

Original Research Article

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Uterine Bacterial Isolates of Early Postpartum Endometritis and its AntibioGram

Leeba Chacko^{1*}, K. Promod¹, Chintu Ravishankar¹, Hiron M. Harshan²,
C. P. Abdul Azeez¹ and K. D. John Martin²

¹Department of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala- 673 576, India

²Department of Veterinary and Animal Sciences, Mannuthy, Thrissur,
Kerala, India

*Corresponding author

ABSTRACT

Bacterial organisms causing endometritis at 31.58 ± 0.13 days postpartum in crossbred dairy cows were investigated along with *in-vitro* antibiotic susceptibility test of seven selected antibiotics viz., enrofloxacin, oxytetracycline, ciprofloxacin, metronidazole, gentamicin, cephalexin and ceftriaxone. The most frequently isolated bacteria in the present study were *E. coli* (27.27%) followed by, *Staphylococcus* spp. (19.48%), *Bacillus* spp. (18.18%), *Streptococcus* spp. (15.58%), *Enterobacter* spp. (11.69%), *Enterococcus* spp. (5.19%) and *Pseudomonas* spp. (2.60%). Significant difference was noted in the sensitivity of different antibiotics ($p < 0.01$). Sensitivity (Z-test) of ciprofloxacin was significantly higher than all other antibiotics under study. Hierarchical cluster analysis of antibiotics under present study revealed highest sensitivity for ciprofloxacin (88.31%) followed by enrofloxacin (54.79%).

Keywords

Endometritis,
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Introduction

Postpartum period has a great influence on the reproductive efficiency of dairy cows and any systemic or reproductive tract infections during this period will adversely affect future production and reproduction of the animal. The financial losses associated with severe uterine infections are due to reduced milk

yield, infertility problems which leads to increased culling rate and cost of treatment involved.

Bacterial contamination of uterus at the time of parturition and early puerperium is inevitable in almost all the cattle in the herd and presence of bacteria in the uterine lumen could be detected during the first two weeks

postpartum (Sheldon *et al.*, 2006). Elimination of uterine contamination is crucial for timely involution of uterus and resumption of reproductive functions in dairy cows. Many of the cows spontaneously eliminate the contamination however, a delay in this process contributes to increased number of days open, more services per conception which in turn affect the reproductive efficiency of the herd. Bacterial invasion of uterus establish infection with resultant inflammatory changes of endometrium and its severity may vary depending on the type of organisms, duration of infection and the uterine layers which were affected and so on.

The most common bacteria associated with bovine endometritis were *Escherichia coli*, *Arcanobacterium pyogenes*, *Fusobacterium necrophorum* and *Prevotella* species. Indeed, *A. pyogenes*, *F. Necrophorum* and *Prevotella* spp. could act synergistically to enhance the chances for uterine diseases and increase the risk of endometritis (Olson *et al.*, 1984; Ruder *et al.*, 1981; Sheldon *et al.*, 2002b). *E. coli* infection in the first week postpartum and *A. pyogenes* infection in the second week postpartum caused severe endometritis (Gilbert *et al.*, 2007). The severity of endometritis depends upon the type of bacteria present, genetic factors, the innate and acquired immunity of the animal and a suitable uterine environment for bacterial growth (Williams *et al.*, 2005). Several antibiotics such as tetracyclines, sulfonamides, aminoglycosides, β -lactams, fluoroquinolones and cephalosporins have been used either alone or in combination for the treatment of postpartum endometritis.

Bovine endometritis has been considered as the most common infertility problem of dairy cows incurring heavy economic loss to dairy industry and livestock farmers worldwide. Postpartum bacterial endometritis alters the

normal cyclicity of cows, interferes with intrauterine transport of spermatozoa and embryo implantation, prolongs calving to first service and conception interval, increased services per conception and thereby decreases the conception rate (LeBlanc *et al.*, 2002 and Hoelker *et al.*, 2012). Hence, accurate and early diagnosis of endometritis is essential for successful treatment to improve reproductive efficiency in dairy cows.

Materials and Methods

The study was conducted at Instructional livestock farm, Pookode, Wayanad under Kerala Veterinary and Animal Sciences University and organized dairy farms of Wayanad district, Kerala. Crossbred dairy cows (n=60) at 28-35 days postpartum, aged between three to eight years, parity between two to six without any history of postpartum reproductive complications, positive for endometritis by vaginal mucus score (Williams *et al.*, 2005) were selected for the study. Cows which were treated with hormones, systemic or intrauterine antibiotic therapy in the current lactation was not included in the study group.

Collection of samples by endometrial cytobrush

Endometrial cytology samples were collected by cytobrush technique (Kasimanickam *et al.*, 2005), with minor modifications. The human cytobrush (SteriUNO[®], India) was modified and fixed sharpened stylet of an artificial insemination (AI) gun. The assembly was inserted into an AI sheath covered by a sanitary plastic sleeve and sterilized by ultraviolet irradiation in a laminar air flow (Klenz Flo, India) for a period of 15 min. The assembly was passed through the cervix and on reaching the uterus, the cytobrush was rolled on to the endometrium. The cytobrush assembly was taken out and the detached

cytobrush placed in a sterile screw cap vial was transported to the laboratory on ice within two hours of collection and incubated for three hours at 37°C on brain heart infusion broth (HiMedia).

Isolation and identification of bacteria

After incubation, a loop full of the sample was inoculated on blood agar and incubated aerobically for 24h at 37°C. In the absence of growth, the plates were further incubated for 48h at 37°C and examined for growth of bacterial colonies before declaring the sample bacteriologically sterile. Isolation and identification of bacteria based on the morphology and cultural characteristics were carried out as per Quinn *et al.*, (1999). The morphological features of the bacterial organisms were studied by Gram's staining technique. Based on the results of Gram's staining, colonies were sub-cultured on MacConkey's agar (HiMedia) and incubated at 37°C for 24 h and growth characteristics were determined. Colonies suggestive of isolates were sub-cultured on brain heart infusion agar (HiMedia). For identification of bacteria, oxidase, catalase, indole, methyl red, Voges Proskauer, citrate utilisation, triple sugar, iron agar, urease, nitrate reduction and sugar fermentation tests were carried out. After the completion of morphological studies and cultural characteristics, the isolates were inoculated on nutrient agar slants and stored at 4°C and sub-cultured periodically to preserve the viability of the isolates. Individual isolates were inoculated in three milliliters nutrient broth and 500µL of defibrinated blood was added and mixed in a vortex shaker. Incubated it for two hours at 37°C and stored at -20°C for long term storage.

***In-vitro* antibiotic susceptibility test**

Antibiotic susceptibility tests were performed by disc diffusion method (Bauer *et al.*, 1996).

Three to four isolated colonies were selected from a pure culture and transferred into sterile nutrient broth and incubated overnight at 37°C. The turbidity of culture was adjusted using solution with half the density of MacFarland standard No.1. Sterile Muller Hinton agar (HiMedia) plates with four millimeter depth were prepared and inoculated by swabbing over its entire surface, within 15 min. after adjusting the density of inoculum. Seven commercially available antibiotic discs *viz.*, enrofloxacin (EX 5 µg), oxytetracycline (O 30 µg), ciprofloxacin (CIP 5µg), metronidazole (MT 5 µg), gentamicin (Gen 10µg), cephalexin (CN 30 µg) and ceftriaxone (CTR 30µg) (HiMedia, Mumbai) were utilised to check the antibiotic susceptibility. The inoculated plates were incubated at 37°C for 18 h. At the end of the incubation period, the plates were examined and the diameter of the zones of complete inhibition was measured in three different directions keeping the midpoint of the disc as the centre of the zone. The mean diameter of the zone of inhibition of each disc was compared with that of standard zone of inhibition chart provided by the disc manufacturing company and the isolates were grouped as resistant (R), fully susceptible (S) or intermediately susceptible (I) for each selected antibiotics.

Statistical analysis

The data obtained were subjected to statistical analysis as per the procedure described by Snedecor and Cochran (1994) using statistical software SPSS version 24.0.

Chi square test for multiple proportions followed by Z-test for testing the proportion of two different groups to test the sensitivity of different antibiotics. Hierarchical cluster analysis was done to identify the antibiotics with homogenous response. Results of the cluster analysis were represented by using dendrogram.

Results and Discussion

Isolation and identification of bacteria

The bacterial organisms isolated from the uterine cytobrush samples of postpartum cows with apparent signs of endometritis at 31.58 ± 0.13 dpp are depicted in table 1. The results of the present study revealed that the most predominant organism isolated from uterus of endometritis affected postpartum cows was *E. coli* (27.22%) followed by *Staphylococcus* spp. (19.48%) and *Bacillus* spp. (18.18%). Baishya *et al.*, (1998), Williams *et al.*, (2007), Wagener *et al.*, (2014), Crivei *et al.*, (2016), Abreham *et al.*, (2017) and Manimaran *et al.*, (2019) reported *E. coli* as the major organism isolated from uterine infections. *E. coli* is considered as one of the earliest pathogen invading the uterus. LPS produced by *E. coli* impaired the hormonal balance and altered the resumption of cyclical activity (Williams *et al.*, 2008). Hence, control of *E. coli* infection could prevent endometritis at later postpartum period. While Abd El-Kader and Shehata (2001) reported *Corynebacterium* spp. while Bhat *et al.*, (2013) and Liu *et al.*, (2013) found *Staphylococcus* spp. as the main causative agent associated with postpartum uterine infection. The most common bacteria isolated were *Streptococcus acidominimus* and *E. coli* and the correlation between cytologic and bacteriologic findings was low (Baranski *et al.*, 2012). Sens and Heuwieser (2013) found alpha-hemolytic *Streptococcus* to be strongly associated with reduced reproductive performance. Perusal of literature revealed that the uterine bacterial flora varied widely depending upon the stage of sampling, severity of infection, country and even differs with region of study. The majority of bacteria isolated from the uterus are opportunistic pathogen and host defense mechanism helps in combating the infection during early postpartum period. Immune state of the animal and the bacterial load played a

key role in the development of uterine diseases in postpartum dairy cows (Sheldon and Dobson, 2004). Uterine infection is also a risk factor for delayed ovulation (Opsomer *et al.*, 2000). Furthermore, ovarian function is perturbed in cows with uterine bacterial contamination after parturition (Sheldon *et al.*, 2002). A recent study by de Boer *et al.*, (2015) found that cows with any bacterial growth at 21 days after parturition, irrespective of bacterial species, were less likely to conceive.

In-vitro antibiotic susceptibility test of uterine bacterial isolates

The *in-vitro* antibiotic susceptibility pattern of different bacteria isolated from uteri of bovine postpartum endometritis cases with selected antibiotics were presented in table 2. The overall results of the *in vitro* antibiotic susceptibility test indicated that the maximum number of isolates were sensitive to ciprofloxacin (88.31%), followed by gentamicin (72.73%), oxytetracycline (63.64%), enrofloxacin (54.79%), ceftriaxone (25.97%) and cephalixin (19.48%). None of the isolates were sensitive to metronidazole. Chi square value indicates that there exists significant difference in the sensitivity of different antibiotics ($p < 0.01$). Z-test for testing the proportion of two different groups was done as pair wise comparison which revealed that the sensitivity of ciprofloxacin was significantly higher than all other antibiotics under study. No significant difference in the sensitivity was noted in the case of enrofloxacin, oxytetracycline and gentamicin. However, these antibiotics had significantly higher sensitivity as compared to cephalixin and ceftriaxone. Moreover, no significant difference in the sensitivity was observed between cephalixin and ceftriaxone.

Hierarchical cluster analysis was done for grouping the antibiotics with similar response.

Results of the cluster analysis showing similarity in antibiotic susceptibility pattern of seven antibiotics namely enrofloxacin, oxytetracycline, metronidazole, ciprofloxacin, gentamicin, cephalixin and ceftriaxone given as dendrogram and were represented in Fig. 1. The antibiotics which showed less than 20 per cent variability in their response were grouped as homogeneous. Cephalixin and ceftriaxone showed almost similar pattern of response. Overall sensitivity of these two antibiotics were only 16.88 and 25.97 per cent, respectively. Hence, it could be concluded that they were similar only in terms of resistance and not in terms of sensitivity.

Ciprofloxacin and enrofloxacin exhibited only less than 15 per cent variability in their response while all the other antibiotics showed more than 20 per cent of variability. The percentage response revealed that ciprofloxacin exhibited 87.01 per cent sensitivity. Even though gentamicin recorded the second highest (72.73 per cent) sensitivity, dendrogram exhibited that the similarity between ciprofloxacin and gentamicin was less when compared to ciprofloxacin and enrofloxacin.

The cross tabulation between the response of isolates to enrofloxacin and ciprofloxacin revealed that among the 46 isolates which were sensitive to enrofloxacin, 45 isolates (97.80 per cent) were sensitive to ciprofloxacin also (Table 3). However, on cross tabulation between the response of the recovered isolates to ciprofloxacin and gentamicin, it was observed that among the 56 sensitive isolates of gentamicin, only 47 isolates (83.90 per cent) were sensitive to ciprofloxacin (Table 4).

Many other studies also supported the present finding of highest sensitivity for ciprofloxacin as 75 per cent (Moharana *et al.*, 2000), 83.33 per cent (Ingawale *et al.*, 2003), 93.75 per

cent (Mane *et al.*, 2009), 75 per cent (Reddy *et al.*, 2012), 71.05 per cent (Bhat *et al.*, 2013) while Nath *et al.*, (2014) and Rohit *et al.*, (2019) reported lower sensitivity of 45.90 per cent and 63.50 per cent, respectively than the present study. Zaman *et al.*, (2015) also observed highest sensitivity for ciprofloxacin and enrofloxacin (100%).

The overall sensitivity to oxytetracycline (63.64%) reported in the present study was lower than that of Bhat *et al.*, (2013) (71.05%). While Reddy *et al.*, (2012) observed that only 25.00 per cent of isolates were sensitive. Resistance to oxytetracycline was reported by Takamtha *et al.*, (2013). Manimaran *et al.*, (2019) reported that although gentamicin showed 100 per cent sensitivity to *E. coli*, the MIC observed indicate that oxytetracycline was more sensitive.

More number of isolates were sensitive for gentamicin than the current study as reported by Mane *et al.*, (2009) (84.3%), Malinowski *et al.*, (2010) (96%), Reddy *et al.*, (2012) (83.33%) and Bhat *et al.*, (2013) (85.53%). However, Kusam *et al.*, (2008) reported lower sensitivity. Results comparable with the present study (77.08%) were reported by Ingawale *et al.*, (2003). Arora *et al.*, (2000) also reported highest sensitivity for gentamicin. Contrary to the present study, Moharana *et al.*, (2000) reported 79.16 per cent sensitivity for enrofloxacin, 75.36 per cent by Kusam *et al.*, (2008), 91.67 per cent by Reddy *et al.*, (2012), 73.68 per cent by Bhat *et al.*, (2013), 73.53 per cent Joshi *et al.*, (2013) and 60.32 per cent by Rohit *et al.*, (2019) which were higher than observed in the present study (54.79%). While Udayavel *et al.*, (2013) observed lower sensitivity (32%) to enrofloxacin. Bhat *et al.*, (2013), Kavyashree (2013), Udhayavel *et al.*, (2013) and Nath *et al.*, (2014) reported that large number of isolates was sensitive to

ceftriaxone (67.11%, 40.26%, 64.00% and 89.20%, respectively) than observed in the present study. Less number of isolates (10.17) were sensitive in the studies by Rohit *et al.*, (2019).

The overall sensitivity for cephalexin reported in the current study (19.48%) was lower than that of Kusam *et al.*, (2008) and Takamtha *et al.*, (2013) while higher than that of Reddy *et al.*, (2012) and Bhat *et al.*, (2013).

Quinolones rapidly inhibit DNA synthesis by promoting cleavage of bacterial DNA in the DNA-enzyme complexes of DNA gyrase and type IV topoisomerase, resulting in rapid bacterial death (Hooper, D.C., 1999). Fluoroquinolones are considered as the most efficacious antibacterials which include enrofloxacin, norfloxacin, ciprofloxacin, orbifloxacin, ofloxacin, danofloxacin, flumequine, difloxacin, marbofloxacin and other newer drugs. The quinolones inhibit the bacterial enzyme DNA-gyrase (topoisomerase), responsible for the supercoiling of DNA so that the DNA can twist in a number of chromosomal domains and seal around an RNA core. The quinolones are usually bactericidal therefore, susceptible organisms lose their viability within 20 min of

exposure to optimal concentrations of the newer fluoroquinolones (Manoj *et al.*, 2014).

The wide range of varying response to different antibiotics may be attributed to the variable environmental conditions, management system, condition of uterus of the animals under study and days postpartum. But, Devriese and Dutta (1981) reported that MIC of antibiotic as the only one important aspect in the *in vivo* situation than *in vitro* antibiotic testing.

In the current study, the most common bacterial species isolated from this region in the present study were *E. coli* and *Staphylococcus* spp. and the antimicrobial drug sensitive for majority of the isolates in this region was ciprofloxacin followed by enrofloxacin. The antibiotics selected for *in-vitro* antibiotic susceptibility studies were frequently used for uterine infections under *in-vivo* conditions. Also, identification of bacterial organisms causing uterine infection and adoptions of treatment measures is the ideal way for the treatment of uterine infection in postpartum animals. Rapid microbiological diagnosis is necessary for adoption of treatment under field conditions.

Table.1 Types of bacteria isolated from the uterus of cows with endometritis

SI. No	Bacterial species	Number of isolates (per cent)
1	<i>Escherichia coli</i>	21 (27.27)
2	<i>Staphylococcus</i> spp.	15 (19.48)
3	<i>Bacillus</i> spp.	14 (18.18)
4	<i>Streptococcus</i> spp.	12 (15.58)
5	<i>Enterococcus</i> spp.	9 (11.69)
6	<i>Enterobacterspp.</i>	4 (5.19)
7	<i>Pseudomonas</i> spp.	2 (2.60)
	Total	77

Table.2 Sensitivity pattern of bacterial isolates from postpartum endometritis

Bacterial species	Total number of isolates (per cent)	Number of isolates sensitive						
		Enrofloxacin	Oxytetracycline	Ciprofloxacin	Metronidazole	Gentamicin	Cephalexin	Ceftriaxone
<i>Escherichia coli</i>	21 (26.25)	11	12	20	0	14	3	5
<i>Staphylococcus spp.</i>	15 (18.75)	8	9	12	0	10	2	3
<i>Bacillus spp.</i>	14 (17.50)	7	10	13	0	11	4	5
<i>Streptococcus spp.</i>	12 (15.00)	9	8	10	0	9	3	3
<i>Enterococcus spp.</i>	9 (11.25)	7	5	6	0	6	2	2
<i>Enterobacter spp.</i>	4 (5.00)	3	3	4	0	4	1	1
<i>Pseudomonas spp.</i>	2 (2.50)	1	2	2	0	2	0	1
Total isolates	77	46	49	68	0	56	15	20
Sensitivity (per cent)		59.74^b	63.64^b	88.31^a	0.00	72.73^b	19.48^c	25.97^c

$\chi^2 = 115.716^{**}$; p-value < 0.01

** Significant at 0.01 level; sensitivity of the antibiotics having different letter as superscript differ significantly

Table.3 Cross tabulation between enrofloxacin and ciprofloxacin

Antibiotics		Ciprofloxacin			Total
		R	I	S	
Enrofloxacin	R	1	4	3	8
	I	2	3	18	23
	S	1	0	45	46
Total		4	7	66	77

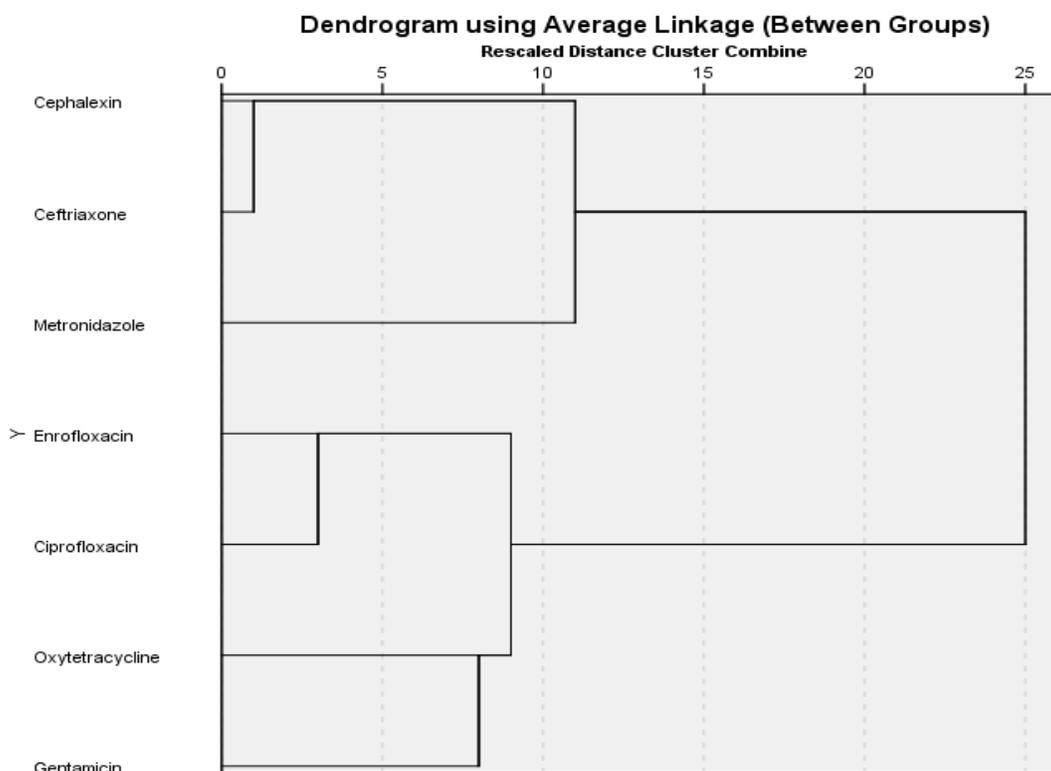
R-Resistant, I- Intermediate, S-Sensitive

Table.4 Cross tabulation between gentamicin and ciprofloxacin

Antibiotics		Ciprofloxacin			Total
		R	I	S	
Gentamicin	R	1	1	10	12
	I	0	0	9	9
	S	3	6	47	56
Total		4	7	66	77

R-Resistant, I- Intermediate, S-Sensitive

Figure.1 Dendrogram of antibiotics



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