RESEARCH ARTICLE Indian Journal of Animal Research, Volume 60 Issue 2: 292-299 (February 2026)

Prevalence of Uterine Infections and Development of a Multiplex PCR Assay for their Detection in Cattle

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10.18805/IJAR.B-5720

ABSTRACT

Background: Uterine infections are a leading cause of infertility and economic loss in dairy cattle, often resulting from complex polymicrobial infections. Rapid and accurate detection of the causative pathogens is essential for effective management. This study focuses on assessing the prevalence of uterine infections and developing a multiplex PCR assay for simultaneous detection of key bacterial pathogens in cattle.

Methods: A total of 1,905 cattle from the Vindhyan region were screened for uterine discharge and categorized as clear with tiny flakes, cloudy, mucopurulent, purulent, or haemorrhagic. Sixty uterine samples were aseptically collected for molecular analysis. Conventional PCR was employed to detect *Escherichia coli*, *Fusobacterium necrophorum*, *Trueperella pyogenes*, *Clostridium tertium* and *Histophilus somni*. A multiplex PCR (mPCR) assay was subsequently developed for simultaneous detection of *E. coli*, *C. tertium* and *H. somni* and its sensitivity, specificity and accuracy were assessed to evaluate diagnostic performance.

Result: The overall prevalence of uterine infections was 10.44%, consisting of 60.80% subclinical endometritis, 26.63% clinical endometritis and 12.56% metritis. The prevalence rates of *E. coli*, *C. tertium* and *H. somni* were 85%, 92.9% and 73.33%, respectively. Conventional PCR detected *E. coli* in 54, *F. necrophorum* in 01, *T. pyogenes* in 70, *C. tertium* in 44 and *H. somni* in 12 of the 60 uterine samples. Using mPCR, 51 samples were positive for *E. coli*, 44 for *C. tertium* and 15 for *H. somni*. The sensitivity, specificity and accuracy of the mPCR were 100%, 66.6% and 95% for *E. coli*; 100% each for *C. tertium*; and 80%, 100% and 95% for *H. somni*, respectively, indicating its high diagnostic efficiency.

Key words: Multiplex PCR, PCR, Prevalence, Uterine infections, Uterine pathogens.

INTRODUCTION

Efficient reproductive performance is fundamental to profitable dairy farming, as it directly impacts herd productivity. However, uterine infections remain a major constraint, contributing significantly to infertility, subfertility and economic loss. Uterine infections, broadly categorized as endometritis, metritis and pyometra depending on their severity and clinical presentation (Azawi, 2008), impair both fertility and productivity. Their economic impact is substantial, arising from delayed conception, increased insemination frequency, prolonged calving intervals, reduced milk yield and premature culling (Deka *et al.,* 2021). The losses are especially severe for smallholder farmers where livestock constitute a primary source of income and nutrition.

The prevalence of uterine infections in cattle varies considerably, with studies indicating that nearly half of dairy cows experience some degree of uterine infection during the postpartum period (Comlekcioglu *et al.,* 2024). The incidence is notably higher among high-yielding dairy breeds (Várhidi *et al*., 2024). Subclinical uterine infections, often overlooked during routine herd management, pose a major challenge as they silently impair fertility. Prevalence data, therefore, are critical for designing effective reproductive management programs. Uterine infections in cattle are typically caused by a variety of bacterial pathogens,

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How to cite this article: Narwade, P.S., Kumar, P.R., Kumar, P.P., Balamurugan, B., Jena, D., Ravi, S.K., Mishra, R.M., Ghosh, M. and Kumar, A. (2026). Prevalence of Uterine Infections and Development of a Multiplex PCR Assay for their Detection in Cattle. *Indian Journal of Animal Research.* **60(2):** 292-299. doi: 10.18805/IJAR.B-5720.

Submitted: 14-11-2025 **Accepted:** 09-12-2025 **Online:** 19-12-2025

including *Escherichia coli* (*E. coli*),*Trueperella pyogenes* (*T. pyogenes*), *Fusobacterium necrophorum* (*F. necrophorum*), *Prevotella melaninogenica* (*P. melaninogenica*) *Staphylococcus aureus* (*S. aureus*), *Streptococcus uberis* (*S. uberis*), *Bacteroides* spp. and *Proteus* spp. usually as mixed infections (Drillich and Wagener, 2018; Jeon *et al.,* 2018). *Clostridium tertium* (*C. tertium*), *Histophilus somni* (*H. somni*) and *Shigella* spp. were less frequently associated with bovine uterine infections in earlier literature, however emerging as potential pathogens of uterine infections. The complex and polymicrobial etiology of uterine infections poses significant challenges for their diagnosis and management. Traditional diagnostic methods, such as clinical examination, vaginal mucus scoring, uterine cytology and bacteriological culture, have long been used in veterinary practice. However, these methods are often constrained by limited sensitivity, delayed results and the inability to clearly distinguish pathogenic organisms from commensals (Barlund *et al.,* 2008).

Recent advances in molecular diagnostics have transformed the understanding of bovine uterine infections. Techniques such as PCR, real-time PCR (RT-PCR), multiplex PCR (mPCR) and sequencing-based methods offer enhanced sensitivity, specificity and rapid detection compared to traditional approaches (Aghamiri *et al.,* 2014; Kumar *et al*., 2021; Selim *et al*., 2024; Tahir *et al.,* 2025). These molecular approaches allow for accurate identification of pathogens, detection of mixed infections and characterization of virulence-associated genes, supporting precise diagnosis. Furthermore, PCR-based assays allow direct detection of bacterial DNA from uterine samples, reduces dependence on viable cultures and facilitates large-scale epidemiological surveillance. These advantages highlight the growing importance of molecular diagnostics in the context of bovine uterine infections. Therefore, the present study was designed to investigate the prevalence of uterine infections in cattle in Vindhyan region of Uttar Pradesh, India and to develop and evaluate the utility of molecular diagnostic approaches in their detection.

MATERIALS AND METHODS

Study area and sample collection

A total of 1,905 cattle were screened across the Vindhyan region of Uttar Pradesh, of which, 199 were diagnosed with uterine infections. Among the infected cattle, 60 uterine samples were collected aseptically from Mirzapur, Bhadohi, Varanasi and Sonbhadra districts of Vindhyan region. The uterine fluid was immediately transferred into sterile collection vials. The collected samples were initially preserved at 4°C in 20% glycerol stock to maintain microbial viability and subsequently stored at -20°C until further processing.

Categorization of samples and case definition

The collected samples were categorized based on physical characteristics of the uterine contents, which included clear with tiny flakes, cloudy, mucopurulent, purulent and bloody discharges. Cows exhibiting clear with tiny flakes were categorized as subclinical endometritis. Animals showing cloudy or mucopurulent discharge were categorized as clinical endometritis whereas, cows with purulent or bloody discharge were categorized as metritis.

DNA extraction

Genomic DNA from the sample collected was extracted using a GSure® Bacterial DNA Isolation Kit (GCC Biotech, India) as per the manufacturer's protocol.

Primers

The nucleotide sequence of *E. coli fimH* gene, *F. necrophorum llkA* gene, *T. pyogenes plo* gene, *C. tertium* 16S rRNA gene were retrieved from the NCBI GenBank database and the specific pairs of oligonucleotide primers were designed using primer blast of NCBI. The primers for *H. somni* 16S rRNA gene was taken from published literature. The designed and published primers were subsequently synthesized commercially at Barcode Biosciences Pvt. Ltd. (Bengaluru, India). The details are presented in Table 1.

Table 1: Primers pairs designed to amplify target genes.

Bacteria	Primer sequence (5′→3′)	Target gene	Amplification size (bp)
E. coli	F: CTGGTCGGTAAATGCCTGGT	*FimH*	604 bp
	R: CCCCAGGTTTTGGCTTTTCG		
F. necrophorum	F: CTTTTGTTGGAGCATGGGGC	*IlkA*	406 bp
	R: CTCCTGTCACTTGGGTGTCC		
T. pyogenes	F: GCGCAAGAGACCGACGACGAGACG	*Plo*	1010 bp
	R: GTTCCTGCCGTGCCCGTTTC		
C. tertium	F: GTGAGGTAACGGCTCACCAA	16S rRNA	988 bp
	R: CTCGCGGTATCGCATCTCTT		
H. somni	F: GAAGGCGATTAGTTTAAGAG	16S rRNA	313 bp
	R: ACTCGAGCGTCAGTATCTTC		

Reaction conditions of PCR

PCR conditions were optimized for specific and efficient amplification. Each reaction was performed in a 25 µL volume, containing 12.5 µL of 2X PCR master mix, 1 µL each of forward and reverse primers, 3 µL of diluted DNA template and 7.5 µL of nuclease-free water. The amplification protocol included an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation, primer annealing and extension. Denaturation was optimized at 95°C for 1 minutes. Annealing temperatures were optimized separately for each primer pair: 65°C for 1 min for the *fimH* gene, 52°C for 45 s for the *IlkA* gene, 57°C for 30s for *plo* gene, 55°C for 30 s for the *C. tertium* 16S rRNA gene and *H. somni* 16S rRNA gene. Extension was performed at 72°C for 1 min in each cycle, followed by a final extension at 72°C for 10 min. PCR products (10 µL) were resolved on a 1% agarose gel, stained with ethidium bromide and visualized using a gel documentation system.

Sequencing of amplicon

Representative products were purified and subjected to Sanger sequencing. Chromatograms were analyzed using Chromas (version 2.6.6) software and sequences were compared with GenBank entries using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Reaction conditions of multiplex PCR

Three bacteria namely *E. coli, C. tertium* and *H. somni* were selected for mPCR and reaction conditions were standardized. Each mPCR reaction was carried out in a 25 µL volume, containing 12.5 µL of 2X PCR master mix, 6 µL of primers (1 µL each of forward and reverse primers for each gene), 3 µL of diluted DNA template and 3.5 µL of nuclease-free water. The amplification protocol consisted of an initial denaturation at 95°C for 10 min, followed by denaturation 95°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 1 min, repeated for 30 cycles in a thermocycler. The final extension was carried out at 72°C for 10 min to ensure complete synthesis of amplicons. Each mPCR product (10 µL) was subjected to electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized using a gel documentation system.

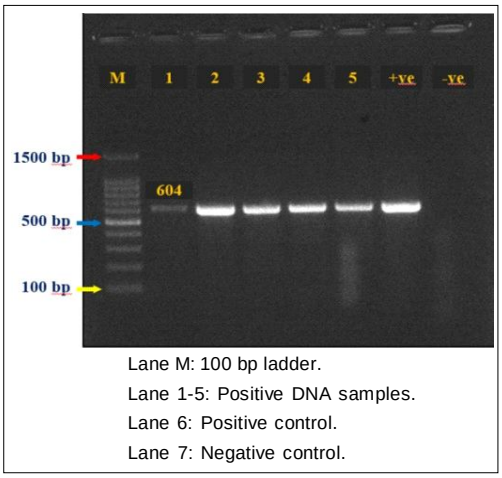
Ethical approval

All procedures for sample collection and laboratory analysis were performed in accordance with the institutional guidelines for animal experimentation. Ethical clearance was obtained from the Institutional Animal Ethics Committee (IAEC), Faculty of Veterinary and Animal Sciences, Banaras Hindu University, under approval number IAEC/BHU/FVAS/2024/173.

RESULTS AND DISCUSSION

Prevalence of uterine infection

Out of a total of 1,905 cattle screened across the Vindhyan region of Uttar Pradesh, 199 animals were diagnosed with uterine infections. The diagnosis was based on a combination of reproductive history, clinical signs and the physical characteristics of the uterine discharge. The overall prevalence of uterine infection was found to be 10.44%. The overall and district-wise prevalence of subclinical endometritis, clinical endometritis and metritis is summarized in Table 2, while the prevalence of *E. coli*, *C. tertium* and *H. somni* in the region is presented in Table 4. These findings indicate a considerable burden of uterine infections among cattle in the region, emphasizing the need for improved reproductive health monitoring and timely diagnosis and therapeutic interventions. Reports show substantial variability in the prevalence of uterine infections in cattle across studies and regions, with some studies reporting rates as high as 50% (Sheldon *et al.,* 2008; Comlekcioglu *et al.,* 2024; Mekibib *et al.,* 2024). The prevalence of metritis has been reported to range from 7.2% to 40% (Várhidi *et al.,* 2024; Nguyen *et al.,* 2025), while clinical endometritis ranges from 5% to 35.5% and subclinical endometritis from 11% to 72% (Galvao, 2012; Aghamiri *et al.,* 2014; Sarkar *et al.,* 2016; Kumar Chetan *et al*., 2021). The prevalence of E. coli in bovine uterine infections has been reported to range from 33.4% to 72.18% (Bicalho *et al.,* 2010; Dutta *et al.,* 2019; Shafique *et al.,* 2021), whereas literature on the prevalence of *C. tertium* and *H. somni* is megre. Variations in reported prevalence rates likely reflect differences in diagnostic methods, criteria for classifying uterine infections, postpartum stages, as well as factors such as parity and herd management practices (Lewis 1997; Aghamiri *et al.,* 2014). Uterine infections are frequently associated with predisposing reproductive disorders such as dystocia, uterine prolapse, abortion and retained fetal membranes (Bell and Roberts, 2007; Singhal *et al*., 2011; Honparkhe *et al*., 2025), which compromise uterine defense mechanisms and facilitate bacterial colonization of the endometrium. These conditions collectively contribute to impaired uterine health and subsequent fertility problems in affected animals.



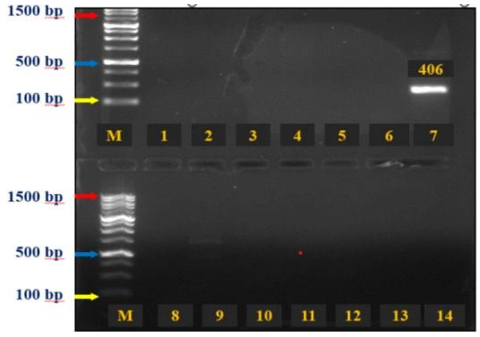
Lane M: 100 bp ladder.
Lane 1-5: Positive DNA samples.
Lane 6: Positive control.
Lane 7: Negative control.

Fig 1: PCR amplification of *E. coli* (*fimH*) gene at 604 bp.

Development of multiplex PCR assay

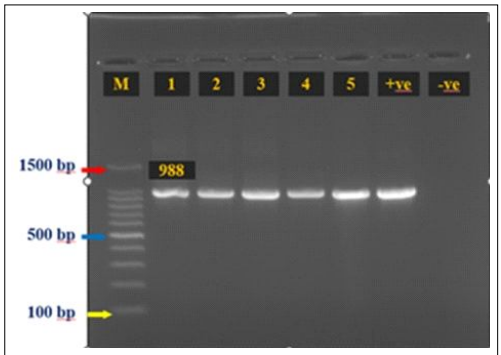
In order to develop mPCR assay, conventional PCR was first performed to identify the *E. coli, C. tertium, F. necrophorum, T. pyogenes* and *H. somni* as a gold standard test. In the present study *E. coli* (Fig 1; Table 3) emerged as the predominant uterine pathogen, followed by *C. tertium* (Fig 4; Table 3) across all categories of uterine discharge. In addition, *F. necrophorum* (Fig 2; Table 3), *T. pyogenes* (Fig 3; Table 3) *and H. somni* (Fig 5; Table 3) were also detected, highlighting the complex microbial interactions contributing to uterine pathology. All the amplicon was confirmed by Sanger sequencing except *Plo* gene of *T. pyogenes* which may be due to non-specific amplification. *E. coli* is often regarded as the primary etiological agent of the uterine infection (Sheldon *et al.,* 2002; Williams *et al*., 2005; Mekibib *et al.,* 2024), gaining access to the uterine lumen during or shortly after parturition. Subsequently, *T. pyogenes* and *F. necrophorum* may establish synergistic infections (Sheldon *et al.,* 2008; Haimerl *et al.,* 2018). The identification of *C. tertium* and *H. somni* in the current study is noteworthy, as these organisms are less frequently associated with bovine uterine infections in earlier literature (Olson, 1984; Jeon *et al.,* 2015; Saad *et al.,* 2022; Molín *et al.,* 2024) suggesting potential regional variation in pathogen ecology or identification techniques. The observed variation in bacteriological findings across studies may also be attributed to differences in sampling stage, diagnostic criteria, geographical conditions, animal breed and management practices. Collectively, these findings indicate that while *E. coli* continue to play a dominant role in uterine infections, emerging bacteria such as *C. tertium* and *H. somni* may be gaining epidemiological significance in specific herds or regions, warranting further investigation using molecular and metagenomic approaches.

Based on PCR and Sanger sequencing result, a mPCR assay was successfully developed for the simultaneous detection of *E. coli*, *C. tertium* and *H. somni* (Fig 6 and 7; Table 4). The developed mPCR accurately identified all three pathogens in 90% (54/60) of samples, while 10% (6/60) were correctly identified for two of the



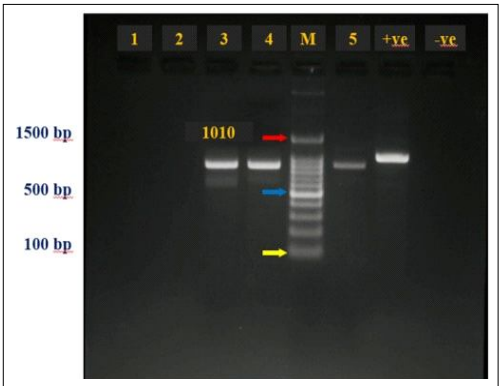
Lane M: 100 bp ladder.
Lane: 1-6 and 8-13: Negative DNA samples.
Lane 14: Negative control.

Fig 2: PCR amplification of *F. necrophorum* (*llkA*) gene at 406 bp.



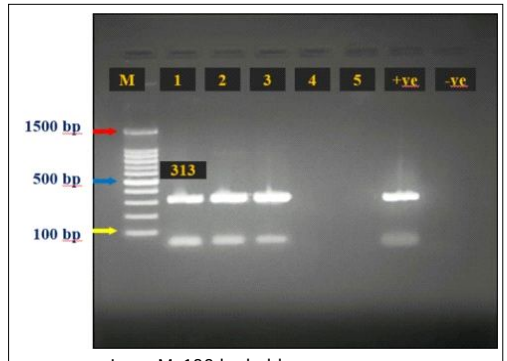
Lane M: 100 bp ladder.
Lane: 1-5: Positive DNA samples.
Lane 6: Positive control.
Lane 7: Negative control.

Fig 4: PCR amplification of *C. tertium* 16S rRNA gene at 988 bp.



Lane 1 and 2: Negative DNA samples.
Lane 3, 4 & 5: Positive DNA samples.
Lane 6: Positive control.
Lane 7: Negative control.

Fig 3: PCR amplification of *T. pyogenes* (*plo*) gene at 1010 bp.



Lane M: 100 bp ladder.
Lane 1-3: Positive DNA samples.
Lane 4 and 5: Negative DNA samples.
Lane 6: Positive control.
Lane 7: Negative control.

Fig 5: PCR amplification of *H. somni* 16S rRNA gene at 313 bp.

Table 2: Prevalence of uterine infection across the vindhyan region.

Districts covered in vindhyan region	Total no. of animals observed	No. of animals with uterine infection	Nature of discharge observed	No. of animals with specific nature of discharge	Nature of disease	% of uterine infections
Mirzapur	1224	158	Clear	114	Subclinical endometritis	72.15
			Cloudy	28	Clinical endometritis	22.15
			Mucopurulent	07		
			Purulent	04	Metritis	5.69
			Hemorrhagic	05		
Varanasi	418	16	Clear	02	Subclinical endometritis	12.5
			Cloudy	02	Clinical endometritis	37.5
			Mucopurulent	04		
			Purulent	03	Metritis	50.0
			Hemorrhagic	05		
Bhadohi	210	09	Clear	02	Subclinical endometritis	22.22
			Cloudy	02	Clinical endometritis	22.22
			Mucopurulent	00		
			Purulent	03	Metritis	55.55
			Hemorrhagic	02		
Sonbhadra	53	16	Clear	03	Subclinical endometritis	18.75
			Cloudy	04	Clinical endometritis	25.0
			Mucopurulent	06		
			Purulent	03	Metritis	18.75
			Hemorrhagic	00		

Overall prevalence

Subclinical endometritis	Clinical endometritis	Metritis	Overall uterine infection
121/199=60.80%	53/199=26.63%	25/199=12.56%	199/1905=10.44%

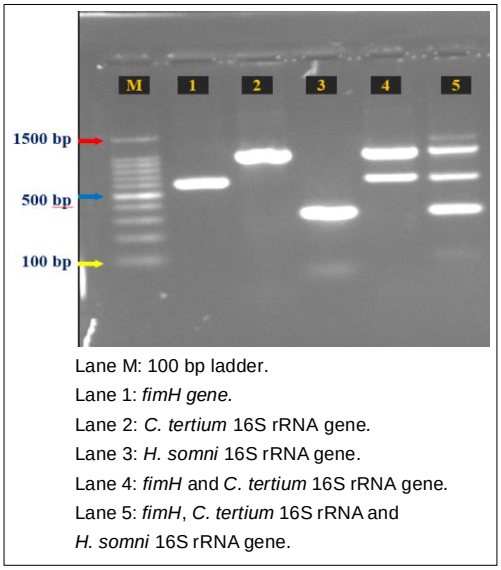
Table 3: Findings of uterine pathogens using PCR across the vindhyan region.

Nature of discharge	Total no. of samples	Pathogenic microorganism	Positive samples	Negative samples	%
Clear	22	*E. coli*	20	02	90.9
		F. necrophorum	00	22	0.0
		T. pyogenes	00	22	0.0
		C. tertium	10	12	83.4
		H. somni	03	19	13.6
Cloudy	14	*E. coli*	12	02	85.7
		F. necrophorum	00	14	0.0
		T. pyogenes	00	14	0.0
		C. tertium	13	01	92.8
		H. somni	02	12	16.7
Mucopurulent	08	*E. coli*	06	02	75.0
		F. necrophorum	00	08	0.0
		T. pyogenes	04	04	50.00
		C. tertium	07	01	87.5
		H. somni	02	06	25.0
Purulent	08	*E. coli*	08	00	100.0
		F. necrophorum	01	07	12.5
		T. pyogenes	03	05	37.5
		C. tertium	06	02	75.0
		H. somni	02	06	25.0
Hemorrhagic	08	*E. coli*	08	08	100.0
		F. necrophorum	00	08	0.0
		T. pyogenes	00	08	0.0
		C. tertium	08	00	100.0
		H. somni	03	05	37.5

Table 4: Prevalence, sensitivity, specificity and accuracy of mPCR.

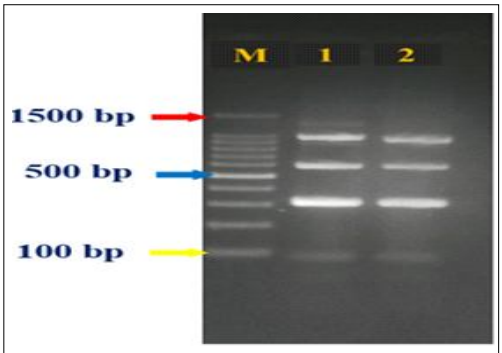
Bacterial pathogens	mPCR	Conventional PCR +ve	Conventional PCR -ve	Prevalence (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	*Kappa*
E. coli	+ve	51	3	85 (73.43, 92.90)	100 (93.02, 100)	66.7 (29.93, 100)	94.44 (87.09, 97.72)	100 (54.07, 100)	95 (86.08, 98.96)	0.77
	-ve	0	6							
C. tertium	+ve	44	0	73.33 (60.34, 83.93)	100 (91.96, 100)	100 (79.41, 100)	100 (91.96, 100)	100 (79.41, 100)	100 (94.04, 100)	1
	-ve	0	16							
H. somni	+ve	12	0	25 (14.72, 37.86)	80 (51.91, 95.67)	100 (92.13, 100)	100 (73.54, 100)	93.75 (84.50, 97.63)	95 (86.08, 98.96)	0.86
	-ve	3	45							

(Results are presented at 95% confidence interval).



Lane M: 100 bp ladder.
Lane 1: *fimH gene.*
Lane 2: *C. tertium* 16S rRNA gene.
Lane 3: *H. somni* 16S rRNA gene.
Lane 4: *fimH* and *C. tertium* 16S rRNA gene.
Lane 5: *fimH*, *C. tertium* 16S rRNA and *H. somni* 16S rRNA gene.

Fig 6: mPCR amplification of *fimH*, *C. tertium* 16S rRNA and *H. somni* 16S rRNA gene.



Lane M: 100 bp ladder.
Lane 1 and 2: *fimH*, *C. tertium* 16S rRNA and *H. somni* 16S rRNA gene.

Fig 7: mPCR amplification of *fimH*, *C. tertium* 16S rRNA and *H. somni* 16S rRNA gene.

three target pathogens. The assay showed high sensitivity and accuracy, with moderate specificity for *E. coli*, whereas *C. tertium* and *H. somni* exhibited high sensitivity, specificity and accuracy (Table 4). Traditionally, *T. pyogenes*, *E. coli*, *F. necrophorum* and *P. melaninogenica* are considered the major uterine pathogens (Sheldon and Dobson, 2004; Azawi, 2008); however, recent studies have highlighted emerging pathogens such as *Clostridium*, *Bacteroides*, *Porphyromonas*, *Prevotella*, *Histophilus*, *Shigella* and *Helcococcus* (Jeon and Galvao, 2018; Basbas *et al.,* 2023; Becker *et al.,* 2023). Reports on the detection of bovine uterine pathogens using mPCR are megre. Dong-bo *et al.* (2011) developed an mPCR for simultaneous detection of *S. aureus*, *E. coli* and *B. cereus* in bovine endometritis, reporting 23.95% triple infections and 17.15% co-infections with *S. aureus* and *E. coli*, which is lower than the *E. coli* detection rate in our study. Similarly, Dung *et al.* (2024) observed 100% sensitivity for *Klebsiella*

pneumoniae, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *E. coli* and 63.6% for *Staphylococcus aureus*, with specificity ranging from 87.5% to 97.6%. Multiplex PCR, employing multiple primer sets in a single reaction, enables rapid and simultaneous detection of multiple pathogens from a single sample (Zhao *et al.*, 2019; Aziz *et al.*, 2021; Kumar *et al.*, 2021).

CONCLUSION

In conclusion, the overall prevalence of uterine infection in cattle was recorded 10.45 % whereas *E. coli* was the most prevalent uterine pathogen in Vindhyan region. Further, a multiplex PCR was developed for simultaneous detection of *E. coli, C. tertium* and *H. somni*. in cattle. This method will be helpful to diagnose mixed infection of uterine pathogens in cattle. Further refinement in the methodology may be undertaken to improve the assay. To the best of our knowledge, this is the first report describing the simultaneous detection of *E. coli*, *C. tertium* and *H. somni* from mixed uterine infections.

ACKNOWLEDGEMENT

The present study was supported by "Faculty Incentive Grant" of Institute of Eminence (IoE), Banaras Hindu University. Authors are highly thankful to Faculty of Veterinary and Animal Sciences and Banaras Hindu University for providing the necessary fund and facilities for undertaking this project.

Disclaimers

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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