusively in *Cfv* strains (Abril *et al*., 2007), and for *Cff* targeting *SapB2* gene developed by Wang *et al.* (2002) were employed in the present study.

The current communication reports the results of the serological and molecular diagnosis of Campylobacteriosis associated with bovine abortion in organised and unorganised livestock sectors in and around Thrissur district, Kerala.

Materials and methods

Sampling: Aborted materials, serum, CVM and semen were collected from a total of 160 cattle and buffaloes maintained in organized and unorganized farms of Kerala in India. Serum was also recovered for blood samples in separate tubes for ELISA.

Isolation: Stomach contents and pooled sample consisting of liver, lung, kidney, spleen and placenta, CVM and semen were collected aseptically in Cary-Blair medium and inoculated on the same day on Brucella agar base supplemented with five to ten per cent sheep blood and incubated at 37°C for 72 hr under micro-aerophilic conditions. Similarly, reference samples received in the form of swab were subjected for inoculation.

Glycine Tolerance Test: The test was performed as described by OIE (2018). Briefly, a cell-suspension of McFarland no.1 was inoculated onto a Brucella base agar with or without 1% glycine medium and was incubated microaerobically at 37°C for 48 h. The growth in the presence of glycine has been considered to be a presumptive test for *Cff*.

Reference strains: Two reference isolates (received as swab) were used in this study. *Campylobacter foetus* subsp. *foetus*

and *Campylobacter foetus* subsp. *venerealis* were kindly provided by Institute for Veterinary Bacteriology, University of Bern, Switzerland.

Genomic DNA extraction: HiPurA™ Multi-Sample DNA Purification Kit, Hi Media, was used for the extraction DNA from clinical samples. DNA concentration of stock solutions was measured using Nano drop (Thermo Scientific). The purity of the extracted DNA was checked by measuring the ratio of absorbance (OD of DNA preparation at 260 and 280 nm). Finally, the extracted DNA was stored at -20° C until use.

***Campylobacter foetus* subsp. *foetus* identification by PCR and qPCR:** In the present study, genus and subspecies specific PCR and qPCR were standardized using the reference sample DNA extracted from *Cff* (ATCC 27374) and *Cfv* (NCTC 10354).

PCR was carried out using the already published *Campylobacter* genus specific *16S rRNA* gene primers by Linton *et al.* (1996). The sequences of primers were: forward primer 5'- GGATGACACTTTTCGGAGC -3' and reverse primer 5'- CATTGTAGCACGTGTGTC -3'. *Campylobacter foetus* subsp. *foetus* identification was performed using specific primers published by Wang *et al.* (2002). The forward primer was 5'-GCAAATATAAATGTAAGCGGAGAG-3' and reverse primer was 5'-TGCAGCGGCCCCACCTAT-3'.

Primers for real-time PCR were designed based on *sapB2* gene sequence for *Cff* (GeneBank Accession no. CP008808.1) using NCBI primer designing software. The forward primer was 5'-TTTAGGAGCCGTATCAGCAA -3' and reverse primer was 5'- TCACCAGCAAGAGCTCCTAT -3'.

***Campylobacter foetus* subsp. *venerealis* identification by PCR and qPCR:** The PCR and qPCR was conducted using the primers specific for *Cfv ISCfe-1* gene (Abril *et al*, 2007). The forward primer was 5'- AGGCGAAGAGAATGTTAAATTTGAA-3' and reverse primer was 5'-CCATAAAGCCTAGCTGAAAAAACTG-3'.

PCR was performed in a reaction volume of 12.5 µL containing approximately 100 ng/µL of genomic DNA, 10 p*M*/µL of forward and reverse primer and 6.25 µL of PCR master mix ((2X, Thermo Scientific).

Identification of the PCR product was done in a submarine agarose gel electrophoresis system using one per cent agarose containing ethidium bromide, using Tris Borate EDTA buffer at a voltage of 50V. The gel was visualized under a UV trans-illuminator and results were documented using a gel documentation system (Biorad).

SYBR green PCR protocol:

Reactions were carried out in Eco™ Real-Time PCR System (Illumina) using 48-well plates, sealed by an adhesive seals after pipetting all reagents into the wells. Reaction mixture and thermal cycling parameters are given in (Tables 2 to 4). SYBR green real-time PCR amplification plots and melt curves were viewed and analysed.

***C. foetus* antibody ELISA:** Conventional method of ELISA was performed as per the method described by Seyyal *et al*. (2000) with slight modifications. The immunogenicity of whole cell antigen of *C. foetus* was confirmed by AGPT and the protein concentration was standardized by Lowry's method. Hyper immune serum raised against *C. foetus* in New Zealand White male rabbits. Pre inoculation bleeding for preparation of control non-immune sera was done. A typical injection schedule consisted of 0.5 mL antigen (2 x 10^8 cells per mL) on the 1st day of inoculation. One millilitre antigen (2 x 10^8 cells per mL) was inoculated on the 4th day, and 2 mL antigen on the 7th, 10th, 14th and 16th days (Seyyal *et al.*, 2000). The rabbits were bled by cardiac puncture one week after the last injection. The sera were separated from blood collected from two New Zealand white male rabbits and stored at -20°C.

Checker board analysis was performed for standardizing the concentration of antigen, sera and commercial rabbit anti-bovine IgG peroxidase conjugate. The DAB

Table 1: Amplification cycle for *Campylobacter foetus* subsp. *foetus* and *Campylobacter foetus* subsp. *venerealis.*

Initial denaturation	94ºC ºC for 2 min.	1 cycle
Denaturation	94ºC ºC for 30sec.	30 cycles
Annealing	61.4ºC ºC for 30sec.	
Extension	72ºC ºC for 30 sec.	
Final extension	72ºC ºC for 4 min.	1 cycle

Table 2. Real-time PCR Reaction mix for the detection of *Campylobacter foetus* subsp. *foetus* and *Campylobacter foetus* subsp. *venerealis*

Components	Volume
Orion 2X Real time PCR smart mix	6.25 µL
Forward primer (10 p*M*/µl)	0.5 µL
Reverse primer (10 p*M* /µl)	0.5 µL
Template DNA	4.0 µL
Nuclease free water	1.25 µL
Total	**12.5 µL**

Table 3. Amplification cycle for *Campylobacter foetus* subsp. *venerealis* used for real-time PCR

Steps	Temperature	Time	Number of cycles
Initial denaturation	95ºC ºC	10 min	1
Denaturation	95ºC ºC	30 sec	40
Annealing/Extension	61.4ºC ºC	30 sec	40

Table 4. Amplification cycle for *Campylobacter foetus* subsp. *foetus* used for real-time PCR

Steps	Temperature	Time	Number of cycles
Initial denaturation	95ºC ºC	10 min	1
Denaturation	95ºC ºC	30 sec	40
Annealing/Extension	60.4ºC ºC	30 sec	40

(50X) was diluted in distilled water in the ratio of 1:20 and used.

The extracted whole cell antigen was diluted in the coating buffer (0.05 *M* carbonate - bicarbonate buffer, pH 9.6), and 100 µL was added to each well. The plates were incubated at 4°C overnight, washed three times with ELISA washing solution (Phosphate buffered saline, pH. 7.4, containing 0.5 per cent Tween-20) and dried by shaking (Shaker Incubator at 180 rpm, 55ºC for 20 min). One per cent bovine serum albumin in PBS-T was used to block the wells which were then subjected to incubation for one hour at 37°C and again washed three times with PBS-T. One hundred microlitres each of 50 serum samples of bovine having history of recent abortion (each diluted in the ratio of 1:100) were loaded into the wells in duplicates and incubated for one hour at 37°C and washed as before. One hundred microlitres of 1:10,000 commercial rabbit anti-bovine IgG peroxidase conjugate diluted (1:10,000) was added in each well. After incubation for one hour at 37°C, the plates were washed with ELISA washing solution and followed by loading of wells of each plate with 100 µL of DAB (50X) diluted in distilled water in the ratio of 1:20. The plates

were incubated at room temperature for 10 min, after which the reaction was stopped using 50 µL of 1.25 M H_2SO_4. The optical density (OD) was measured on an ELISA reader (Biorad) at 450 nm.

Results and discussion

Even though the isolation of causative agent is considered as the gold standard test for the diagnosis, the difficulties associated with the isolation of *C*. *foetus* on non-selective media have limited its practical use in the timely diagnosis (Sanhueza *et al*., 2014). The attempts made for isolation of *C*. *foetus* spp. in Brucella agar was unsuccessful for all the samples tested under the present study and *Proteus* was the major contaminant grown in the culture during the study. Meanwhile it was reported that reduced viability and fragile nature of the agent in clinical samples could also be affecting the culture results (Clark and Dufty, 1978; Brooks *et al*., 2004). Furthermore, chances of contamination with ubiquitous, faster growing microorganisms, such as *Pseudomonas* spp. and *Proteus* spp. often contaminate the samples and make it difficult to detect the presence of *C*. *foetus* (Monke *et al.,* 2002). Many of the contaminating materials like faeces, urine or other contaminants may potentially interfere with the direct culturing (Radstrom *et al.*, 2004). Thus the viability of *C*. *foetus* outside the host is important which might also get affected by the exposure to atmospheric levels of oxygen that have a toxic effect on the bacteria. These might be the reasons for unsuccessful isolation in the present study. The suppression of growth of fastidious *Campylobacter* by the contaminants like *Proteus* could also be one of the reasons for failure of isolation.

PCR offers a reliable and accurate technique for the species and subspecies identification of *C*. *foetus* (Hum *et al*., 1997) and many researchers have used this technique successfully in detecting *C*. *foetus* spp. DNA from clinical samples (Wang *et al*., 2002; Van Bergen *et al*., 2005). The present study also attempted to standardize genus, subspecies specific PCR and subspecies specific real time PCR for directly detecting antigens from clinical samples. The PCR primers based on *SapB2* gene and *ISCfe-1* gene were standardized at an annealing temperature of 61.4℃ for 30 seconds. One hundred and ten clinical specimens were subjected to PCR and all of them were found to be negative for the presence of genus specific as well as subspecies specific *Campylobacter* DNA. Positive controls showed respective amplicon size of 816bp, 435bp and 84bp in submarine agarose gel electrophoresis (Fig. 1-3).

Also, *SapB2* gene and *ISCfe-1* gene based real time PCR was standardized at an annealing temperature of 60.4℃ and 61.4℃ for 30 seconds using conventional PCR. Of the 160 samples, all the samples processed and tested were found to be negative. Positive controls showed an average Tm value of 86.4℃ for *SapB2* and 83.4℃ for *ISCfe-1* respectively (Fig. 4-7). Then, the amplicons were visualized in three per cent agarose gel which showed band approximately at 87 bp and 84 bp level respectively (Fig. 8).

According to Hosseinzadeh *et al*. (2013), any sample could contain a mixture of semen, faeces, urine, blood or other contaminants. Many of these contaminating materials may potentially interfere with DNA extraction techniques and are also inhibitory to PCR. This might be probable reasons for unsuccessful PCR results in the present study.

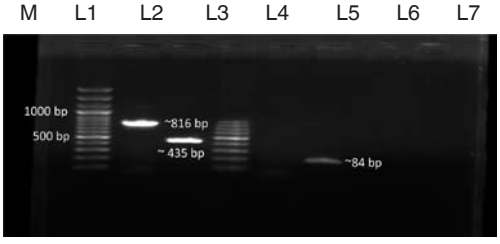

Fig.1 Agarose gel showing amplified product of *Campylobacter* genus and *C. foetus* subspecies specific PCR

M – 100 bp DNA ladder

L1 – *Campylobacter* genus (Positive control - 816bp)

L2 – *C. foetus* subsp. *foetus* (Positive control - 435bp)

L3 – 50 bp DNA ladder

L4 – Negative control

L5 – *C. foetus* subsp. *venerealis* (Positive control – 84bp)

L6 –L7 – Clinical samples

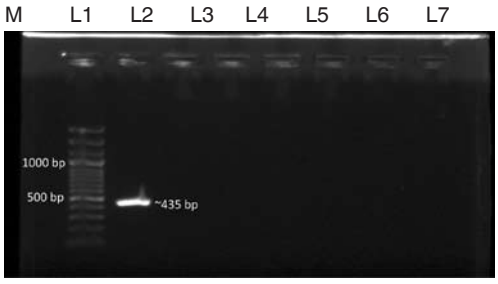

Fig.2 Agarose gel electrophoresis of PCR amplified product of *C. foetus* subspecies *foetus* specific PCR

M – 100 bp DNA ladder

L1 – *C. foetus* subsp. *foetus* (Positive control)

L2 – Negative control

L3 to L7 – Clinical Samples

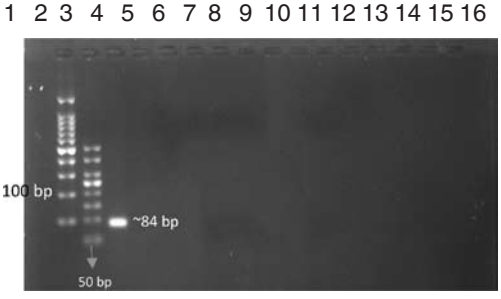

Fig.3 Agarose gel electrophoresis of PCR amplified product of *C. foetus* subsp. *venerealis* specific PCR

1 – 100 bp DNA ladder

2 – 50 bp DNA ladder

3 – *C. foetus* subsp. *venerealis* (Positive control)

4 – Negative control

5 to 16 – Clinical Samples

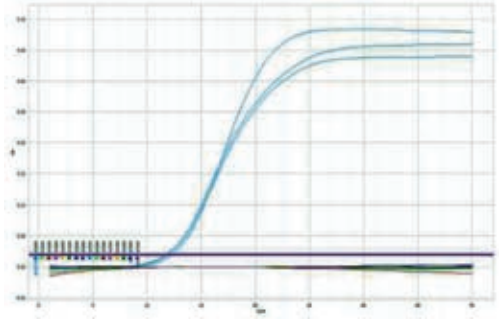

Fig. 4 Amplification plot of real time PCR assay for *SapB2* gene from positive control and clinical samples

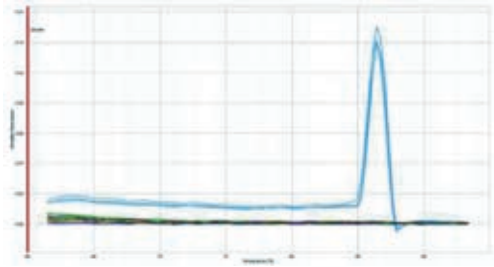

Fig. 5 Melt curve of real time PCR assay for *SapB2* gene from positive control and clinical samples

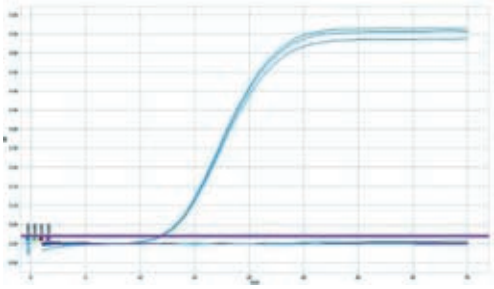

Fig. 6 Amplification plot of real time PCR assay for *ISCfe-1* gene from positive control and clinical samples

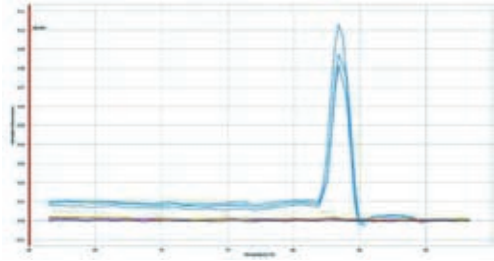

Fig. 7 Melt curve of real time PCR assay for *ISCfe-1* gene from positive control and clinical samples

Furthermore, the report of no campylobacteriosis case from 160 clinical samples processed might be due to insignificant endemic levels in the location, where the study was conducted. This assumption is supported by the results of Mani (2014), where he could not detect any *C. foetus* organism from the samples of infertility and abortion from bovines in the same area.

Similarly, ELISA was standardized for detecting antibodies from clinical samples. Initially the optimum concentrations of the antigen, antibody and IgG-HRP conjugate was calculated employing checker board analysis. It was observed that antigen concentrations (10

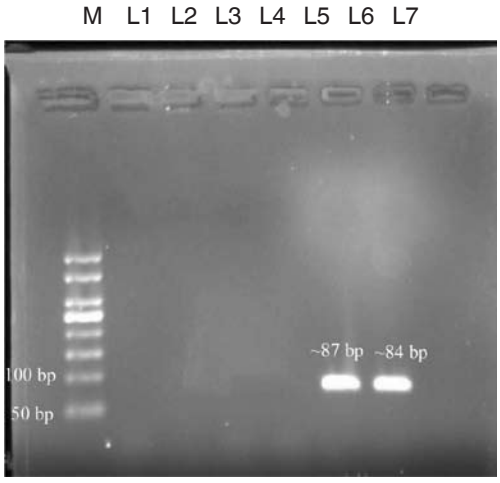

Fig. 8 Standardisation of *SapB2* and *ICSfe-1* gene using conventional PCR with product size 87bp and 84bp

M – 50 bp DNA ladder

L1-L4 – Negative control

L5 – *C*. *foetus* subsp. *foetus* (Positive control-87bp)

L6 – *C*. *foetus* subsp. *venerealis* (Positive control – 84bp)

µg /100 µL) of 50 ng per well for *SapB2* were found optimum at a test sera dilution of 1:100.

In order to determine the cut off value for demarcating the negative and positive sera samples, the ELISA was conducted employing the whole cell antigen antibody and IgG-HRP conjugate and the cut off values obtained were 0.12. A total of 50 suspected bovine serum samples were screened using the ELISA and all the serum samples were found to be negative. The high frequency of negative samples observed while performing ELISA might be due to factors such as better awareness regarding contagious disease among farmers, improved husbandry practices, climatic and geographic aspect of the area under study.

The work did not provide any proof of absence of the organism but the weight of evidence indicates that infection was highly unlikely to be widespread in herds. Artificial insemination is often considered as a simple control method for BGC, but is impractical for many herds where natural service takes place as a part of breeding. As per the breeding policy, only artificial insemination is practiced in Kerala in bovines, which might be one of the factors that prevent extensive spread of BGC.

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