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Molecular biological tools applied for identification of Pathogens causing female reproductive tract infection

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Abstract

Metritis and endometritis are the uterine diseases causing severe economic loss to the dairy farmers due to failure of conception at the appropriate time. Various microorganisms are responsible for causing metritis and endometritis, among which Arcanobacterium pyogenes, coliforms and the Gram – negative anaerobes, Fusobacterium and Bacteroides species are frequently involved.

The aim of the present study was to isolate some commonly found bacteria like Escherichia coli and Staphylococcus aureus from the uterus of cattles. The reproductive tracts secreations from the adult cattles were collected from Gynic clinic of RAJUVAS, Bikaner. Uterine fluid was subjected for isolation of E. coli and S.aureus on eosin methylene blue (EMB) agar and mannitol salt agar (MSA) respectively; confirmation of isolates by Gram staining, biochemical test and polymerase chain reaction (PCR) using fim H, hlyA, kpsMTII, hlyE, astA gene of E.coli and coa & spa gene of S.aureus respectively.

INTRODUCTION

Metritis, endometritis, pyometra, RFM and some non-specifc infections of the uterus are the most important diseases causing infertility in the dairy cattle. Many times these reproductive disorders have common etiology and predispose to each other. Most important causes of subfertility in dairy cows are reported to be metritis and endometritis[1]. Endometritis, the inflammation of the superfcial layers of uterus, alters the uterine health leading to conception failure and repeat breeding condition in bovines. These infections of uterus may occur, during or immediately after parturition, coitus or while carrying out artificial insemination. Inflammation of the uterus slows down the uterine involution and delays the onset of activities of the ovaries lead to economic loss due to systemic illness, loss of milk and meat production and marked drop in fertility. 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 reproductive failures by causing endometritis, repeat breeding, anoestrous etc[2]. Bacteria causing uterine contamination are nonspecific and belong to a great number of bacterial species[3,4]. A number of bacteria, namely Streptococcus spp., Staphylococcus spp. and Bacillus spp., have been isolated from the uterus of cows without signs or puerperal metritis[5] while Trueperella pyogenes, Escherichia coli, Fusobac-terium necrophorum, Prevotella melaninogenicus, Bacteroides spp. and Clostridium, spp. were detected in the uterus from cows with endometritis[6,5,7] . The Gram negative bacterium Escherichia coli and Staphylococcus aureus are the normal environmental pathogens. They are responsible for ascending infections during early postpartum period and associated with impaired reproductive performance in bovines[8,9,10] . E. coli alone or in combination with other pathogens commonly infects uterus which is influenced by certain environmental factors like poor hygienic practices during animal management[11,9,10].

Diagnosis of chronic endometritisis based on hysteroscopy of the uterine cavity, endometrial biopsy with plasma cells being identified histologically, while specific treatment is determined based on microbial culture. Apart from conventional detection methods, molecular tools of polymerase chain reaction (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 animal[11,12,9]

Thus, the present study was carried out to assess the incidence of E. coli and S. aureus on reproductive pathology of cattles. The study included cultural isolation of E. coli and S. aureus from the affected uterus of cattles, identification of the bacterium by biochemical tests and molecular tool PCR.

MATERIALS AND METHODS

Sample collection and processing: 100 uterine secretion samples, of cattles were collected in Bikaner district of Rajasthan from clinical cases of endometritis.

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. Immediately samples were transferred to 5 ml tubes containing buffered peptone water (BPW) and incubated for 18 h at 37^0C for enrichment. Then the enriched samples were further cultured on selective medium (Levine EMB and MSA agar respectively) and incubated for overnight at 37°C. Colonies showing typical metallic sheen and golden yellow colour respectively were subjected for further confirmation of the presence of E.coli and S.aureus by Gram staining and biochemical tests namely catalase test, indole, methyl red, Voges-Proskauer test, nitrate reduction, citrate utilization, urease production, oxidase test and sugar fermentation tests etc (Table 1).

Nucleic acid detection: The extraction DNA was done from the bacterial colonies of 25 cases using snapchill method. In brief, a loopful of colonies were suspended in 500µl of 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 gene (fim H, hlyA, kpsMTII, hlyE, astA) of E.coli and coa & spa of S. aureus were used for confirmation and by PCR method as described earlier[13] (Table 3). Briefly, reaction mixture of 20 µl was prepared using 10 µl 2x Mastermix (Thermoscientific, USA), 1 µl of MgCl2, 1 µl

of 10 pmol of each primer, 3µl 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. Then amplicons were 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 different molecular weight.

RESULTS AND DISCUSSION

E. coli and S. aureus were isolated from 27 cases and 32 cases respectively, out of 100 (27 % & 32% respectively) uterine washings/fluid samples on the EMB agar with typical metallic sheen and on MSA agar with typical golden yellow colour and confirmed by biochemical tests (Table 1). The increased incidence of E.coli & S.aureus in uterus might be due to unhygienic practices during artificial insemination and during parturition results in uterus contamination with dung, which is the main source for bacterias. In the present study, the isolation rate of E. coli from the cattles uterus was in accordance with Azawi et al. (2008), who reported 18.4% isolation rate.

The uterine culture 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[13,10]. 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). Five E. coli VF genes are associated with uterine infection namely fimH, astA, kpsMII, hlyE, and hlyA. While two S. aureus VF genes coa and spa were 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 and S. aureus negative cows [10,16]. In the present study, out of 27 cases, 19 cases (68%) showed positive for E. coli genes- fimH, astA, kpsMII, hlyE, and hlyA while 16 cases out of 32 cases (50%) showed positive for S. aureus coa & spa genes by PCR. 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[12]. In contrast, Silva et al. (2009) reported that uterine E. coli was just an 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[17].

The virulence factor Capsular polysaccharides (kpsMTR) gene is associated with the pathogenic E. coli (PEC). Capsules are mainly a polysaccharide structure covering bacteria which acts to protect the bacterium from the host immune system[18] .The virulence gene kpsMTII allowing bacteria to escape phagocytosis. Recently, the presence of kpsMTII was found to be associated with postpartum metritis in dairy cows[9].

Another significant VF gene is hly (hemolysin), which is a heat-labile extracellular protein synthesized by a large proportion of ExPEC[19].

The hly toxin is responsible for poring the membrane and lysis several different mammalian cells and affects erythrocytes, leukocytes[20]. Escherichia coli hemolysin is potentially cytotoxic to monocytes, lymphocytes and

macrophages, leading them to autolysis and death. The virulence gene hlyA activity on polymorphonuclear granulocytes and liberates leukotrienes, histamine, and ATP[21] and is neutralized by specific antiserum.

The astA gene is responsible for production of enteroaggregative Escherichia coli heat stable enterotoxin 1 (EASTl). Recently, Bicalho et al. (2010) reported that an important EAEC virulence factor {astA) was also prevalent among the Luec isolates and when present it increased the chances of metritis significantly.

Like above five Protein A, a surface protein of S. aureus binds to the IgG molecules by their Fc portion and inhibits phagocytosis of bacteria and thus contributes to the development of the disease. It is encoded by spa gene which is considered as one of the important virulence factors in development and severity of endometritis.

Coagulase, a major virulence factor for this organism is encoded by coa gene which has been reported to be polymorphic because of presence of tandem repeats at its 3' end, the number of which may vary among the isolates. This property of coa gene is being utilized by scientists to discriminate different strains of S. aureus[22] .

The author also observed a close correlation between the presence of the EAST1 toxin marker and Fl Fimbriae genes and conclude out that cows with an astA carrying lUEC were 12 times more likely to develop postpartum metritis and 4.6 times more likely to develop endometritis compared with E. coli negative cows. Abe et al. (2008) demonstrated that UPEC can carry VF genes from diarrheagenic E. coli, especially VF associated with EAEC. It is unknown whether ExPEC strains have acquired EAEC genes or whether some EAEC are involved in extra intestinal infections such as UTI[23]. Thus, our study suggests that EASTl may play an important role in the pathogenesis of postpartum uterine infections.

Figure 1: S. aureus colony on MSA agar

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	+	+	-	-	+	+	-

While biochemical test like Catalase, oxidase and sugar fermentation test were positive for S.aureus

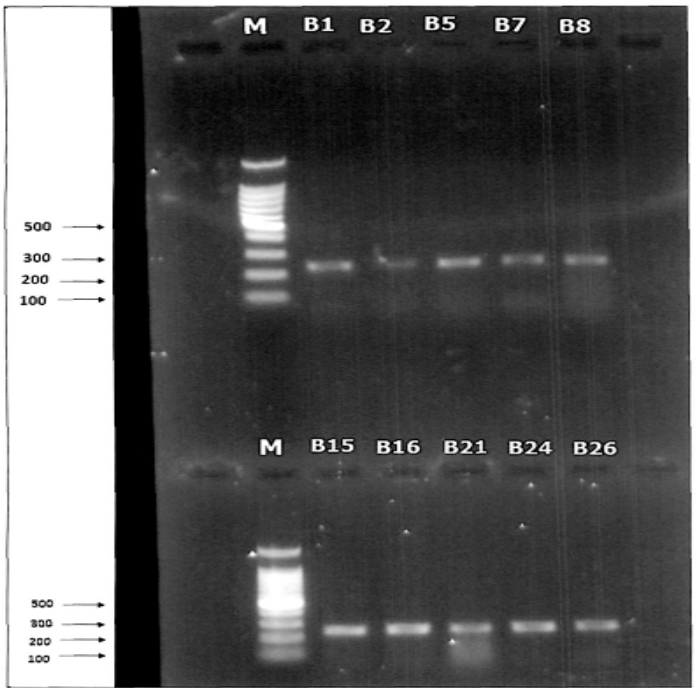
Table 2: Prevalence of different types of virulence genes in Escherichia coli

S. No.	Virulence gene	Positive (%)	Negative (%)
1.	fimH	100	0
2.	kpsMTII	66.4	33.3
3.	hlyE	6.7	93.3
4.	hlyA	46.7	53.3
5.	astA	76.7	23.3

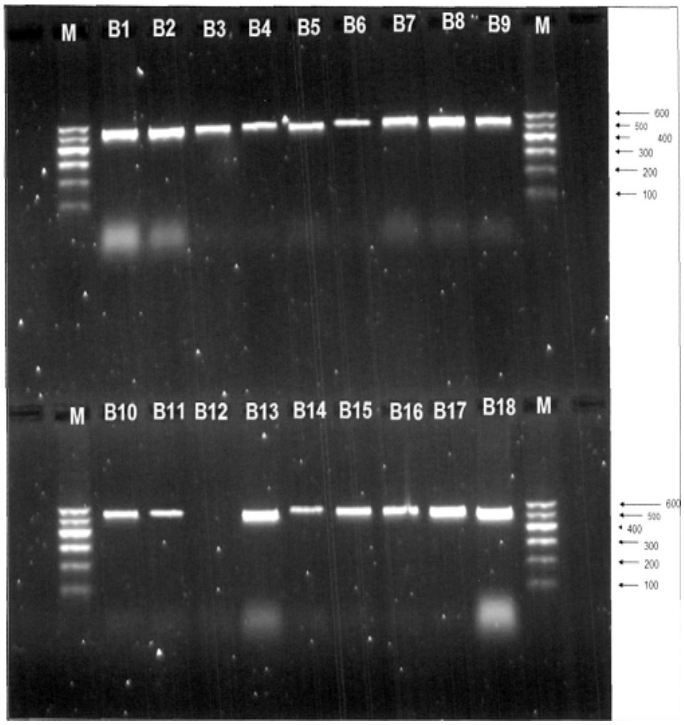
Many researchers studied the uterine E.coli in buffaloes. But E.coli and S.aureus together with allthese genes were not evaluated in any study of female reproductive tract infection of cattles.

Table 3: Details of primers used in this study

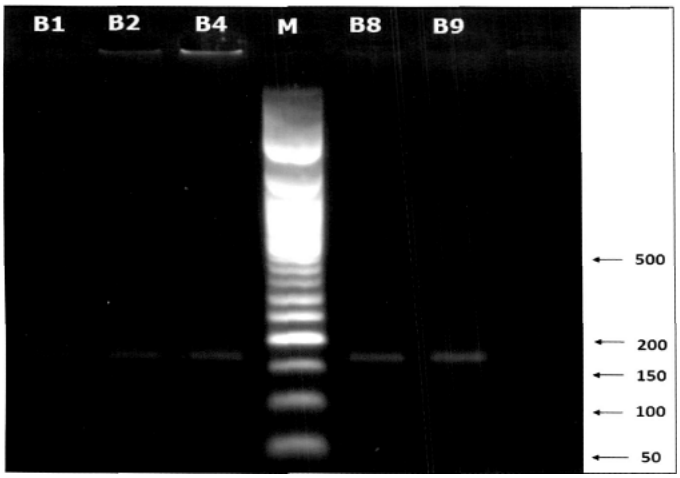
Primer	Sequence(5'-3')	Gene	Reference
P1	5'TCG AGA ACQ GAT AAG CCG TGG3'	FimH	Zahraa et al. (2013)
P2	5'GCA GTC ACC TGC CCT CCG GTA3'	FimH	Zahraa et al. (2013)
P3	5'GCG CAT TTG CTG ATA CTG TTG 3'	KpsMTII	Zahraa et al. (2013)
P4	5'CAT CCA GAC GAT AAG CAT GAG C 3'	KpsMTII	Zahraa et al. (2013)
P5	5'GAA ACC GCA GAT GGA GCA TT3'	HlyE	Silva et al. (2009)
P6	5'CGC CCG CAG CAA TAG AAT AG 3'	HlyE	Silva et al. (2009)
P7	5'ACG ATG TGG TTT ATT CTG GA3'	HlyA	Fagan et al.(1999)
P8	5'CTT CAC GTG ACC ATA CAT AT 3'	HlyA	Fagan et al.(1999)
P9	5'CCA TCA ACA CAG TAT ATC 3'	AstA	Yamamoto et al. (1996)
P10	5 GTC GCG AGT GAC GGC TTT GT3'	AstA	Yamamoto et al. (1996)
P11	5' CAA GCA CCA AAA GAG GAA 3'	Spa	Frenay et al. (1996)
P12	5' CAC CAG GTT TAA CGA CAT 3	Spa	Frenay et al. (1996)
P13	5' ATAGAGATGCTGGTACAGG 3'	Coa	Hookey et al. (1998).
P14	5' GCTTCCGATTGTTCGATGC 3'	Coa	Hookey et al. (1998).



Agarose gel electrophoresis of PCR amplification products of *E. coli kpsMTII* gene (1.5% agarose, 100V, 90 min.).The amplified DNA in each sample was 272 pb of *kpsMTII* gene.



Agarose gel electrophoresis presenting the PCR results obtained with *hlyE* -F and *hlyE*-R primers. The amplified DNA in each sample except (sample B12) was 543 pb of *hlyE* gene.



Agarose gel electrophoresis presenting the PCR results obtained with *hlyA* -F and *hlyA*-R primers. The amplified DNA in each sample was 165 pb of *hlyA* gene.

Conclusion

The present study reported the higher incidence of E. coli and fimH, astA, kpsMII, hlyE, and hlyA genes and S. aureus with coa & spa genes were significantly associated with reproductive disorder (endometritis)

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