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ORIGINAL ARTICLE

Biofilm forming potentiality of *Escherichia coli* isolated from bovine endometritis and their antibiotic resistance profiles

Ismail Abd Elhafez Radwan Raheel, Walid Hamdy Hassan, Shaaban Salem Radwan Salem, Hala Sayed Hassan Salam
Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt

ABSTRACT

Objective: The objectives of this study were to determine the biofilm-forming capability and antimicrobial susceptibility of *Escherichia coli* recovered from bovine endometritis samples.
Materials and Methods: A total of 120 uterine specimens were collected from cows suffering from endometritis for bacteriological examination. Antimicrobial susceptibility testing was carried out for all isolated *E. coli* by using the disc diffusion method. The isolates were phenotypically studied for biofilm-forming ability by cultivation on yeast extract -casamino acids Congo red agar (CRA). Some randomly selected isolates were chosen for the molecular identification of some virulence and resistance genes.
Results: A total of 58(48.3%) *E. coli* isolates could be isolated from the 120 samples. Antimicrobial susceptibility testing exhibited that 91.4%, 79.3%, 79.3%, 74.1%, and 58.6% of the isolates were sensitive to gentamicin, amoxicillin-clavulanic acid, ciprofloxacin, cephalexin, and sulfamethoxazole-trimethoprim, respectively. On the other hand, 91.4% and 70.7% isolates were resistant to cefotaxime and doxycycline, respectively. Cultivation on CRA revealed that 46.6% of isolates were biofilm producers. The molecular detection of resistance and virulence genes declared that all isolates harbored bla_{TEM}, *sul*1, *tet*A, *qnr*S, $bla_{CTX\text{-}M}$, and *fim*H with a percentage of 100%, *pap*C (40%), and *hly*A (10%). *Fim*H was the most prevalent biofilm-associated gene.
Conclusion: The present study highlights the high prevalence of multi-drug- resistant *E. coli* associated with bovine endometritis. The detection of the *fim*H gene is circumstantial evidenced that this gene has a crucial role in biofilm formation in intrauterine pathogenic *E. coli*.

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Biofilm *E. coli*; endometritis; resistance; virulence

Introduction

Endometritis is known to be one of the major diseases, which upset the reproductive performance of cattle and reduce livestock productivity [1]. Following parturition, the invasion of the endometrium with different bacterial species (more than 200) occurs, but not all these bacteria considered as pathogens [2]. The initial step in developing bovine endometritis is the infection of the endometrium with *Escherichia coli* preceded by further bacteria such as *Arcanobacterium pyogenes* [3]. In a study, *E. coli* was regarded as the main associated bacteria in clinical and subclinical endometritis samples [4].

Moreover, cows with positive uterine *E. coli* cultures did not become pregnant to the same degree as cows without *E. coli* in their uteri [5]. The crucial pathogenicity characters of *E. coli* include epithelial cell adhesion, flagella-mediated motility, exotoxins, and lipopolysaccharides. Endometrial pathogenic *E. coli* strains were more adherent and invasive for the endometrial cells *in vitro* than that isolated from the uteri of clinically healthy animals and triggered the ultimate inflammatory response [3]. Carniello et al. [6] stated that the means of bacterial protection other than the expression of resistance genes include the production of a large quantity of extracellular polymeric substance (EPS) throughout the process of biofilm formation. This EPS is composed mainly of exopolysaccharides that form the main structure of biofilm and serve in bacterial resistance to antibiotics and host immunity [7].

Three major components, including surface, microbes, and slime EPS, constitute the output of biofilm so that it

Correspondence Hala Sayed Hassan Salam ✉ hala_saydh@yahoo.com Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt.

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can be discarded by removing either of these components [8]. The process of biofilm formation occurs by cell-to-cell communication that is known as quorum sensing, where the accumulation of signaling molecules in the extracellular environment occurs, leading to the regulation of specific gene expression [9]. Ahmadi et al. [10] have observed the opaque liquid or some particles in uterine lavage fluid by normal saline of repeat breeder cows at estrus phase that leads to a question about the nature of these particles and the possibility of the presence of bacterial biofilm. Biofilm development is a multistep process, where it begins with the bacteria preliminary adherence to the substratum and permanent attachment followed by their colonization, in which gene alteration and protein expression occur, subsequently the exponential growth phase. The formation of EPS and water channels promotes the supply of nutrients, which results in the maturation of biofilms [9].

For biofilm formation, a set of genes is required for the initial bacterial adhesion, maturation, and production of EPS [11]. Some recent studies recognized that genes encoding certain *E. coli* virulence factors, such as *fim*H, *pap*C, *and hly*A, are responsible for bacterial adhesion and associated with bovine endometritis [12–14], where *Fim*H (a type 1 pilus component) is *E. coli* specific gene and considerably associated with metritis and endometritis in cattle [13]. Consequently, the ability to produce biofilm in endometrial pathogenic *E. coli* hinders antimicrobial therapy. Hence, the current work aimed to investigate biofilm-forming capability and antimicrobial resistance of *E. coli* recovered from bovine endometritis in Egypt at Beni-Suef and Fayum governorates.

Materials and Methods

Ethical approval

The study was approved from Beni-Suef University, Institutional Animal Care and Use Committee (BSU-IACU/http://www.bsu.edu.eg).

Samples

A total of 120 uterine samples, including uterine discharges, vaginal swabs, and uterine lavages, were collected for bacteriological examination under complete aseptic conditions. They were collected from various dairy farms in Beni-Suef and Fayoum governorates in the period from February to June 2019. The samples were sent to the laboratory with a minimum of delay to avoid the dryness of samples.

Isolation and biochemical identification of E. coli

A loopful from each sample was inoculated into the tryptone soya broth (TSB) and incubated at 37°C for 16–18 h. After the incubation period, one loopful from the TSB culture was inoculated onto MacConkey agar to be incubated at 37°C for 24–48 h. Pink colonies were picked up for morphological and biochemical identification using oxidase, indole production, methyl red, Voges Proskauer, citrate utilization, and urease tests as well as growth on triple sugar iron agar as described by Quinn et al. [15].

Antimicrobial susceptibility testing of E. coli isolates

The standard disk diffusion technique was used against seven different antimicrobial disks, according to Clinical and Laboratory Standards Institute (CLSI) [16]. The suspensions of the isolates equivalent to 0.5 McFarland standards turbidity were prepared, and Mueller Hinton agar plates were inoculated. Antimicrobial disks [amoxicillin-clavulanic acid (30 µg), cephalexin (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), gentamicin (10 µg) and sulfamethoxazole-trimethoprim (25 µg)], representing the antimicrobials mostly used in the treatment of uterine affections under field conditions, were applied on the plates. The tested isolates were categorized as sensitive, intermediate sensitive, or resistant, according to CLSI [16].

Biofilm formation of identified E. coli isolates

Congo red (CR) assay for bacteria, as described by Zhou et al. [17], was used for the detection of biofilm formation on yeast extract-casamino acids (YESCA) CR agar plates after pre-enrichment of the isolates on Luria–Bertani agar medium. For good induction of curli production, the isolates were grown on YESCA CR agar plates at 26°C for 48 h; after that, the color of the bacterial colonies was checked, where the red-stained colonies considered as positive for curli production, and on the other hand, pink or white colonies considered as negative.

Detection of resistance and virulence genes of E. coli isolates

Ten *E. coli* isolates were selected for genotypic characterization by polymerase chain reaction (PCR) to detect the presence of several virulence and resistance-associated genes such as *fim*H, *pap*C, *hly*A, bla_{TEM}, *sul*1, *tet*A, *qnr*S, and bla_{CTX-M} using their specific forward and reverse primers as shown in Table 1. The selected isolates exhibited a multidrug resistance pattern, which was resistance to at least one agent in three or more antimicrobial classes [18]. As well, they were representing different resistance patterns and positive for phenotypic biofilm formation. The positive control DNA was obtained from confirmed positive *E. coli* field isolate in RLQP (Reference laboratory for veterinary quality control on poultry production, Dokki, Giza, Egypt). On the contrary, a negative control is a PCR mixture free from the DNA template.

Table 1. Oligonucleotide primers used for amplification of virulence and resistance-associated genes.

Annealing temp.	Product	Reference	Primer sequence(5′-3′)	Target Gene
50°C	508-bp	[40]	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	*fim*H
60°C	1,177-bp	[41]	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	*hly*A
58°C	501-bp	[42]	TGATATCACGCAGTCAGTAGC CCGGCCATATTCACATAA	*pap*C
54°C	516-bp	[43]	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	bla_{TEM}
60°C	433-bp	[44]	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	*sul*1
50°C	576-bp	[45]	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	*tet*A
55°C	417-bp	[46]	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	qnrS
54°C	593-bp	[47]	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	bla_{CTX-M}

Results

Escherichia coli prevalence

A total number of 58 *E.coli* isolates were recovered from 120 uterine samples by a ratio of 48.3%.

Antimicrobial susceptibility of recovered E. coli

The antimicrobial susceptibility testing of *E. coli* isolates (*n* = 58) showed that 91.4%, 79.3%, 79.3%, 74.1%, and 58.6% of them were sensitive to gentamicin, amoxicillin-clavulanic acid, ciprofloxacin, cephalexin, and sulfamethoxazole-trimethoprim, respectively. On the contrary, 91.4% and 70.7% were resistant to cefotaxime and doxycycline, respectively. The detailed results of each antimicrobial are shown in Table 2. Of the 58 isolates, 36 (62.07%) were classified as multidrug resistant (MDR).

Biofilm formation on YESCA CR agar

Of the total tested isolates (*n* = 58), 27 *E. coli* isolates (46.6%) were grown as red colonies on YESCA CR agar and described as biofilm positive. In comparison, 31 isolates (53.4%) were grown as white colonies and described as negative for biofilm formation, as shown in Figure 1.

Association between antimicrobial resistance and biofilm formation

Of 27 biofilm-producing *E. coli* isolates, 22(81.5%) were recorded as MDR.

Detection of antimicrobial resistance and virulence genes of E. coli

Ten *E. coli* isolates were tested using PCR for the detection of *fim*H, *pap*C, *hly*A, bla_{TEM}, *sul*1, *tet*A, *qnr*S, and bla_{CTX-M}. All of them harbored *fim*H gene, four of them (40%) contain *pap*C gene, only one isolate (10%) exhibited *hly*A gene, and all of them carried all the tested antimicrobial resistance genes (bla_{TEM}, *sul*1, *tet*A, *qnr*S, and bla_{CTX-M}) (Figs. 2–9).

Discussion

The present study revealed that *E. coli* is one of the most significant bacteriological risk factors of bovine endometritis. It was isolated by a percentage of 48.3%, where many other studies confirmed by Kasimanickam et al. [19], who isolated *E. coli* by 45%. The high prevalence of *E. coli* in bovine endometritis may be connected to the existence of these bacteria in enteric microflora, in addition to the proximity of the rectum and external genital tract, which donate to uterine contamination by these enteric bacteria [20].

The antimicrobial sensitivity and resistance patterns of *E. coli* isolates by the disk diffusion method against seven diverse antimicrobial agents of five different classes were studied.

Cefotaxime was the most antimicrobial agent showing resistance by the percentage of 91.4%, followed by doxycycline (70.7%).

Table 2. Antimicrobial susceptibility of different *E. coli* isolates (*n* = 58).

Antimicrobial class	Antimicrobial disk	Resistant		Intermediate		Sensitive	
		No.	%	No.	%	No.	%
β-lactamase stable	Amoxicillin-clavulanic acid	7	12.1	5	8.6	46	79.3
β-lactams	Cephalexin	15	25.9	–	–	43	74.1
Cephalosporins	Cefotaxime	53	91.4	–	–	5	8.6
Fluoroquinolones	Ciprofloxacin	7	12.1	5	8.6	46	79.3
Tetracyclines	Doxycycline	41	70.7	5	8.6	12	20.7
Aminoglycosides	Gentamicin	–	–	5	8.6	53	91.4
Potentiated sulfonamide	Sulfamethoxazole-trimethoprim	24	41.4	–	–	34	58.6

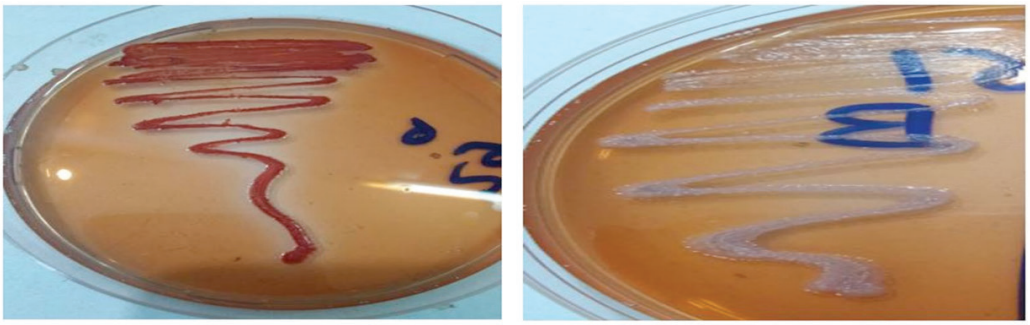

Figure 1. Cultivation of *E. coli* on YESCA CR agar. Left side = *E. coli* colonies appeared red (biofilm positive). Right side = *E. coli* colonies seemed to be white on YESCA CR agar (biofilm negative).

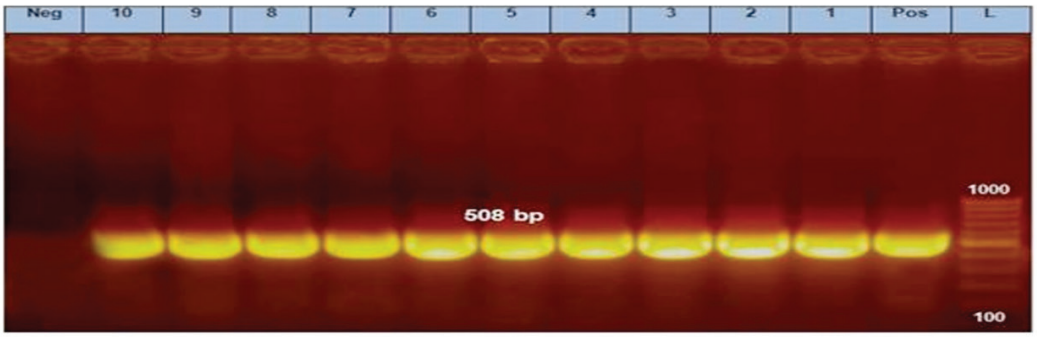

Figure 2. PCR amplification of the *fim*H gene at 508-bp fragment. Lanes 1–10 showed positive amplification of the *fim*H gene. Pos = Positive control; Neg = negative control; L = 100-bp DNA molecular size ladder.

On the other hand, *E. coli* found to be 91.4% sensitive to gentamicin, 79.3% to both amoxicillin-clavulanic acid and ciprofloxacin, 74.1% to cephalexin, and 58.6% to sulfamethoxazole-trimethoprim.

The percentage of cefotaxime resistance of intrauterine pathogenic *E. coli* was 70.8% in a study that was performed by Ma et al. [14]. The high percentage of cefotaxime resistance in this study may be related to the extensive use of third-generation cephalosporin ceftiofur in the treatment of endometritis.

In the present study, *E. coli* showed high resistance to doxycycline, whereas Zhao et al. [21] recorded a high resistance, to a certain degree, (46%) against this antibiotic. This high resistance in this study may be related to the widespread use of the broad-spectrum antibiotic oxytetracycline in uterine irrigation as one of the methods for the treatment of endometritis, either clinical or subclinical.

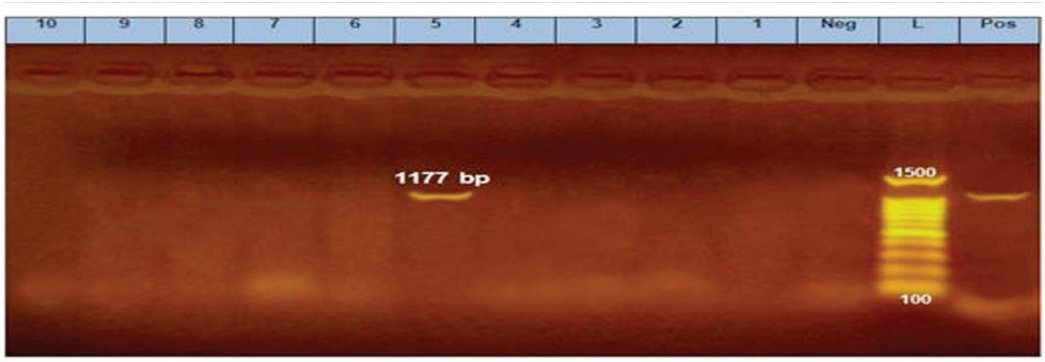

Figure 3. PCR amplification of the *hly*A gene at 1177-bp fragment. Lane 5 showed positive amplification of the *hly*A gene. Pos = Positive control; Neg = negative control; L = 100-bp DNA molecular size ladder.

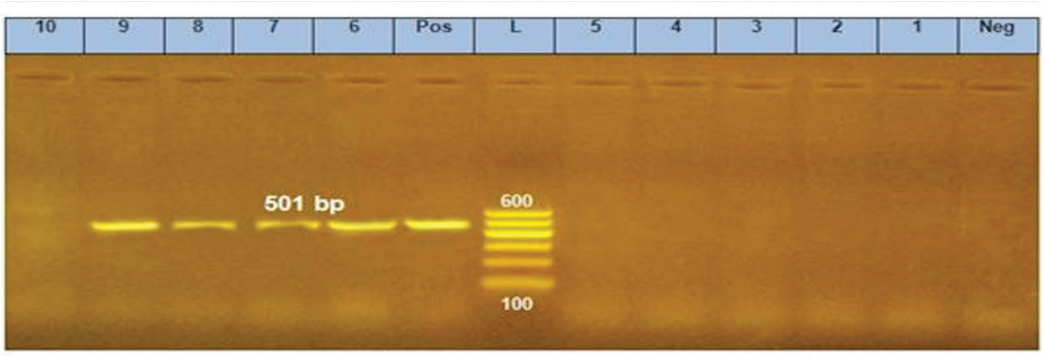

Figure 4. PCR amplification of *pap*C gene at 501 bp fragment. Lanes 6–9 showed positive amplification of *pap*C gene. Pos = Positive control; Neg = negative control; L = 100-bp DNA molecular size ladder.

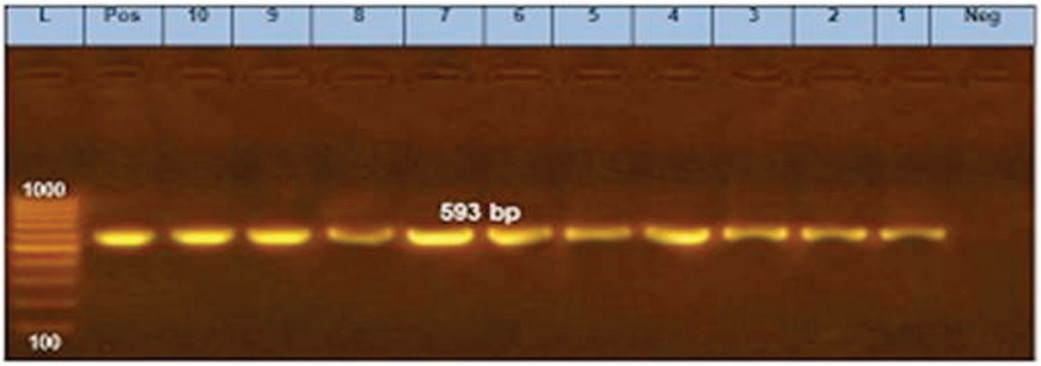

Figure 5. PCR amplification of the bla_{CTX-M} gene at 593-bp fragment. Lanes 1–10 showed positive amplification of the bla_{CTX-M} gene. Pos = Positive control; Neg = negative control; L = 100-bp DNA molecular size ladder.

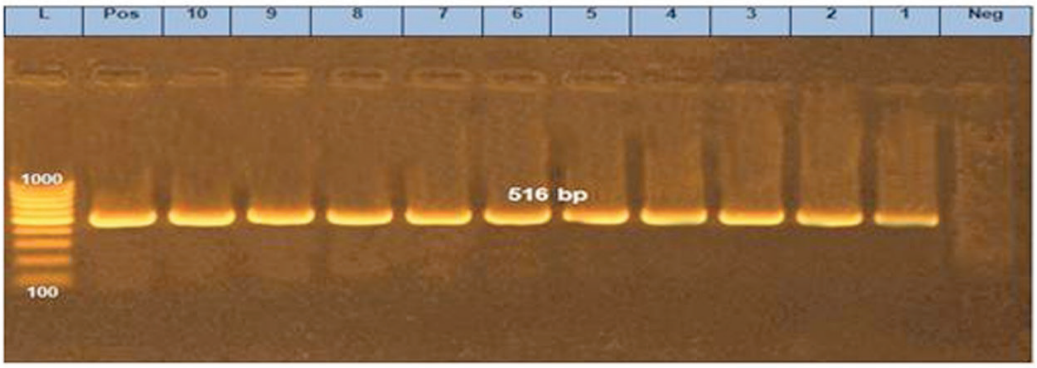

Figure 6. PCR amplification of the *bla*$_{TEM}$ gene at 516bp fragment. Lanes 1–10 showed positive amplification of the *bla*$_{TEM}$ gene. Pos = Positive control; Neg = negative control; L = 100-bp DNA molecular size ladder.

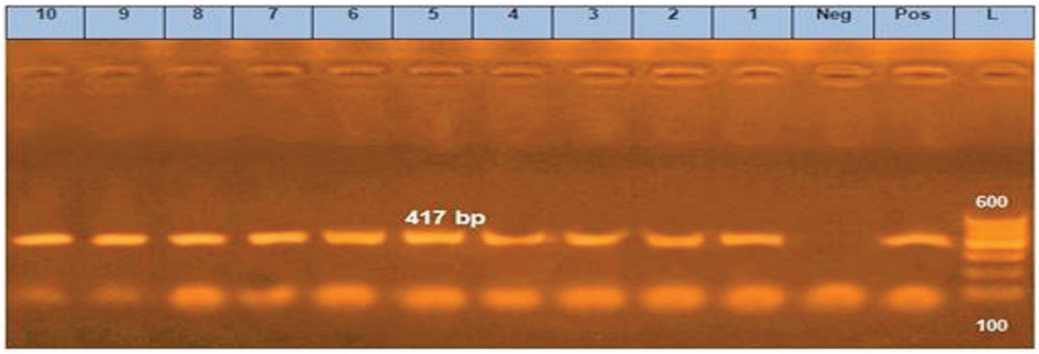

Figure 7. PCR amplification of *a* gene at 417-bp fragment. Lanes 1–10 showed positive amplification of *a* gene. Pos = Positive control; Neg = negative control; L = 100-bp DNA molecular size ladder.

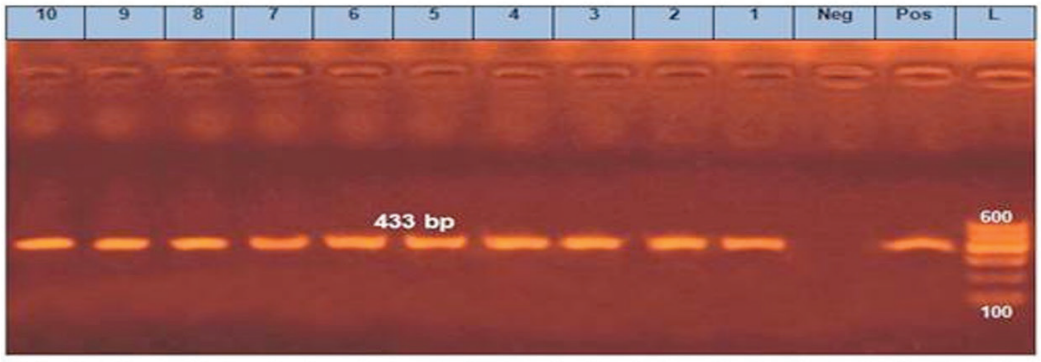

Figure 8. PCR amplification of the *sul*1 gene at 433-bp fragment. Lanes 1–10 showed positive amplification of the *sul*1 gene. Pos = Positive control; Neg = negative control; L = 100-bp DNA molecular size ladder.

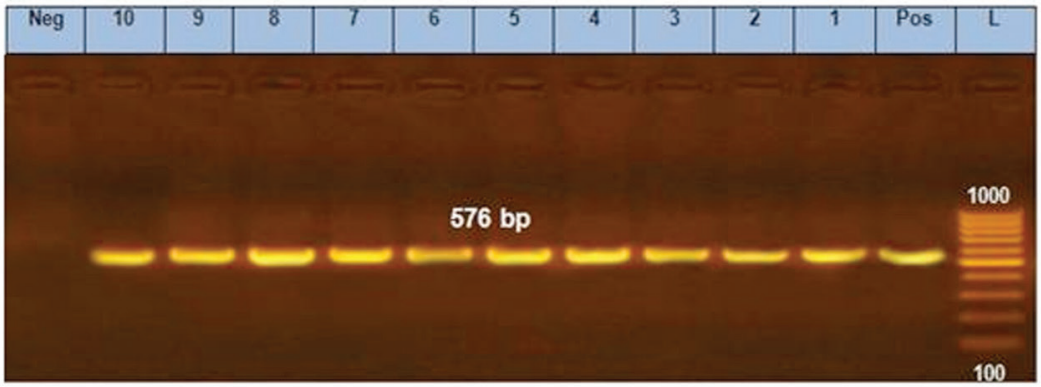

Figure 9. PCR amplification of *tet*A gene at 576-bp fragment. Lanes 1–10 showed positive amplification of *tet*A gene. Pos = Positive control; Neg = negative control; L = 100-bp DNA molecular size ladder.

The same results of high sensitivity to gentamicin and ciprofloxacin were obtained by Zhao et al. [21], where they recorded 60.1% and 77.1% sensitivity against them, respectively.

Brodzki et al. [22] found that *E. coli* isolated from bovine uteri was sensitive to amoxicillin-clavulanic acid by the percentage of 100% supporting the results of high sensitivity to this antibiotic. On the contrary, Li-ming et al. [23] found that *E. coli* was highly resistant to amoxicillin.

The present study declared that intrauterine pathogenic *E. coli* is highly sensitive to cephalexin, but Dutt et al. [24] found that *E. coli* isolates were 100% resistant to it.

In this study, the sensitivity against sulfamethoxazole-trimethoprim was 58.6%, whereas Zhao et al. [25] reported 100% resistance against it. Based on the finding of this study, 62.07% of *E. coli* isolates were resistant to 3–5 categories of antimicrobials. Ma et al. [14] reported that all intrauterine pathogenic *E. coli* were MDR. Furthermore, Zhao et al. [21] isolated 148 *E. coli* isolates from the cases of bovine endometritis, and 132 (89.2%) out of them were MDR.

Biofilm formation is a mechanism for bacterial resistance and also for bacterial virulence [26], where it increases the antimicrobial resistance up to 1,000 folds to inactivate organisms developing inside a biofilm, and high antimicrobial concentrations are required [27]. This resistance may be due to the inadequate concentration of antimicrobials that reach certain parts of the biofilms and metabolic inactivity, in addition to the existence of active antibiotic degradation mechanisms that contribute to the cessation of drug accumulation to a sufficient concentration [10].

For the detection of biofilm in *E. coli* isolates, Reichhardt et al. [28] concluded that CR dye can bind to curled whole cells, without inhibition of growth, and can be used to comparatively measure the whole-cell curliation, where *E. coli* accumulate extracellular adhesive amyloid fibers termed curli which enable the bacterial adhesion and encourage the biofilm formation.

The current study reported that 46.6% of the recovered *E. coli* isolates were phenotypically positive for biofilm formation. Moori Bakhtiari et al. [29] reported that 53.3% and 16.6% of *E. coli* isolates were moderately and strongly biofilm producers, respectively.

Cephalosporin resistance is linked to the genes that encode for β-lactamases such as bla_{TEM}, $bla_{CTX\text{-}M,}$ and bla_{CMY} [30]. Moreover, $bla_{CTX\text{-}M}$ genes are the most common type of extended-spectrum β-lactamases with high clinical significance [14]. In this study, bla_{TEM} and $bla_{CTX\text{-}M}$ were identified in all selected *E. coli* isolates, whereas Zhao et al. [21] detected them by a percentage of 30.4%, as well they concluded that bla_{TEM} gene was predominant in *E. coli* isolates that were resistant to quinolones, whereas, in this study, *qnrS* gene also detected in all selected isolates.

*Sul*1 gene is a plasmid-borne sulfonamide resistance gene that is linked to the universal and long-known sulfonamide resistance in Gram-negative bacteria [31]. This gene determined in all selected *E. coli* isolates. Similarly, *tet*A gene detected in all the studied isolates that encodes the synthesis of the protein responsible for the efflux pump process, which is the most common resistance mechanism for tetracycline and its analogs [32].

In this study, the virulence-associated *fim*H gene identified in all tested isolates that were phenotypically positive for biofilm formation. The same high gene prevalence was also mentioned by Bicudo et al. [20], where it was detected in more than 90% of uterine isolates of cows, which reinforces the effect of this adhesion in early uterine contamination. *Fim*H is a Type 1 pili correlated to adherence, invasion, and biofilm formation in the epithelial cells of host tissues [33]. Moreover, Bicudo et al. [20] clarified that *fim*H has an essential role in the establishing of *E. coli* in

the endometrium, increasing the risk of endometritis and the failure in the consequent pregnancy when detected in cows at 1–3 days postpartum. In addition, the treatment of endometrial pathogenic *E. coli* with mannose resulted in a reduction of their ability to adhere to the endometrial cells, which confirms the expression of the *fim*H gene [34].

The *pap*C gene which also encodes for bacterial adhesion detected in 4 out of the 10 selected isolates (40%). In a study conducted by Kassé et al. [35], *pap*C gene was detected by 9% in *E. coli* isolates associated with postpartum metritis in cattle.

Alpha-hemolysin (*hly*A) gene identified only in one of the selected isolates (n = 10) by a percentage of 10%, where it is a pore-forming cytotoxin that is responsible for lysis of the cell wall of the host cells including leukocytes, erythrocytes, and endothelial cells [36].

Silva et al. [12] did not find any relation between *hly*A and *fim*H genes in the occurrence of bovine metritis, and on the other hand, Bicalho et al. [37] proposed a relationship between the presence of *hly*A gene and the presence of *fim*H gene in the occurrence of bovine metritis, but the expression of hemolysin must be considered an extra mechanism of *E. coli* pathogenicity, favoring the development of extra-intestinal infections, included in bovine endometritis [35].

In the present study, of 27 biofilm-producing *E. coli* isolates, 22 (81.5%) were recorded as MDR that declares the correlation between the antimicrobial resistance and the biofilm formation, and similar results were also obtained by Neupane et al. [38] and Karigoudar et al. [39].

Conclusion

The present study highlights the high incidence of MDR *E. coli* associated with bovine endometritis. The detection of *fim*H gene is circumstantial evidence that this gene has a significant role in biofilm formation in intrauterine pathogenic *E. coli*. Moreover, there was a high antimicrobial resistance of *E. coli* isolates in addition to its correlation with biofilm formation.

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Conflict of interest

All the authors contributed equally. Other than this, there was no conflict of interest among the authors.

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