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Isolation, characterisation and biofilm-forming ability of *Escherichia coli* from postpartum dairy cows with endometritis[#]

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Abstract

Postpartum uterine infections are major cause of infertility in dairy cows, with Escherichia coli being one of the most common pathogens. Its ability to colonise the endometrium, release endotoxins and form biofilms contributes to persistent infections and reduced treatment efficacy. This study aimed to isolate E. coli from postpartum cows with endometritis and evaluate their biofilm-forming ability. Uterine samples were collected from 70 crossbred cows between 28 and 35 days postpartum, yielding 83 bacterial isolates. Among these, 35 (42.16%) were confirmed as E. coli based on cultural and biochemical characteristics. Biofilm formation was assessed using Congo Red Agar (CRA) and microtitre plate (MTP) assays. CRA assay detected biofilm production in 60 per cent of isolates, while MTP assay, identified 68.57 per cent as biofilm producers. Moderate biofilm formers were the most common phenotype across both assays. The presence of biofilm-forming E. coli highlights their role in the persistence of uterine infections and their resistance to host defenses and conventional antibiotics. These findings emphasize the importance of incorporating biofilm-targeted strategies in the management of postpartum endometritis to improve reproductive performance in dairy cows.

Keywords: Congo red agar method, microtitre plate assay, biofilm detection assays

Reproductive efficiency is a cornerstone of profitable dairy production, directly influencing milk yield, calving intervals, and herd replacement rates. However, the postpartum period is often complicated by uterine infections, particularly endometritis, which remains one of the leading causes of infertility in dairy cows (Sheldon et al*.,* 2004 Wei et al*.,* 2024). Endometritis is most commonly associated with bacterial pathogens, of which *Escherichia coli* is a predominant opportunistic invader of the postpartum uterus (Raheel et al., 2020; Wei et al., 2024).

A major factor contributing to the pathogenicity of *E. coli* is its ability to form biofilms, structured communities of bacteria enclosed in a self-produced extracellular matrix (Raheel et al*.,* 2020; Gonzalez Moreno et al., 2020). Biofilm formation enhances bacterial adherence to endometrial surfaces, provides protection against host immune responses and significantly reduces susceptibility to antibiotics (Chacko, 2020; Wei et al., 2024). This makes infections difficult to eradicate and increases the risk of persistent or recurrent endometritis (Kasimanickam et al., 2016).

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Detection of biofilm-producing bacteria is, therefore, critical for understanding the pathogenesis of uterine infections and for guiding appropriate therapeutic interventions (Mathur et al*.,* 2017). The Congo Red Agar (CRA) method is a simple qualitative approach for detecting biofilm formation based on colony morphology and colour changes (Freeman et al*.,* 1989; Turu and Canli, 2025). In contrast, the Microtitre Plate (MTP) assay, which employs crystal violet staining to quantify biofilm biomass, is widely regarded as the gold standard for biofilm detection due to its higher sensitivity and reproducibility (Christensen et al., 1985; Stepanovic et al*.,* 2000).

The present study was undertaken to isolate and identify *E. coli* from postpartum cows diagnosed with endometritis and to assess their biofilm-forming ability using CRA and MTP assays (Chaitanya et al*.,* 2021). This work provides insights into the prevalence of biofilm-producing *E. coli* in postpartum uterine infections and underscores the importance of considering biofilm-associated resistance in treatment strategies (Raheel et al., 2020; Wei et al., 2024).

Materials and methods

Uterine samples were collected from postpartum crossbred dairy cows between 28 and 35 days postpartum using cytobrush technique (Kashimanikam et al., 2005). After proper cleaning and disinfection of the perineal area, a sterile cytobrush was introduced through the cervix into the uterine body under aseptic conditions. The brush was gently rotated against the endometrium to collect secretions and cells, withdrawn, aseptically detached and transferred into sterile screw-cap vials. Samples were transported on ice to the laboratory within two hours of collection for microbiological analysis.

Samples were enriched in Brain Heart Infusion (BHI) broth, incubated aerobically at 37 °C for three hours, streaked on Brain heart infusion agar (BHA), and incubated at 37 °C for 24 hours. Colonies were subjected to Gram staining. Suspected colonies were further plated on MacConkey agar and then Eosin Methylene Blue (EMB) agar. Lactose-fermenting pink colonies on MacConkey agar and colonies with a characteristic metallic green sheen on EMB agar were selected for further confirmation for *E. coli*. Biochemical characterisation of the isolates was carried out using conventional tests such as catalase, oxidase, IMViC (Indole, Methyl Red, Voges–Proskauer, Citrate) and Triple Sugar Iron (TSI) agar. Isolates exhibiting the typical biochemical reaction pattern of *E. coli* were confirmed as *E. coli* (Markey et al., 2014).

E. coli isolates were tested for antibiotic sensitivity (Bauer et al*.,* 1966). Bacterial suspensions, adjusted to 0.5 McFarland standard, were spread evenly on Mueller-Hinton agar plates. Antibiotic disks were placed onto the surface and plates were incubated at 37°C for 16-18 hours. Zones of inhibition around the disks were measured to assess bacterial susceptibility.

The biofilm-forming ability of the isolates was assessed by the Congo Red Agar (CRA) method and microtitre plate (MTP) assay. In the CRA test, BHI agar supplemented with 5 per cent sucrose and 0.08 per cent Congo red dye was inoculated with isolates and incubated at 37 °C for 24 h; black, dry colonies indicated biofilm producers, while pink colonies were considered non-producers (Freeman et al*.,* 1989). The CRA method classifies *E. coli* biofilm formation based on colony colour and texture. Weak biofilm formers produce light black or dark red colonies, moderate formers show partially crystallised black colonies, and strong biofilm producers have intense, dry, crystalline black colonies (Turu & Canli, 2025).

For the MTP assay, isolates grown in trypticase soy broth (TSB) with 1 per cent glucose were inoculated in triplicate into sterile 96-well plates and incubated at 37 °C for 24 h. Wells were washed with phosphate buffered saline, fixed with methanol, stained with 0.1 per cent crystal violet and destained with 33 per cent glacial acetic acid. Optical density (OD) at 590 nm was measured using an ELISA plate reader (Thermo Scientific Multiskan GO®, USA) to quantify biofilm formation (Christensen et al., 1985; Stepanovic et al., 2000). The MTP assay categorises biofilm formation based on OD values, where weak biofilm producers show OD slightly above the control, moderate producers have intermediate OD levels and strong producers exhibit significantly higher OD values. This method reflects differences in the metabolic activity of biofilm cells and allows reliable quantification (Mathur et al., 2017).

Results and discussion

Out of the 70 uterine cytobrush samples analysed, a total of 83 bacterial isolates were obtained, out of which 35 (42.16%) were confirmed as *E. coli*, while the remaining 48 (57.84%) were other bacterial species. The *E. coli* isolates were identified as Gram-negative bacilli, producing smooth, circular, grey-white colonies on BHI agar and exhibiting the characteristic metallic green sheen on EMB agar. Biochemical characterisation, including catalase, oxidase, IMViC test and triple sugar iron reactions, confirmed the isolates were *E. coli* (Figure 1, 2).

In the present study, *E. coli* was isolated as 35 isolates out of a total of 83 bacterial isolates (42.16%) recovered from 70 uterine cytobrush samples, emphasizing its significant role in postpartum uterine infections, consistent with previous findings (Arora et al., 2000; Dohmen et al., 2000; Sarkar et al*.,* 2006; Wei et al*.,* 2024). Its persistence is likely due to its abilities to adhere, colonise, produce endotoxins and create a favorable environment for secondary pathogens such as *T. pyogenes* (Williams

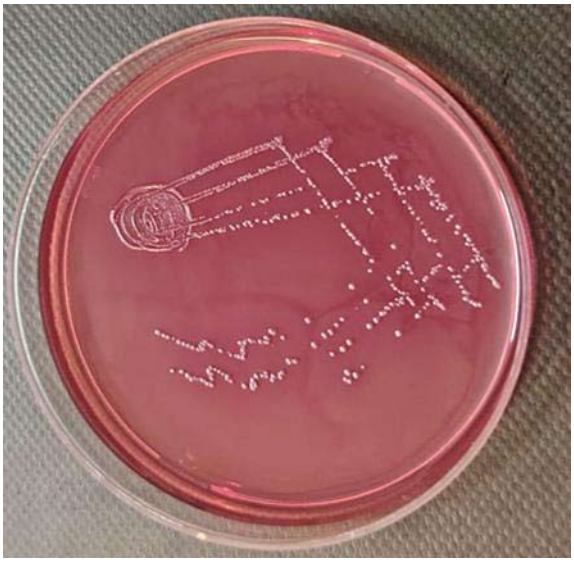

Fig. 1. Colonies on MacConkey agar showed flat, smooth, circular, pink to red colonies

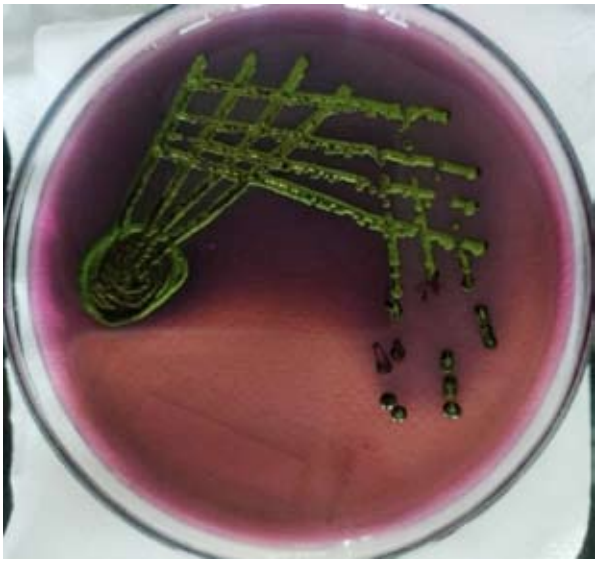

Fig.2. *E. coli* Colonies on EMB agar showed green metallic sheen.

et al*.,* 2005). Dhaliwal et al. (2001) observed that bacterial invasion of the uterus during parturition and immediate puerperium is typically cleared within one to two weeks postpartum in healthy cows; however, failure to eliminate these bacteria can lead to endometritis and negatively impact reproductive performance. Early colonisation of the endometrium may cause tissue damage, inflammation, delayed uterine involution, disrupted ovarian function and prolonged days open (Sheldon et al., 2006; Sheldon & Dobson, 2004). These findings underscore the critical role of *E. coli* in postpartum uterine health and its implications for fertility management.

Virulence factors, including adhesins, fimbriae, toxins and biofilm-associated proteins, contribute to its pathogenicity and persistence (Bicalho et al., 2012; Wei et al*.,* 2024) as reflected by the biofilm-forming ability observed in this study. Although *S. aureus* can be predominant in some contexts (Bhat et al., 2013; Liu et al., 2013), *E. coli* remains the most consistent early postpartum pathogen. Recent studies report its high prevalence in puerperal metritis (>95%) with virulence and antibiotic resistance genes, further complicating treatment (Wei et al., 2024). The presence of uterine bacteria 21 days postpartum also reduces early conception rates, underscoring the need for timely identification and effective management of infections (De Boer et al., 2015).

The biofilm-forming potential of the confirmed *E. coli* isolates was evaluated using CRA and MTP assays. In the CRA assay, isolates were classified based on colony colour and morphology on Congo red agar after 24-48 hours incubation: pink/red colonies as non-producers, light black/pinkish-black as weak producers, black as moderate producers and very dark black with thickened/ crystal-like surfaces as strong producers (Freeman et al., 1989; Stepanović et al., 2000).

The CRA assay revealed that 21 of the 35 isolates (60.00 %) were capable of biofilm formation, with six (17.14%) classified as weak, 10 (28.57 %) as moderate and five (14.29 %) as strong biofilm producers, while 14 isolates (40.00 %) were non-producers (Table 2).

The MTP assay identified a slightly higher proportion of biofilm producers, with 24 isolates (68.57%) showing positive results. Among these, 10 (28.57%) were weak, 11 (31.43%) were moderate and three (8.57%) were strong biofilm formers, whereas 11 isolates (31.43%) were non-producers (Table 2). The isolates were categorized as weak, moderate, or strong biofilm formers according to established optical density (OD) thresholds relative to the cutoff value (ODc) in the MTP assay, where ODc represents the mean OD of negative controls plus three standard deviations. Specifically, isolates with OD values exceeding ODc but not surpassing twice ODc qualified as weak producers, those between two and four times ODc were deemed moderate producers, and those surpassing four times ODc were classified as strong producers, while values at or below ODc indicated non-producers. (Christensen et al., 1985; Stepanović et al., 2000).

Comparison of the two methods indicated that the MTP assay detected a higher number of biofilm-forming isolates compared to the CRA assay, although the differences were not statistically significant. Both methods consistently demonstrated that moderate biofilm production was the most common phenotype. Interestingly, CRA identified a greater proportion of strong biofilm formers than the MTP assay (Table 2). Overall, the results suggest that both CRA and MTP are reliable techniques for assessing biofilm formation in *E. coli*, with minor variations in sensitivity.

Fig. 3. Colonies of *E. coli* on Congo red agar (CRA) assay, colonies appeared black, dry colonies (biofilm positive)

In the present study, biofilm-forming ability of *E. coli* isolates was evaluated using both CRA and microtitre plate (MTP) assays, which revealed that 60.00 per cent and 68.57 per cent of isolates, respectively, were biofilm producers. The higher detection rate by MTP compared to CRA assay is consistent with earlier reports, as MTP provides a more sensitive and quantitative measurement of biofilm biomass, while CRA serves only as a qualitative screening tool and is subject to interpretive variation (Chaitanya et al., 2021). The predominance of moderate biofilm formers observed in this study aligns with findings by Chacko (2020), who also reported a higher proportion of moderate biofilm producers in uterine bacterial isolates by MTP assay.

Comparisons with previous studies highlight some variations. Raheel et al. (2020) reported 46.60 per cent biofilm-positive *E. coli* isolates from bovine endometritis using CRA, which is lower than the 60.00 % observed in the present study.

Beeham et al. (2015), in contrast, reported a predominance of weak and strong biofilm producers in equine endometritis, suggesting that the biofilm-forming potential of *E. coli* may differ across host species. Gonzalez Moreno et al. (2020) also demonstrated that isolates from cows with endometritis exhibited significantly stronger biofilm-forming ability compared to isolates from healthy cows, reinforcing the role of biofilms in disease pathogenesis. Similarly, Fiamengo et al. (2020) found that 75 per cent of *E. coli* isolates from canine pyometra produced biofilms, contributing to chronic infection and antimicrobial resistance.

The overall results of the *in vitro* antibiotic susceptibility test in the present study indicated that the maximum number of *E. coli* isolates were sensitive to levofloxacin (68.57 %), followed by ciprofloxacin (51.43 %), gentamicin (37.14 %), enrofloxacin (34.28 %), cephalexin (28.57 %) and oxytetracycline (22.86 %). None of the isolates were sensitive to metronidazole (0 %). Chi-square test for multiple proportions revealed that there existed a significant variation ($p< 0.01$) in the sensitivity of *E. coli* isolates towards different antibiotics.

Bacteria within biofilms exhibit greatly increased antibiotic resistance compared to their free-floating counterparts. This heightened resistance is due to biofilms acting as a physical barrier that limits antibiotic penetration and by fostering genetic and metabolic adaptations that enhance survival. These changes contribute to persistent infections that are difficult to eradicate and promote the development of multidrug-resistant strains.

The role of biofilms in persistence and severity of uterine infections is well documented. Biofilm communities protect bacteria from host immune defenses and antibiotic penetration, thereby enabling recurrent and chronic infections (Raheel et al., 2020; Ahmadi et al., 2017). In equine endometritis, *Pseudomonas aeruginosa* isolates from clinical cases form adherent biofilms on the endometrial surface, characterised by elevated Pel exopolysaccharide and cyclic di-GMP levels, which locally suppress neutrophil infiltration while maintaining stromal

Table 1. Comparison of biofilm forming property of *E. coli* isolates by Congo red assay and microtitre plate assay

Biofilm Formation Category	Congo red assay		Microtitre (MTP) Assay	
	No of isolates	per cent (%)	No of Isolates	Per cent (%)
Weak biofilm formers	6	17.14	10	28.57
Moderate biofilm formers	10	28.57	11	31.43
Strong biofilm formers	5	14.29	3	8.57
Non-biofilm formers	14	40	11	31.43
Total isolates	**35**	**100**	**35**	**100**
Chi Square value = 1.908^{ns}; p-value = 0.592				

ns non-significant

Table2. Overall antibiotic susceptibility of *E. coli* isolates of postpartum dairy cows with endomtritis

ANTIBIOTICS	Number of *E. coli* isolates		
	Sensitive (%)	Intermediate (%)	Resistant (%)
Enrofloxacin	12 (34.28) [a]	15 (42.86)	8 (22.86)
Levofloxacin	24 (68.57) [b]	7 (20.00)	4 (11.43)
Ciprofloxacin	18 (51.42) [c]	12 (34.29)	5 (14.29)
Gentamicin	13 (37.14) [a]	15 (42.85)	7 (20.01)
Cephalexin	10 (28.57)	19 (54.28) [d]	6 (17.15)
Oxytetracycline	8 (22.85)	9 (25.71)	18 (51.44) [e]
Metronidazole	0 (00.00)	0 (00.00)	35(100.00) [f]

χ^2 Value = 39.36 p-value = 0.00003

inflammation (Ferris et al., 2017). In repeat breeder dairy cows, periodic acid-Schiff (PAS) staining detects biofilms in 60 per cent of uterine samples irrespective of cytological inflammatory cell counts, establishing a direct association with subfertility (Ahmadi et al*.,* 2017)

The detection of biofilm formation among *E. coli* isolates in the present study further underscores its clinical significance, as biofilm-producing strains are more difficult to eliminate and may contribute to prolonged uterine inflammation, impaired fertility and treatment failures. Molecular studies have demonstrated the association of biofilm-related virulence genes such as *fimH, Agn43a* and *Agn43b* with enhanced adhesion, colonisation and antimicrobial resistance in *E. coli* (Kasimanickam et al., 2017; Raheel et al., 2020; Wei et al., 2024). These findings highlight that biofilm formation is not only a survival strategy but also a key pathogenic determinant in postpartum uterine infections.

Taken together, the results of this study confirm that biofilm formation is a widespread trait among uterine *E. coli* isolates, with MTP being more reliable than CRA for detection. The predominance of moderate biofilm producers suggests that these strains can persist in the uterine environment, contributing to chronic infections and reduced fertility outcomes. Understanding biofilm dynamics and associated genetic determinants is therefore essential for developing targeted therapeutic strategies aimed at disrupting biofilms and improving the management of postpartum uterine infections in dairy cows.

Conclusion

The present study demonstrated that *E. coli* is a major bacterial species associated with postpartum endometritis in dairy cows, with an isolation rate of 42.16 per cent. A substantial proportion of these isolates exhibited biofilm-forming ability, highlighting their potential role in the persistence and chronicity of uterine infections. Comparative evaluation of biofilm detection methods revealed that the microtitre plate assay was more sensitive than the Congo red agar method, though both proved useful in characterizing biofilm production. The predominance of moderate biofilm producers indicates that such strains may be well adapted to survive in the uterine environment, thereby complicating therapeutic interventions and fertility restoration. These findings reinforce the importance of considering biofilm-associated resistance when designing treatment strategies. Future research focusing on molecular determinants of biofilm formation and on effective antibiofilm therapies could offer promising avenues for improved control of postpartum uterine infections and enhanced reproductive efficiency in dairy herds.

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Conflict of interest

The authors declare that they have no conflict of interest

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