

Characterization of bacterial flora associated with canine pyometra

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Journal of Livestock Science (ISSN online 2277-6214) 17: 759-765

Received on 24/4/26; Accepted on 15/9/26; Published on 18/9/26

doi. 10.33259/JLivestSci.2026.759-765

Abstract

Canine pyometra is a critical reproductive disorder in intact female dogs, driven by progesterone-induced uterine changes that facilitate bacterial colonization while suppressing local immune responses. This study characterized the bacterial flora from 15 female dogs categorized into mild, moderate and severe cases using ultrasonography. Microbial identification was performed via conventional culture, BD PhoenixTM automated systems and molecular validation through 16S rRNA PCR. *Escherichia coli* was the most prevalent isolate (86.67%), consistently present in all severe cases, followed by *Staphylococcus spp.* (53.33%). Other identified organisms included *Pseudomonas spp.*, *Klebsiella spp.* and *Proteus spp.* Molecular analysis successfully confirmed these findings, yielding taxon-specific amplicons of 1476 bp for *E. coli* and 876 bp for *Staphylococcus spp.* antimicrobial susceptibility testing revealed that Imipenem (100% sensitivity) and Gentamicin were the most effective agents. Conversely, significant resistance was observed for Ceftriaxone and Enrofloxacin, with *E. coli* showing 0% sensitivity to Ceftriaxone. These results emphasize the necessity of 16S rRNA-based molecular confirmation for diagnostic accuracy and highlight the urgent need for culture-guided antimicrobial therapy to address rising antimicrobial resistance in bacteria causing canine pyometra.

Keywords: Amplicons; Ceftriaxone; *Escherichia coli*; Gentamicin; Imipenem; *Staphylococcus spp.*

Introduction

Pyometra is a serious and potentially life-threatening uterine disorder of intact female dogs, characterized by bacterial infection and the accumulation of purulent material within the uterus. The condition typically develops during the luteal phase of the oestrous cycle, when prolonged progesterone exposure induces cystic endometrial hyperplasia, suppresses uterine immune defences and facilitates bacterial colonization (Xavier et al., 2023). Clinically, pyometra presents in open- and closed-cervix forms, with the latter often associated with rapid systemic deterioration and increased mortality even with surgical. Despite improvements in diagnostic imaging and surgical techniques, canine pyometra continues to pose a significant challenge in small animal practice, particularly where antimicrobial resistance complicates management (Paudel et al., 2023). Bacterial infection is central to the pathogenesis and progression of pyometra, with ascending infection from the lower reproductive tract regarded as the principal route of uterine contamination. Progesterone-mediated reduction in uterine contractility and local immune response further enhances bacterial persistence, reinforcing the need for accurate microbial identification and appropriate antimicrobial therapy (Xavier et al., 2022).

Among the bacterial species implicated in canine pyometra, *Escherichia coli* is most frequently isolated, either alone or in combination with other organisms (Ylhäinen et al., 2025). Its pathogenicity is attributed to virulence factors that promote adhesion to the endometrium and facilitate tissue invasion. Other opportunistic bacteria, including *Staphylococcus spp.*, *Pseudomonas spp.*, *Klebsiella spp.* and *Proteus spp.* have been variably reported in uterine infections. Conventional bacteriological methods remain fundamental for bacterial isolation but may be limited by time requirements and reduced precision in species-level identification. Automated microbial identification systems and molecular techniques, particularly PCR targeting conserved genes such as 16S rRNA, offer rapid and reliable confirmation of bacterial isolate (Xavier et al., 2022). In parallel, antimicrobial therapy plays a critical role in pyometra management; however, the rising incidence of antimicrobial resistance among uterine pathogens emphasizes the importance of culture-based antimicrobial susceptibility testing for rational antibiotic selection (Bertero et al., 2024).

Although several studies have reported bacterial isolation from canine pyometra, comprehensive region-specific data integrating disease severity, identification using conventional, automated and molecular methods, and antimicrobial susceptibility patterns remain limited (Ylhäinen et al., 2025). In this context, the present study was undertaken to characterize the bacterial flora associated with mild, moderate and severe canine pyometra through combined conventional culture, BD Phoenix™ automated identification and molecular confirmation by 16S rRNA PCR, with simultaneous evaluation of antimicrobial susceptibility profiles of the isolates, aiming to generate clinically relevant evidence to support targeted antimicrobial therapy and improve therapeutic outcomes in canine pyometra.

Materials and methods

The present study involved 15 intact female dogs presented to the Outpatient Department of Veterinary Gynaecology and Obstetrics, Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Jabalpur, Madhya Pradesh. These animals were from Jabalpur and surrounding regions, exhibited clinical symptoms reported by owners, including purulent vaginal discharge, lethargy, anorexia, polyuria and polydipsia etc. Following a thorough physical examination, the dogs were clinically and ultrasonographically diagnosed with open-cervix pyometra. Diagnosis was based on clinical signs and transabdominal ultrasonographic findings, as per criteria for canine pyometra described by Zheng et al., (2023). Based on ultrasonographic assessment of uterine distension and echogenicity of uterine contents, animals were categorized into mild (n = 5), moderate (n = 5) and severe (n = 5) pyometra, following severity-based classification approaches reported in studies. Pus samples were aseptically collected using sterile cotton swabs introduced into the vaginal canal under proper restraint and hygienic conditions. Swabs were gently rotated to ensure adequate sample collection and immediately transferred into sterile vials containing phosphate-buffered saline (PBS). Samples were transported to the bacteriology laboratory under chilled conditions for further processing, as recommended for uterine microbiological investigations by Ylhäinen et al. (2025).

Isolation and identification of bacteria

For primary enrichment, samples were inoculated into brain heart infusion (BHI) broth and incubated aerobically at 37°C for 18-24 h. Following enrichment, cultures were streaked onto nutrient agar, blood agar, mannitol salt agar and MacConkey agar plates and incubated at 37°C for 24-48 h. This culture strategy is routinely employed for the isolation of aerobic uterine pathogens in canine pyometra (Ylhäinen et al., 2025). Bacterial colonies were examined for morphological characteristics. Distinct isolates were purified and identified using the BD Phoenix™ M50 automated microbial identification system employing Gram-positive and Gram-negative identification panels, which provides rapid and reliable bacterial identification in veterinary clinical microbiology. Identified isolates were preserved on nutrient agar slants at 4°C for further analysis.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (Hindler & Matuschek, 2023; Zimmer, 2024; Sweeney, 2024; Diaz-Campos & Burbick, 2024). Bacterial suspensions were adjusted to 0.5 McFarland turbidity and uniformly inoculated on agar plates. The antimicrobial discs used included ceftriaxone (30 µg), enrofloxacin (10 µg), levofloxacin (5 µg), tetracycline (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg) and imipenem (10 µg), reflecting antibiotics commonly evaluated in canine pyometra and uterine infections (Sroithongkham et al., 2024). Plates were incubated at 35°C for 24 h and zones of inhibition were measured and interpreted according to CLSI standards.

Molecular confirmation of bacterial isolates

Genomic DNA from *E. coli* isolates was extracted using the QIAamp DNA Mini Kit, whereas DNA from *Staphylococcus spp.* was isolated using the InstaGene™ Matrix (Bio-Rad), following the manufacturers' protocols. The application of standardized commercial kits for bacterial DNA extraction has been widely reported in molecular investigations of canine pyometra. DNA concentration and purity were determined using a NanoDrop spectrophotometer (ND-2000c) by measuring absorbance at 260 and 280 nm (A260/280). The quality of the DNA was evaluated by electrophoresis on a 0.8% agarose gel for 60 minutes at 80 V. The extracted DNA was diluted in autoclaved Milli-Q water in 1.5 mL Eppendorf tubes to obtain a final concentration of 30 ng/µL. Samples exhibiting A260/280 ratios between 1.7 and 1.9 were selected for polymerase chain reaction (PCR) analysis.

Molecular confirmation of isolates was performed by PCR targeting the 16S rRNA gene using taxon-specific primers, yielding amplicons of 1476 bp for *E. coli* and 876 bp for *Staphylococcus spp.* (Table 1). PCR amplification was carried out in a 25.0 µL reaction mixture comprising 12.5 µL of DreamTaq™ Green PCR Master Mix, 1.0 µL (10 pmol) of each forward and reverse primer, 3.0 µL (approximately 90 ng) of diluted genomic DNA and 7.5 µL of nuclease-free water. The thermal cycling conditions included an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation, annealing (55°C for *E. coli* and 58°C for *Staphylococcus spp.*) and extension, with a final extension at 72°C. The PCR products were visualized by electrophoresis on a 1.5% agarose gel prepared in 0.5× TBE buffer and stained with ethidium bromide (500 ng/µL). 5.0 µL of each amplified product was loaded per lane. A DNA ladder (Fermentas Life Science; 100–1500 bp for *E. coli* and 100–1000 bp for *Staphylococcus spp.*), along with negative control, was run in separate lanes. Electrophoresis was performed at a constant voltage of 80 V for 60 min at 37°C using 0.5× TBE buffer. The amplified DNA fragments were visualized under a UV transilluminator and documented using a gel documentation system (Gel Doc, Bio-Rad, USA). General PCR amplification and gel-electrophoretic procedures followed standard molecular laboratory practices (Khan et al., 2024).

Results

Isolation and identification of bacteria

Pus samples obtained from 15 female dogs were subjected to comprehensive bacteriological examination to isolate and identify the associated pathogens, following their categorization into mild (n=5), moderate (n=5), and severe (n=5) groups based on transabdominal ultrasonographic findings. To obtain pure bacterial isolates, samples were inoculated onto selective and differential culture media, including Nutrient agar, Blood agar and MacConkey agar, with species-level identification subsequently confirmed using the BD Phoenix™ automated identification system. Out of the 15 samples examined, *E. coli* was the most prevalent isolate, detected in 13 cases (86.67%) while *Staphylococcus spp.* was isolated from 8 samples (53.33%) (Table 2). Other organisms found in the samples included *Pseudomonas spp.* in five cases (33.33%), *Klebsiella spp.* in three (20.00%), and *Proteus* and *Enterobacter spp.* in two cases (13.33%) respectively whereas *Bacillus licheniformis* was found in one sample (6.67%). Representative isolates exhibiting prominent colony characteristics on different culture media are displayed in Fig. 1. Distribution by disease severity revealed that *E. coli* was consistently isolated from all five severe cases, followed by *Staphylococcus spp.* (3), *Klebsiella spp.* (2), *Pseudomonas spp.* (2), *Proteus spp.* (1), and *Bacillus licheniformis* (1). In moderate cases, *E. coli* also remained the most frequent isolate (5), followed by *Staphylococcus spp.* (2), *Pseudomonas spp.* (2), *Enterobacter spp.* (2), and single instances of *Klebsiella spp.* and *Proteus spp.* Among mildly affected dogs, *E. coli* and *Staphylococcus spp.* were each detected in 3 samples, while *Pseudomonas spp.* was isolated in one instance. Following identification, antimicrobial susceptibility testing was conducted using the disk diffusion method to evaluate sensitivity and resistance patterns against commonly used antibiotics.

Antibiotic sensitivity test (AST)

The results of the antimicrobial susceptibility testing (AST) for *Escherichia coli* and *Staphylococcus spp.* are detailed in Table 3 and 4. Among the *E. coli* isolates (n=13), the highest mean zone of inhibition was recorded for Imipenem (35.62±1.0 mm), followed by Levofloxacin (18.5±0.9 mm), Gentamicin (17.0±0.7 mm), Tetracycline (16.5±1.2 mm), Enrofloxacin (14.5±0.8 mm), Ciprofloxacin (14.0±0.6 mm) and Ceftriaxone (12.0±0.5 mm). Similarly, *Staphylococcus spp.* isolates (n=8) exhibited the maximum mean zone of inhibition

Table 1 - Sequences of primers used for molecular identification of *Escherichia coli* and *Staphylococcus spp.*

Target organism	Gene	Primer sequence (5'-3')	Product size (bp)	Reference
<i>Escherichia coli</i>	16S rRNA	F: TGGGAACGGCGAGTCGGAATAC	1476	Patil et al. (2025)
		R: GGCGCAGGGGATGAAACTCAAC		
<i>Staphylococcus spp.</i>	16S rRNA	F: GTGCCAGCAGCCGCGGTAA	876	Dégi et al. (2021)
		R: AGACCCGGGAACGTATTAC		

Table 2 - Bacterial isolates obtained from severe, moderate and mild pyometra pus samples

Type of Pyometra	No. of animals (n)	Bacterial Isolates (n)						
		<i>E. coli</i>	<i>Staphylococcus spp.</i>	<i>Klebsiella spp.</i>	<i>Pseudomonas spp.</i>	<i>Proteus spp.</i>	<i>Enterobacter spp.</i>	<i>Bacillus licheniformis</i>
Severe	5	5	3	2	2	1	0	1
Moderate	5	5	2	1	2	1	2	0
Mild	5	3	3	0	1	0	0	0

Table 3 - Zone of inhibition in mm (Mean±SE) of common antibiotic against *E. coli* and *Staphylococcus spp.* isolates obtained from pus sample of female dogs suffering from pyometra

Bacterial isolates	CTR 30	EX 10	LE 5	TE 30	GE 10	CIP 5	IMP 10
<i>E. coli</i> (n=13)	12.0±0.5	14.5±0.8	18.5±0.9	16.5±1.2	17.0±0.7	14.0±0.6	35.62±1.0
<i>Staphylococcus spp.</i> n=08)	14.0±0.6	12.0±0.5	20.0±0.7	22.0±0.9	24.0±1.0	13.5±0.5	37.87±1.1

Table 4 - Antibiotic sensitivity of common antibiotic against *E. coli* and *Staphylococcus spp.* isolates obtained from pus sample of female dogs suffering from pyometra

Bacterial Isolates	CTR 30	EX 10	LE 5	TE 30	GE 10	CIP 5	IMP 10
<i>E. coli</i> n=13	00 (00.00)	02 (15.38)	09 (59.23)	04 (30.76)	05 (38.46)	03 (23.07)	13 (100.0)
<i>Staphylococcus spp.</i> n=8	01 (12.50)	00 (00.00)	06 (75.00)	04 (50.00)	07 (87.50)	02 (25.00)	08 (100.0)

Figure in parenthesis indicate percentage.

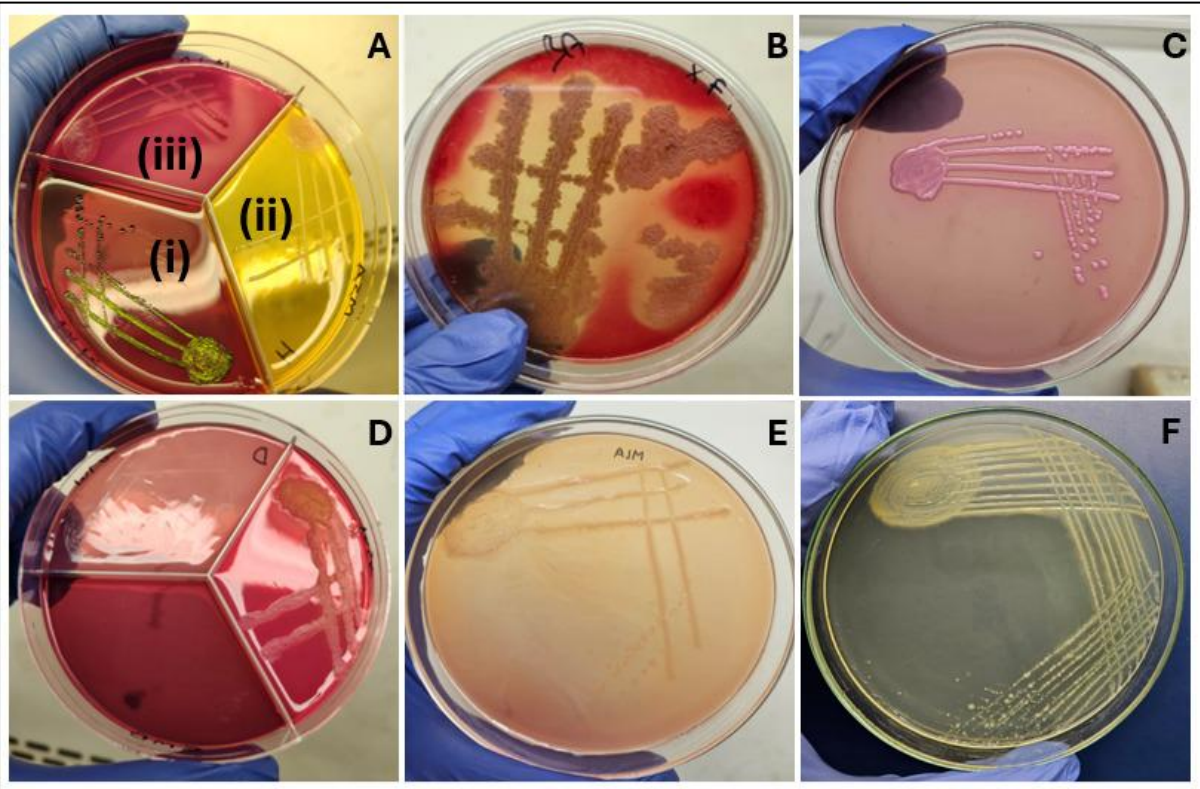


Fig. 1. Representative isolates exhibiting prominent colony characteristics on different culture media - (A) (i) Growth of *E. coli* on Eosin Methylene Blue (EMB) agar showing characteristic metallic green sheen colonies; (ii) Bright pink/red *E. coli* colonies on MacConkey agar; (iii) Differential growth of *Staphylococcus spp.* on Mannitol Salt Agar (MSA). (B) - Large, opaque and dull-grey colonies of *Bacillus licheniformis* on Blood Agar with rough, wrinkled and mucoid surface. (C) Pink colour growth of *Klebsiella spp.* on MacConkey agar with mucoid colonies. (D) Growth of *Staphylococcus spp.* on Blood Agar with heavy, opaque and white colonies. (E) Non-lactose fermenting colonies of *Pseudomonas spp.* on MacConkey agar (F) Growth of *Pseudomonas spp.* on Nutrient Agar with large, opaque and flat colonies.

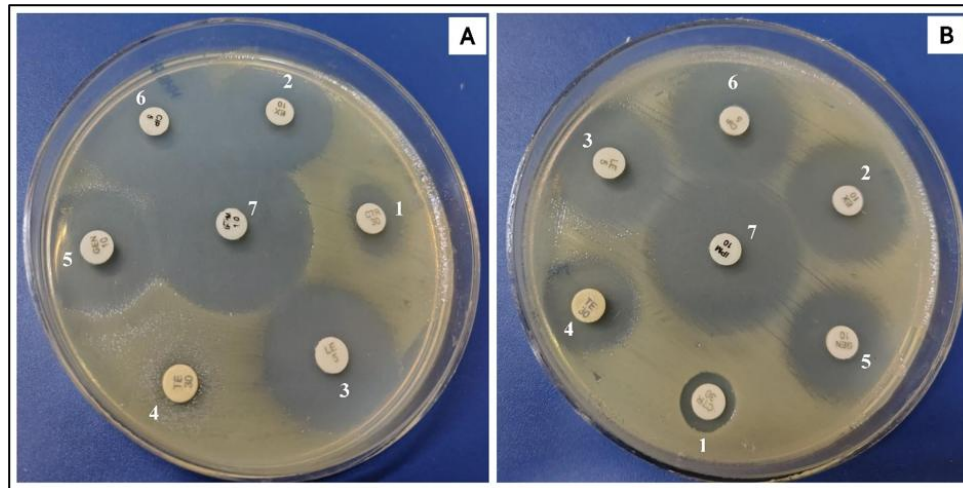


Fig. 2. Antimicrobial susceptibility testing by the disk diffusion method, illustrating distinct zones of inhibition for *E. coli* and *Staphylococcus spp.* isolates against a panel of various antibiotics on Mueller-Hinton agar - 1. Ceftriaxone (30 µg), 2. Enrofloxacin (10 µg), 3. Levofloxacin (5 µg), 4. Tetracycline (30 µg), 5. Gentamicin (10 µg), 6. Ciprofloxacin (5 µg), 7. imipenem (10 µg).

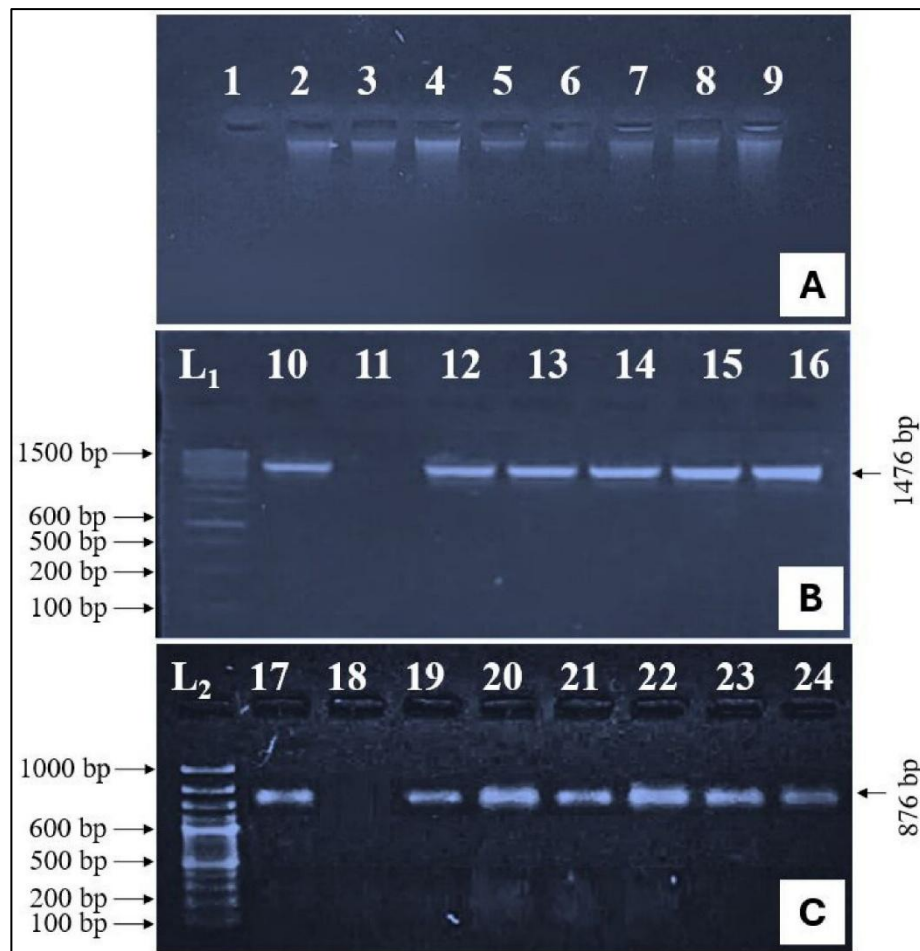


Fig. 3. Molecular characterization of bacterial isolates (a) Genomic DNA of *Escherichia coli* and *Staphylococcus spp.* on 0.8% agarose gel; (b) PCR amplicons (1476 bp) of the 16S rRNA gene of *E. coli* on 1.5% agarose gel; (c) PCR amplicons (876 bp) of the 16S rRNA gene of *Staphylococcus spp.* on 1.5% agarose gel. [Lane L1: 1500 bp ladder; Lane L2: 1000 bp ladder; Lanes 1, 11, 18: Negative controls; Lanes 10-17 Genomic DNA as positive controls, 2-5: Genomic DNA of *E. coli*; Lanes 6-9: Genomic DNA of *Staphylococcus spp.*; Lanes 12-16: 1476 bp amplicons of *E. coli*; Lanes 19-24: 876 bp amplicons of *Staphylococcus spp.*]

with Imipenem (37.87 ± 1.1 mm), followed by Gentamicin (24.0 ± 1.0 mm), Tetracycline (22.0 ± 0.9 mm), Levofloxacin (20.0 ± 0.7 mm), Ceftriaxone (14.0 ± 0.6 mm), Ciprofloxacin (13.5 ± 0.5 mm) and Enrofloxacin (12.0 ± 0.5 mm). In terms of sensitivity percentages, Imipenem demonstrated 100% efficacy against both *E. coli* and *Staphylococcus spp.* isolates. The results of the antimicrobial susceptibility testing, displaying the zones of inhibition for *E. coli* and *Staphylococcus spp.* against various antibiotics, are illustrated in Figure 2. For *E. coli*, this was followed by Levofloxacin (59.23%), Gentamicin (38.46%), Tetracycline (30.76%), Ciprofloxacin (23.07%), and Enrofloxacin (15.38%), though notably, no isolates were sensitive to Ceftriaxone. *Staphylococcus spp.* isolates showed high sensitivity to Gentamicin (87.5%), Levofloxacin (75%), and Tetracycline (50%), with lower sensitivity to Ciprofloxacin (25%) and Ceftriaxone (12.5%), and a complete lack of sensitivity to Enrofloxacin. Overall, Imipenem emerged as the most effective agent against both Gram-negative and Gram-positive pathogens in this study, while Ceftriaxone and Enrofloxacin displayed the lowest therapeutic potential.

Molecular confirmation of bacterial isolates

The extracted genomic DNA from *E. coli* and *Staphylococcus spp.* exhibited OD 260/280 ratios between 1.75 and 1.88, indicating high purity with minimal protein or impurity contamination. Samples falling outside this range underwent reprocessing via phenol-chloroform extraction to ensure quality. DNA concentrations ranged from 180.2 ng/ μ L to 860.3 ng/ μ L, and the integrity of the extracted genomic DNA was verified through 1.0% agarose gel electrophoresis (Fig. 3A). For molecular confirmation, phenotypically identified isolates were subjected to PCR targeting the 16S rRNA gene. All thirteen *Escherichia coli* isolates yielded the expected 1476 bp amplicon, while all eight *Staphylococcus spp.* isolates produced the characteristic 876 bp amplicon, revealing a taxon-specific amplification (Fig. 3B & 3C). These results successfully validated the phenotypic and automated identification findings, confirming *E. coli* and *Staphylococcus spp.* as the predominant bacterial pathogens associated with canine pyometra in this study.

Discussion

PCR confirmation was concordant with phenotypic identification: all 13 *E. coli* isolates yielded the expected 1476-bp 16S rRNA amplicon and all eight *Staphylococcus spp.* isolates yielded the expected 876-bp amplicon. This concordance supports PCR as a useful complementary method for confirming predominant bacterial isolates, while culture remains essential when antimicrobial susceptibility testing is required. Overall, the predominance of *E. coli*, the occurrence of multiple bacterial taxa and the observed variation in antimicrobial susceptibility emphasize the value of etiological diagnosis and susceptibility-guided therapy in canine pyometra.

Antimicrobial susceptibility differed markedly among the tested drugs. Imipenem showed 100% in-vitro susceptibility against both *E. coli* and *Staphylococcus spp.*, while gentamicin showed comparatively high activity, particularly against *Staphylococcus spp.* In contrast, none of the *E. coli* isolates were susceptible to ceftriaxone and none of the *Staphylococcus spp.* isolates were susceptible to enrofloxacin. Variable susceptibility and multidrug-resistant *E. coli* associated with canine pyometra have also been documented in recent studies (Sroithongkham et al., 2024; Ylhäinen et al., 2025), while broader companion-animal surveillance demonstrates substantial resistance among *E. coli* and *Staphylococcus spp.* isolates (Seo et al., 2025). These findings support culture- and susceptibility-guided antimicrobial selection rather than empirical reliance on broad-spectrum drugs. Nevertheless, in-vitro susceptibility should not be equated directly with clinical outcome, because surgery and other clinical factors also influence recovery in canine pyometra (Bertero et al., 2024).

E. coli was recovered from all severe and moderate cases and from three of five mild cases; however, because each severity category contained only five animals, the distribution should be regarded as descriptive and no firm association between a particular organism and disease severity can be inferred. *Escherichia coli* was the predominant bacterial isolate in the present study, being detected in 86.67% (13/15) of pyometra cases, followed by *Staphylococcus spp.* (53.33%); *Pseudomonas spp.*, *Klebsiella spp.*, *Proteus spp.*, *Enterobacter spp.* and *Bacillus licheniformis* occurred less frequently. This pattern agrees with recent studies identifying *E. coli* as the principal bacterial pathogen in canine pyometra (Paudel et al., 2023; Sroithongkham et al., 2024; Ylhäinen et al., 2025). The frequent occurrence of *E. coli* has been associated with extraintestinal pathogenic strains carrying virulence traits that facilitate colonization of the reproductive and urinary tracts (Melo et al., 2022; Xavier et al., 2022). The presence of several additional bacterial genera in the present study also supports the recognized polymicrobial diversity of canine pyometra.

Conclusion

In the present study, *E. coli* and *Staphylococcus spp.* were the predominant bacterial pathogens associated with canine pyometra across all grades of disease severity, with *E. coli* emerging as the dominant isolate, particularly in severe clinical cases, suggesting an ascending infection of gastrointestinal origin. Concordance among conventional culture, automated BD Phoenix™ identification and 16S rRNA based PCR highlights the value of integrating phenotypic and molecular approaches for accurate etiological diagnosis. Antimicrobial susceptibility testing revealed high efficacy of imipenem and gentamicin, whereas reduced sensitivity to commonly used antibiotics, including ceftriaxone and enrofloxacin, reflects the growing challenge of

antimicrobial resistance. Overall, these findings emphasize the importance of culture and molecular confirmation combined with susceptibility guided therapy for effective clinical management and improved therapeutic outcomes in canine pyometra.

Acknowledgement

The authors gratefully acknowledge the facilities and support provided by the Department of Veterinary Gynaecology and Obstetrics and the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, for providing laboratory infrastructure and technical assistance necessary for carrying out this research.

Conflict of Interest

Authors declare no competing interest.

Ethics approval

The study protocol was approved by Institutional Animal Ethical Committee, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh. The study was conducted in accordance with the ethical standards for animal experimentation. Pus samples were collected from dogs clinically presented with pyometra as part of routine diagnostic and therapeutic procedures, without causing any additional harm or invasive intervention to the animals. Informed consent was obtained from the owners prior to sample collection.

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