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ISOLATION, PCR DETECTION, PATHOTYPING AND ANTIBIOGRAM PROFILING OF *ESCHERICHIA COLI* ASSOCIATED WITH ENDOMETRITIS IN BUFFALOES

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ABSTRACT

The aim of the present study was to isolate *Escherichia coli* from the uterus of buffaloes and to determine its *in-vitro* sensitivity to commonly used antibiotics. The reproductive tracts (n=103) from the adult buffaloes were collected from Deonar slaughter house, Mumbai, India. Uterine fluid was subjected to isolation of *E. coli* on eosin methylene blue (EMB) agar; confirmation of isolates by Gram staining, biochemical test and polymerase chain reaction (PCR) using *fimH* gene, and pathotyping to detect enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) and enterotoxigenic *E. coli* (ETEC). The lipopolysaccharide (LPS) concentration from follicular fluid was estimated using chromogenic Limulus amoebocyte lysate assay, histopathology of ovary and uterine tissue samples was done to grade the lesions, and antibiogram of *E. coli* isolates was carried out to determine sensitivity pattern. Out of 103 cases, 25 samples (24.27%) showed positive for *E. coli* and only 2 samples showed positive for atypical EHEC pathotype. The LPS concentration in follicular fluid ranged from 0.5 to 4.69 EU/ml and histopathology showed lesions of sub-acute endometritis. The isolated *E. coli* strains showed good antibiotic sensitivity to tetracycline (100%), cotrimoxazole (100%), gentamicin (90%) and chloramphenicol (88%). However, the isolates were quite resistant to nitrofurantoin (48%) and amoxicillin (41%). In conclusion, the study reported the higher incidence of *E. coli*, and *fimH* gene was significantly associated with reproductive disorder (endometritis) and LPS concentration in buffaloes, and the drugs like tetracycline, cotrimoxazole, gentamicin and chloramphenicol could be considered for preventing and treating clinical endometritis in the field. The present study will provide a platform for development of vaccines or therapeutics for *E. coli* associated endometritis.

Keywords: Buffalo, Uterus, *E. coli*, Endometritis, Pathotyping, Lipopolysaccharide, Antibiotic sensitivity.

INTRODUCTION

India holds largest buffalo population with finest germplasm in the world. Buffaloes contribute significantly to the country's total milk production. Infertility, particularly repeat breeding originating from metritis and endometritis in buffaloes is the major constraint faced by livestock owners (Barile, 2005; Sah and Nakao, 2006, Bicalho and Machado, 2015). There is a gamut of infectious agents such as *Arcanobacterium pyogenes*, *E. coli*, *Fusobacterium necrophorum* and *Provetella spp.* that shows affinity to reproductive tracts, resulting in temporary or permanent reproductive failures by causing endometritis, repeat breeding, anoestrous etc (Azawi, 2013). These infectious agents can be broadly categorised into two groups. The first group includes classical group of pathogens associated with different forms of reproductive failures such as abortions, stillbirths, repeat breeding etc. In the second group, pathogens remain in the animal environment or on the body of animals and cause ascending reproductive infections leads to endometritis and death of embryo (Azawi and Taha, 2002; Azawi *et al.,* 2008).

The Gram negative bacterium *Escherichia coli* is a normal commensal of lower gastrointestinal tract as well as an environmental pathogen. It is responsible for ascending infections during early postpartum period and associated with impaired reproductive performance in bovines (Kaper *et al.,* 2004; Bicalho *et al.,* 2012; Machado *et al.,* 2012a). *E. coli* alone or in combination with other pathogens often infects uterus which is influenced by certain environmental factors mainly poor hygienic practices during animal management (Markandeya and Deshmukh, 2009; Bicalho and Machado, 2015). *E. coli* population comprising of both commensals and pathogenic strains are voided into environment through faecal route, therefore chances of infection of uterus are more likely to happen especially during postpartum period, if proper hygiene is compromised (Azawi, 2008; Katouli, 2010).

The selection of suitable antibiotics for treating uterine infections is a key factor owing to loss of potency in an anaerobic environment of uterus (Sheldon and Dobson, 2004). In the field conditions, it is not always possible for clinician to carry out the antibiotic sensitivity test for each and every animal suffering from reproductive ailment due to lack of time and/or facilities.

This leads to use of inappropriate antibiotics, resulting in development of resistance and animal becomes non profitable, culled and sent for the slaughter (Tiwari *et al.,* 2013; Agarwal and Tomar, 2003; Agarwal *et al.,* 2005) Therefore, determination of antibiogram is essential for selecting antibiotics precisely in treatment of uterine infections. Apart from conventional detection methods, molecular tools of polymerase chain reaction (PCR) and multiplex PCR and others have been found to be effective for rapid and confirmatory diagnosis of *E. coli* and other infectious agents associated with reproductive infections of animals (Vidal *et al.,* 2004; Sheldon *et al.,* 2010; Bicalho *et al.,* 2012).

Thus, the present study was carried out to assess the incidence of *E. coli* on reproductive pathology of buffaloes and to determine the sensitivity to commonly used antibiotics which is essential for field veterinarians to follow a rational treatment. The study included cultural isolation of *E. coli* from the affected uterus of buffaloes, identification of the bacterium by biochemical tests and molecular tool PCR, pathotyping by multiplex-PCR, histopathology of ovary and uterine tissues, LPS estimation and antibiogram.

MATERIALS AND METHODS

Animals under study: A total of 103 genital tracts from adult female buffaloes were collected from Deonar slaughter house, Mumbai. This slaughterhouse receives animals from the organised and unorganised farms as well as from villages located near Mumbai, Thane and Raigarh districts. Therefore, the slaughtered animals can be assumed to be representative of dairy buffaloes of these regions.

Sample collection and processing: Ovarian follicular fluid for estimation of endotoxin (LPS) was collected from medium to large sized follicles of 25 *E. coli* positive cases using non-pyrogenic 22 gauge needles, and centrifuged at 1500 x g and supernatant was stored at -20˚C in endotoxin free vials until processed (Magata *et al*., 2015). For bacteriology, uterine fluid/washing were collected aseptically without opening the uterine tubes. Both the uterine horns having lesions were cleaned with 70% alcohol and injected with 10 to 20 ml of sterile phosphate-buffered saline (PBS) into the lumen using sterile syringe and aspirated back into the same syringe to nullify the chance of environmental contamination.

Samples were immediately transferred to 5 ml tubes containing buffered peptone water (BPW) and incubated for 18 h at 37°C for enrichment. The enriched samples were further cultured on selective medium (Levine EMB agar) and incubated for overnight at 37°C. Colonies showing typical metallic sheen colour were subjected for further confirmation of the presence of *E. coli* by Gram staining and biochemical tests namely catalase test, indole, methyl red, Voges-Proskauer test, nitrate reduction, citrate utilization, urease production and sugar fermentation tests (Table 1).

Nucleic acid detection: The DNA was extracted from the bacterial colonies of 25 cases using snapchill method. In brief, a few loopful of colonies were suspended in 500 µl of molecular grade nuclease free water (NFW) and kept in the water bath at 95°C for 10 minutes. The suspension was immediately transferred to -20°C for 10 minutes. Finally, centrifugation was done at 6000 RPM for 5 minutes and supernatant was used as template for PCR test.

The *fimH* gene was used for confirmation of *E. coli* by PCR by method as described earlier (Bicalho *et al*., 2012) (Table 2). In brief, 20 µl reaction mixture was prepared using 10 µl 2x Mastermix (Thermoscientific, USA), 1 µl of $MgCl_2$, 1 µl of 10 pmol of each primer, 3 µl of template and NFW was added to make volume 20 µl. The cyclic conditions included initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation, annealing and extension steps at 94°C for 1 minute, 60°C for 30 sec and 72°C for 1 minute, respectively. Final extension was done at 72°C for 5 minutes. The amplicon was resolved in 1.25% of agarose (Himedia, India) prepared in 1X TBE buffer using ethidium bromide (0.5 mg/ml) as an indicator dye and visualised in UV trans-illuminator for observing the presence of specific band at 508 bp molecular weight.

Identification of *E. coli* pathotypes by multiplex PCR: All the confirmed *E. coli* isolates were further subjected to multiplex PCR (Vidal *et al*., 2004) for identification of pathotypes viz., Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC) and Enterotoxigenic *E. coli* (ETEC), respectively, by targeting *eae, bfp, stx_1, stx_2,* lt and *StII* genes (Table 2). In brief, the PCR protocol for 25 µL reaction mixture included 2.5 µL of 10X PCR buffer (100 mM Tris-HCl buffer, pH 8.3 containing 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin), 2.5 µL of 2 mM dNTP mix (final concentration of 0.4 mM), 2.0 µL of 50 mM $MgCl_2$ (final concentration of 2.0 mM) and 20 µM of a primer set containing forward and reverse primers (final concentration of 0.4 µM of each primer), 0.25 unit of Taq DNA polymerase, 2 µL of DNA template and NFW to make up the reaction volume. The cycling conditions for PCR included an initial denaturation of DNA at 94°C for 5 min followed by 35 cycles each of 1.5 min denaturation at 94°C for 1.5 min, annealing at 62°C for 1.5 min and extension at 72°C for 1.5 min, followed by a final extension of 5 min at 72°C and hold at 4°C.

LPS estimation from the follicular fluid: The LPS concentrations in follicular fluid were estimated from the *E. coli* positive cases using the QCL-1000 Chromogenic

LAL Endpoint Assay Kit (Lonza, USA) as per manufacturer's instructions.

Histopathology: The ovary and uterine tissue samples (n=103) were collected in 10% neutral buffered formalin for histopathological studies. Tissue samples of 1 to 2 mm thickness were dehydrated in graded alcohol, cleared in xylene and embedded in paraffin blocks. Serial sections of 4-5 µ thickness were taken with rotator microtome on clean grease free slides and subjected to haematoxylin and eosin staining (Luna, 1968).

***In-vitro* antibiotic sensitivity test:** The *E. coli* isolates recovered were subjected to *in-vitro* antimicrobial sensitivity test using disc diffusion method (CLSI, 2004) on Muller-Hinton agar (MHA; Hi-media, India) plates as per method described by Bauer *et al.* (1966). A total of 15 antibiotics with varied concentrations were tested against the *E. coli* isolates. In brief, pure colonies of *E. coli* isolates were incubated in nutrient broth overnight at 37°C. Lawn cultures were prepared on MHA plates. The plates were allowed to dry and antibiotic discs were placed on the agar surface about 1.5 to 2 cm apart. The plates were then incubated at 37°C overnight and diameter of the zone of inhibition was measured in millimetre (mm). The measurements were compared with zone size given in interpretative chart furnished by the manufacturer and the zones were graded as sensitive, intermediate and resistant.

RESULTS AND DISCUSSION

E. coli was isolated from 25 cases, out of 103 (24.27%) uterine washings/fluid samples on the EMB agar with typical metallic sheen and confirmed by biochemical tests (Table 1). The increased incidence of *E. coli* in uterus might be due to unhygienic practices during artificial insemination and during parturition results in contamination of uterus with dung, which is the main source for *E. coli*. In the present study, the isolation rate of *E. coli* from the buffalo uterus was in accordance with Azawi *et al.* (2008), who reported 18.4% isolation rate. The culture of uterine samples yielded a wide range of bacteria during the first 3 weeks of postpartum, including *Arcanobacterium pyogenes*, *E. coli*, *Fusobacterium necrophorum* and *Provetella spp.* which are commonly associated with clinical and subclinical endometritis (Azawi, 2008; Bicalho *et al.*, 2010). Sheldon *et al.* (2002) and Williams *et al.* (2005) suggested that *E. coli* should be classified as potential bacterial pathogen infecting the uterus.

E. coli isolated from bovine uterus within 10 days of postpartum have been shown to express a battery of virulence factors (VF). Six *E. coli* VF genes are associated with uterine infection namely *fimH*, *astA*, *cdt*, *kpsMII*, *ibeA*, and *hlyA*. The VF gene *fimH* was the most prevalent and significantly associated in cows with metritis and increases the risk of endometritis (4.6 fold increase) when compared to *E. coli* negative cows (Bicalho *et al.*, 2010, 2012; Yang *et al.*, 2016). In the present study, out of 25 cases, 17 cases (68%) showed positive for *E. coli fimH* gene by PCR (Fig. 1). The *fimH* protein is a type 1 pili adhesive protein that has vital role for adhesion with mannosidase to establish infections in epithelial surfaces. It was also proved that *fimH* mediates adhesion between endometrial pathogenic *E. coli* and the bovine uterine mucosa, because mannose treatment of *E. coli* decreases their ability to adhere to bovine endometrial cells *in vitro* (Sheldon *et al.*, 2010). In contrast, Silva *et al.* (2009) reported that uterine *E. coli* was just opportunistic environmental bacteria, because none of the VFs (hlyE, hlyA, iuc and eaeA) were detected, however VF gene *fimH* was not evaluated in that study (Silva *et al.*, 2009).

All the 25 *E. coli* isolates recovered from the uterus of buffaloes were subjected to multiplex PCR for pathotyping to detect the presence of EHEC, EPEC and ETEC. Among these 25 isolates, only 2 (8%) showed positive for atypical EHEC pathotypes with only *stx2* gene positive (Fig. 2). This indicates the *E. coli* isolated from uterus may be extra-intestinal pathogenic *E. coli*, especially endometrial pathogenic *E. coli* (EnPEC) and/or the presence of VF gene *fimH* indicates the bacteria may be uropathogenic *E. coli* (Krekeler *et al.*, 2012). Sheldon *et al.* (2010) reported new strains of *E. coli* known as EnPEC that has particular tropism for the endometrial epithelial and stromal cells. They also reported that EnPEC is a diverse serogroup and different from enteroinvasive *E. coli* (EIEC), EPEC, EHEC, uropathogenic *E. coli* (UPEC) and enteroadherent *E. coli* (EAEC).

In the present study, *E. coli* positive cases showed presence of LPS in the ovarian follicular fluid, and concentration ranging from 0.5 to 4.69 EU/ml. The virulence factor *fimH* gene positive *E. coli* cases showed more concentration of LPS than *fimH* gene negative *E. coli* cases. The endotoxin portion (LPS) present in the cell wall of *E. coli* affects host reproductive organs locally as well as systemically. A receptor complex made up of toll like receptor-4 (TLR-4), CD-14 and MD-4 molecules present on uterine mucosa and ovarian cells, mainly responses to LPS of *E. coli* (Beutler *et al.*, 2003). LPS acts at different levels on the endometrial mucosa and causes the cells to secrete increased quantity of prostaglandin E (PGE). Due to luteotropic activity of PGE, it causes abnormal estrous cycle length (Herath *et al.*, 2006). At ovarian level, down regulation of aromatase synthesis leads to reduced estradiol production by thecal cells. LPS also inhibits the production of progesterone by luteal cells. This altered hormonal state and presence of LPS in peripheral plasma affects hypothalamus and pituitary secretions causing disruption in luteinizing

hormone (LH) production and release of LH (Herath *et al.,* 2007). During active infection, *E. coli* sets the inflammatory reaction causing endometritis; however, the portion of LPS remains for longer periods and tends to accumulate in the ovarian follicular fluid by a less understood mechanism. It negatively affects the secretion of the steroids, thereby compromising their functions such as folliculogenesis, ova survival, and ovulation, hampering the normal reproductive functions (Herath *et al.,* 2007; Magata *et al.,* 2014). Also, the presence of *E. coli* and its LPS can modify the uterine immune system which leads to more vulnerable to uterine infections by *Arcanobacterium pyogenes* and other Gram negative rods resulting in reproductive failure (Dohmen *et al.,* 2000). In the present study, the LPS concentration in the follicular fluid was found to be lower as compared to Magata *et al.* (2015) who reported 0.62 to 12.40 EU/ml concentrations of LPS in bovine metritis. Dohmen *et al.* (2000) also reported a positive relationship between the presence of *E. coli* and intrauterine endotoxins during 1–2 days of postpartum. Herath *et al.* (2007) reported that LPS was detectable from the follicular fluid of clinical endometritis case, whereas LPS was undetectable from follicular fluid of healthy animals.

On histological examination, the uterus mainly showed lesions of sub-acute endometritis (Fig. 3). The lesions are more severe in the VF *fimH* gene positive *E. coli* cases than *fimH* gene negative *E. coli* cases. The glandular epithelium of endometrium was severely degenerated and got replaced moderate to severely by inflammatory cells consisting of mononuclear cells. Moderate periglandular fibrosis was noticed in the endometrium suggesting sub-acute endometritis. These findings were in agreement with Sayyari *et al.* (2012) who reported grade II and IV lesions, and extensive periglandular fibrosis in the uterus with *E. coli* as the main bacterial isolate.

Table 1. Biochemical test results for confirmation of *E. coli*

Biochemical test	Indole	Methyl Red	VP	Citrate utilization	Nitrate reduction	Catalase test	Urease production
Reaction/ result	+	+	-	-	+	+	-

Table 2. Details of primers used in this study

Organism	**Target gene**	**Primer sequence 5' – 3'**	**Product size (bp)**	**References**
E. coli	*fimH*	F-5'TGCAGAACGGATAAGCCGTGG 3' R-5' GCAGTCACCTGCCCTCCGGTA 3'	508	Johnson and Stell (2000)
E. coli pathotypes	*eae*	F-5'TCAATGCAGTTCCGTTATCAGTT3' R-5'GTAAAGTCCGTTACCCCAACCTG3'	482	Vidal *et al.* (2004)
	bfp	F-5'GGAAGTCAAATTCATGGGGGTAT3' R-5'GGAATCAGACGCAGACTGGTAGT3'	254	Vidal *et al.* (2004)
	stx1	F-5'CAGTTAATGTGGTGGCGAAGG3' R-5'CACCAGACAATGTAACCGCTG3'	348	Cebula *et al.* (1995)
	stx2	F-5'ATCCTATTCCCGGGAGTTTACG 3' R-5'GCGTCATCGTATACACAGGAGC3'	584	Cebula *et al.* (1995)
	lt	F-5'GCACACGGAGCTCCTCAGTC 3' R-5'TCCTTCATCCTTTCAATGGCTTT3'	218	Vidal *et al.* (2004)
	StII	F-'AAAGGAGAGCTTCGTCACATTTT3' R- 5'AATGTCCGTCTTGCGTTAGGAC3'	129	Vidal *et al.* (2004)

The sensitivity of the *E. coli* isolates tested for the 15 antibiotics is shown Fig. 4. These isolates showed highest sensitivity to tetracycline (100%), ceftazidime/clavulanic acid (100%), co-trimoxazole (100%), cefotaxime/clavulanic acid (94%), gentamicin (90%), chloramphenicol (88%) and ciprofloxacin (88%). The lower susceptibility was shown to nitrofurantoin (48%) and amoxicillin (41%). These findings indicate that tetracycline, ceftazidime/clavulanic acid and co-trimoxazole were the first choice of selection of antibiotics in the field for the prevention and treatment of uterine diseases in cows. In the present study, antimicrobial resistance to broad spectrum antibiotics like nitrofurantoin and amoxicillin might be due to overuse of antibiotics leads to resistance to microbes, which creates major challenge for pharmaceuticals and veterinary practitioners. Gandahi *et al.* (2010) conducted a study on antibiogram of *E. coli* isolated from buffalo uterus and reported high degree of sensitivity to gentamicin (80%) and chloramphenicol (73.3%). They also reported ineffectiveness to tetracyclines (0%). Moges *et al.* (2013) also reported that the *E. coli* isolates were resistant to

tetracyclines (0%) and gentamycin (40%). However, in the present study, the *E. coli* isolates were found to be more sensitive to both gentamicin (90%) and tetracycline (100%), which is in agreement with other workers (Muneer *et al.,* 1991; Bhat and Bhattacharya, 2012). Udhayavel *et al.* (2013) reported limited sensitivity of *E. coli* towards chloramphenicol, which was contrary to the findings of present study, where chloramphenicol showed good effectiveness (88%). Various researchers have also studied the antibiotic sensitivity patterns of *E. coli* in cattle and reported that gentamicin (96%), ciprofloxacin (96%) and cotrimoxazole (73%) are effective, which is similar to the present study (Gani *et al*., 2008; Goncuoglu *et al.,* 2010; Mshelia *et al.,* 2014). In the present study, isolates were quite resistant to nitrofurantoin (48%) which is in agreement with the study carried out by Khushawa *et al.* (2012). The differences between the findings in literature might be due to the type of antibiotics used, company, duration of application and *E. coli* strain.

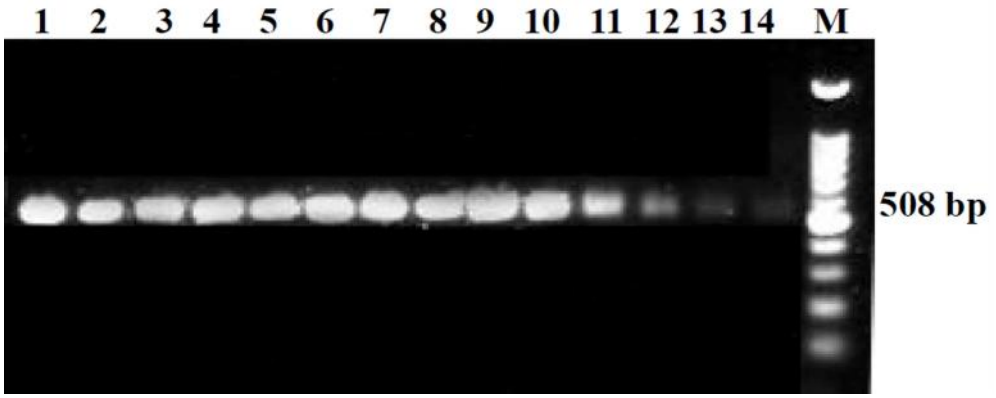

Figure 1. PCR confirmation of *E. coli* from uterine fluid using *fimH* gene showing specific band at 508 bp. Lane M: 100 bp DNA ladder, Lane 1: Positive control, Lane 2 to 13: Uterine fluid samples and Lane 14: Negative control

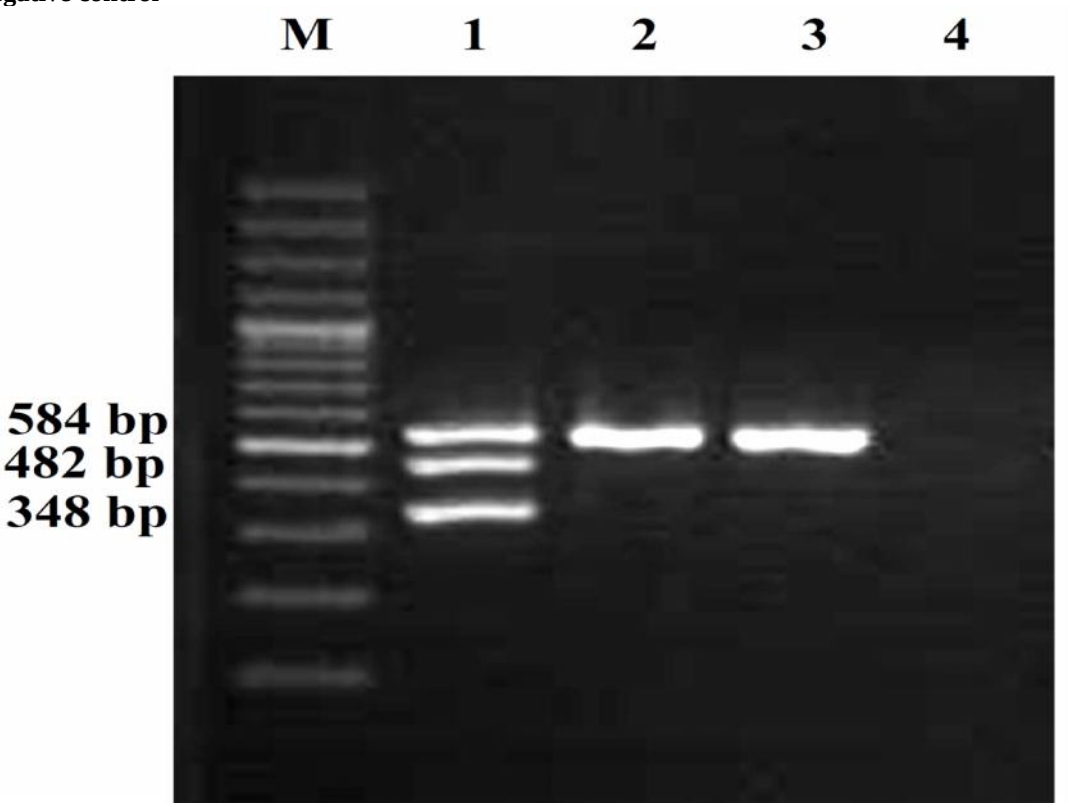

Figure 2. Multiplex PCR for confirmation of *E. coli* pathotypes (EHEC). Lane M: 100 bp to 1.5 Kbp DNA ladder; Lane 1: Positive control-EHEC: *stx1*, *stx2*, *eae*; Lane 2 & 3: atypical EHEC; Lane 4: Negative control

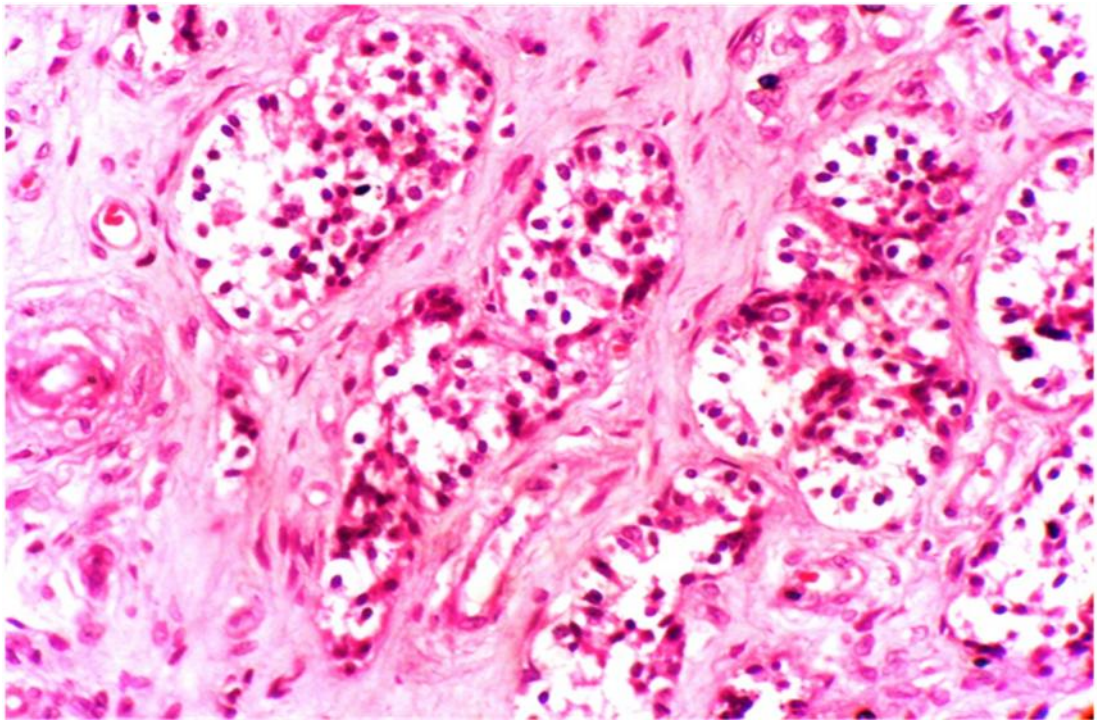

Figure 3. Uterus showing sub-acute endometritis with severe degeneration of glandular epithelium, presence of moderate to severe infiltration of MNCs and mild to moderate periglandular fibrosis. H&E x 200

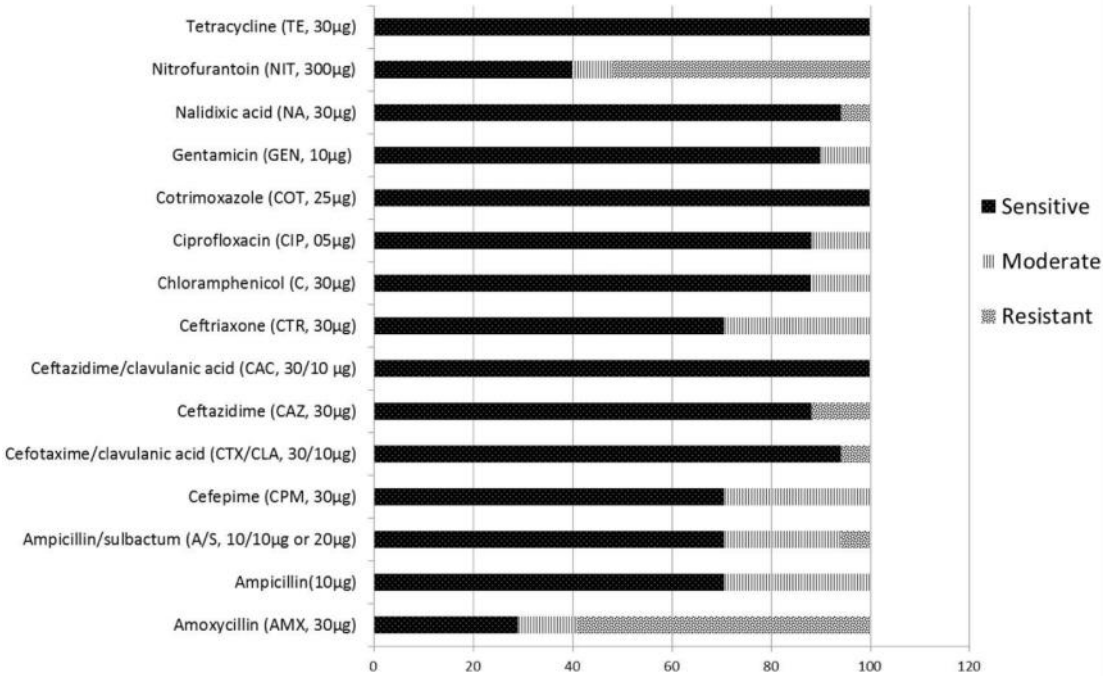

Figure 4. Bar chart showing sensitivity of *E. coli* isolates to different antibiotics

Conclusion: The present study reported the higher incidence of *E. coli* and *fimH* gene was significantly associated with reproductive disorder (endometritis) and LPS concentration in buffaloes and the drugs like tetracycline, cotrimoxazole, gentamicin and chloramphenicol could be considered for preventing and treating clinical endometritis in the field. The study also will provide a platform for development of vaccines or therapeutics for *E. coli* associated endometritis. The present study emphasized that *E. coli* infection occurs

mostly due to lack of hygiene in animal house and surrounding or inappropriate gynaecological examinations, which need to be corrected to minimize the incidences of reproductive failures. In field condition, during the treatment of animals for reproductive problems, it is necessary for clinician to consider the role of *E. coli* infection along with other agents owing to its likelihood to invade and infect the reproductive organs, rather than overlooking it as an environmental microbe.

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