

Original Research Article

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Bacterial Isolation, Culture Sensitivity Test, Endometrial Cytology of Postpartum Cows and Assessment of their Reproductive Performance

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ABSTRACT

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The objectives of this study were to find out the bacteria from postpartum dairy cows, determination of their antimicrobial sensitivity, to diagnose subclinical endometritis via endometrial cytology and evaluation of reproductive performance. Uterine discharge from normally parturited cows (n=23) were examined for aerobic bacterial identification and antimicrobial sensitivity at a weekly interval. Isolates include gram-positive bacteria (n=13), gram-negative bacteria (n=10) and mixed bacteria (n=7). The most frequently found isolates of bacteria were as follow: *E. coli* (32.26%), *Bacillus cereus* (22.58%), *Staphylococcus aureus* (16.13%), *Bacillus cereus* + *Staphylococcus aureus* (9.68%) and *Escherichia coli* + *Proteus vulgaris* (3.23%). From all 13 antimicrobial agents used in the culture sensitivity test, Levofloxacin and Ciprofloxacin were highly sensitive antibiotics and metronidazole was found resistant against both gram-positive and gram-negative bacteria. Endometrial cytology was performed in 18 cows, out of which 6 cows were diagnosed with subclinical endometritis. Reproductive performance of these cows was assessed via evaluation of days to first artificial insemination, number of inseminations per conception and calving to conception interval. In conclusion, this study provided an update on information of bacteria present in uterus after parturition, their status of antibiotic resistance and further effect on reproductive performance of cows.

Introduction

Bacterial contamination is common in the uterus during early postpartum period. 80 to 100% of cows are found to have bacterial contamination of the uterus in the first 2 weeks postpartum (Foldi *et al.*, 2006). Many of these bacteria are eliminated during the first 5 weeks after parturition, but the remaining bacteria cause uterine disease in some cows. Common bacteria isolated from cows with uterine infections are *Escherichia coli*, *Prevotella* spp. (Sheldon *et al.*, 2006).

Other studies also found *Staphylococcus* spp., *Streptococcus* spp. or non-coliform aerobic gram-negative rods (Kaczmarowski *et al.*, 2004; Jadon *et al.*, 2005). To choose an appropriate antimicrobial agent for treatment of postpartum uterine infection, it is essential to know the susceptibility of the pathogen to antibiotics. Antimicrobial resistance has emerged as important problem in human and veterinary medicine and antimicrobial agent misuse is considered the most important

factor for the emergence, selection and dissemination of antimicrobial agent-resistant bacteria (Takamtha *et al.*, 2013). The cytobrush technique has been used to diagnose subclinical endometritis (SCE) and thus, evaluating the relationship between polymorphonuclear cells (PMNs) and conception (Barlund *et al.*, 2008). The presence of neutrophils in the lumen therefore, is an excellent indication of an active inflammatory process (Butt *et al.*, 1993). Postpartum uterine infections can delay the regeneration of endometrium and disrupts the resumption of cyclic ovarian function which leads to the postponement of first insemination (AI), increase in number of inseminations per conception and thus calving interval is prolonged (Foldi *et al.*, 2006). Therefore, the main objective of the present study was to find out the sensitivity of bacteria to various antimicrobial agents, to diagnose subclinical endometritis and assessment of future reproductive performance.

Materials and Methods

The present study was carried out on 23 postparturient dairy cows (Jersey and Jersey crossbred) of Livestock farm, College of Veterinary and Animal Sciences, Palampur.

Collection of uterine discharge

After proper restraining of cows, uterine discharge was collected from parturited cows upto 29 days at a weekly interval. The perineal area was washed with 0.01 percent potassium permanganate solution prior to sample collection followed by swabbing with ethyl alcohol. The discharge was collected using artificial insemination gun and then transferred into a sterile screw capped vial containing sterile swabs. The discharge samples collected were placed upright in the cool box with ice and were transferred to the

microbiological laboratory for immediate processing for isolation of bacteria and antimicrobial susceptibility test.

Bacterial isolation

Primary isolation from uterine discharge was done by directly streaking it on ovine blood agar plates to obtain discrete colonies. The plates were incubated at 37⁰C aerobically and examined for the presence of bacteriological growth after 24 – 72 hours of incubation. The smears were then prepared from single isolated purified colonies and gram stained. The gram negative organisms were grown on MacConkey's Lactose Agar (MLA) and Eosin Methylene Blue (EMB) agar. Single isolated colony of each purified isolate was inoculated in nutrient broth tubes and incubated at 37⁰C for 48 hours to obtain broth cultures for biochemical testing.

Culture sensitivity test (Disc Diffusion Method)

The isolates were tested for their sensitivity to various chemotherapeutic agents by disc diffusion method (Bauer *et al.*, 1966; Jorgensen and Ferraro, 2009). The test was performed using Mueller Hinton agar (Hi-media) by employing 14 antibiotic diffusion discs (Hi-media) viz. Ciprofloxacin (CIP, 5 mcg/disc), Gentamicin (GEN, 10 mcg/disc), Amoxicillin (AMX, 10 mcg/disc), Cloxacillin (COX, 10 mcg/disc), Enrofloxacin (EX, 10 mcg/disc), Penicillin (P, 10 units/disc), Ofloxacin (OF, 5 mcg/disc), Oxytetracycline (O, 30 mcg/disc), Ampicillin (AMP, 10 mcg/disc) Metronidazole (MT, 5 mcg/disc), Levofloxacin (LE, 5 mcg/disc), Cephalexin (CN, 30 mcg/disc), Ceftriaxone (CTR, 30 mcg/disc) and Streptomycin (S, 10 mcg/disc). Now, the zones of growth inhibition around each of the antibiotic discs are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the

isolate and to the diffusion rate of the drug through the agar medium (Jorgensen and Ferraro, 2009).

Endometrial cytology

Endometrial cytology was done (on Day 43 postpartum) via passing the stainless steel tube (covered with plastic sleeve to avoid contamination) through the vagina to the external os of the cervix. The plastic sleeve was perforated and the stainless steel sheath and extension was manipulated through the cervix and into the body of the uterus where the cytobrush was turned clockwise approximately ¼ turn to obtain endometrial cells from the uterus.

The cytobrush was rolled onto a clean glass slide and allowed to air-dry on form. Slides are stained with modified Wright Giemsa stain and then the slide was evaluated using 400x magnification and a differential count, a minimum of 100 cells (endometrial, PMNs and epithelial cells), was performed to provide a quantitative assessment of endometrial inflammation (Barlund *et al.*, 2008).

Assessment of postpartum reproductive performance

Postpartum reproductive performance was assessed by the study of days to first artificial insemination (A.I.), number of inseminations per conception and calving to conception interval (days open). The data was statistically analyzed using Student's t-test with SPSS® 20 level version for windows.

Results and Discussion

Bacterial isolation

Various bacteria isolated from uterine discharge have been shown in table 1.

In this study, *E. coli* (32.26%), *B. cereus* (22.58%) and *S. aureus* (16.13%) were frequently isolated.

Culture sensitivity test

Antibiogram of bacteria isolated from uterine discharge has been shown in tables 2 and 3.

Culture sensitivity test was performed at a weekly interval upto day 29 postpartum. From all the 13 antimicrobial agents used, Levofloxacin, Enrofloxacin and Ciprofloxacin were sensitive (96.43, 93.18, 93.18 %, respectively), whereas, Cloxacillin, Penicillin and Ampicillin were resistant (78.57, 65.91 and 60.72 %, respectively) against *E.coli*. Gram positive aerobic bacteria (*B. cereus*, *S. aureus* and *Streptococci spp.*) were sensitive to Levofloxacin and Enrofloxacin (94.08, 95.83 and 93.88 %, respectively), whereas, Penicillin, Cloxacillin and Amoxycillin were resistant (98.61, 86.67 and 81.51%, respectively). Mixed bacteria were sensitive to Ciprofloxacin, Levofloxacin and Enrofloxacin (84.11, 87.14 and 92.67%, respectively), whereas, Amoxycillin, Cloxacillin and Ampicillin were resistant (80.48, 78.25 and 77.62 %, respectively).

Mean \pm S.E. of PMNs in cows having subclinical endometritis (SCE) was 22 ± 4.77 (%), whereas in cows not having SCE, it was 2.50 ± 0.88 (%) on day 43 after parturition.

Also, there was a significant difference ($P < 0.01$) between infected and non-infected cows (Table 4).

In cows diagnosed with SCE, mean time required for days to first A.I. was 107.67 ± 7.94 days postpartum. In cows having no SCE, mean time required for days to first A.I. was 89.58 ± 4.44 days postpartum and there was a significant difference ($P < 0.05$) between the infected and non-infected cows.

Mean number of inseminations required for conception in cows having no SCE were less *i.e.* 1.58±0.15. In cows diagnosed with SCE, mean number of inseminations per conception were 1.67±0.21, although, there was no significant difference (P>0.05) between infected and non-infected cows. In cows having SCE, mean time required for calving

to conception interval was comparatively shorter (121.67±10.86 days postpartum).

However, mean time required for calving to conception interval, in cows having no SCE, was 101.30±5.86 days postpartum and there was no significant difference (P>0.05) between infected and non-infected cows.

Table.1 Bacteria isolated from uterine discharge during weekly sampling (1, 8, 15, 22, 29 days after parturition)

Bacterial type	Bacterial identification	Number (n)	%
Gram negative	<i>Escherichia coli</i>	10	32.26
Gram positive	<i>Bacillus cereus</i>	7	22.58
	<i>Staphylococcus aureus</i>	5	16.13
	<i>Streptococci spp.</i>	1	3.23
Mixed bacteria	<i>Bacillus cereus + Staphylococcus aureus</i>	3	9.68
	<i>Escherichia coli + Staphylococcus aureus</i>	2	6.46
	<i>Bacillus cereus + Escherichia coli</i>	1	3.23
	<i>Escherichia coli + Proteus vulgaris</i>	1	3.23
	<i>Salmonella paratyphimurium + Edwardsiella tarda</i>	1	3.23
Total		31	100

Table.2 Antibiogram of Gram negative aerobic bacteria isolated from uterine discharge after parturition

Antimicrobial agents	Degree of sensitivity (%)		
	HS	MS	R
Enrofloxacin	72.73	20.45	6.82
Ciprofloxacin	70.45	22.73	6.82
Ofloxacin	56.82	38.64	4.54
Gentamicin	25	63.64	11.36
Ceftriaxone	50	36.36	13.64
Oxytetracycline	3.57	50	46.43
Streptomycin	10.71	46.43	42.86
Cephalexin	21.42	39.29	39.29
Cloxacillin	3.57	17.86	78.57
Ampicillin	7.14	32.14	60.72
Penicillin	0	34.09	65.91
Levofloxacin	75	21.43	3.57
Amoxicillin	7.14	35.72	57.14

Table.3 Antibiogram of Gram positive aerobic bacteria isolated from uterine discharge after parturition

Antimicrobial agents	Degree of sensitivity (%)		
	HS	MS	R
Enrofloxacin	84.44	11.39	4.17
Ciprofloxacin	53.33	40.55	6.12
Ofloxacin	50.83	45	4.17
Gentamicin	15	80.83	4.17
Ceftriaxone	31.94	53.33	14.73
Oxytetracycline	39.72	33.06	27.22
Streptomycin	1.67	75.28	23.06
Cephalexin	11.55	61.62	26.83
Cloxacillin	2.78	15.71	81.51
Ampicillin	7.68	24.42	67.90
Penicillin	1.39	11.94	86.67
Levofloxacin	94.08	1.75	4.17
Amoxicillin	2.78	28.67	68.33

Table.4 Relationship between endometrial cytology and postpartum reproductive performance (mean ± S.E.)

Subclinical Endometritis (on day 43 postpartum)				
Number of cows	PMNs (Neutrophils)	Days to first AI	No. of AI per conception	Calving to conception interval
n=6	22±4.77 ^a	107.67±7.94 ^x	1.67±0.21	121.67±10.86
No subclinical endometritis				
n=12	2.50±0.88 ^b	89.58±4.44 ^y	1.58±0.15	101.30±5.86

^{a, b} Values with different superscripts within the same column are significantly different (P<0.01)

^{x, y} Values with different superscripts within the same column are significantly different (P<0.05)

Dairy cows are highly susceptible to uterine infections after parturition because of housing conditions and production demands. Diseases like SCE, clinical endometritis, metritis and pyometra are associated with subfertility and infertility and can result in longer intervals from calving to first conception or ultimately lead to involuntary culling of animals failing to conceive (Sheldon *et al.*, 2008). The present study revealed the bacteria isolated from uterine discharge among which *E. coli* (32.26%) and *B. cereus* (22.58%) were frequently found after parturition. Our study was supported by findings of Takamtha *et al.*, (2013) who isolated *E. coli* (24.07%),

Staphylococcus spp. (11%), *Bacillus* spp. (20.37%) and *Streptococcus* spp. (14.81%). Also, Virakul *et al.*, (1995) reported *Staphylococcus aureus* (28.2%), *Corynebacterium pyogenes* (23.1%) and *E. coli* (17.9%) in 30 days postpartum dairy cows while Ngarmkum *et al.*, (1993) found *Staphylococcus aureus* (32.14%), *Acinetobacter anitratus* (25.0%), *E. coli* (21.43%) and *Corynebacterium pyogenes* (14.29%).

In our study, Fluoroquinolone group (Levofloxacin, Enrofloxacin and Ciprofloxacin) of antibacterial agents were

found highly sensitive to Gram negative and Gram positive bacteria. Culture sensitivity trends were same in case of mixed bacteria. These results were in agreement with some other observations where percent sensitivity to enrofloxacin and ciprofloxacin has been reported as 92 and 96%, respectively (Patel *et al.*, 2009; Mshelia *et al.*, 2014). β -lactam antibiotics were mostly resistant against all bacterial isolates. Also, Oxytetracycline was found resistant against Gram negative, Gram positive and mixed group of bacteria (46.43, 27.22 and 64.42%, respectively). In our findings, Cloxacillin was found less sensitive (18.49%) against Gram positive bacteria. Sadig *et al.*, (2010) also reported that Gram positive bacteria were resistant to cloxacillin. In contrary, high sensitivity to penicillin and ampicillin (90 and 100%, respectively) was reported which was against our study where sensitivity to penicillin and ampicillin was low (13.33 and 32.10%, respectively). Brooks *et al.*, (2001) also found that *Staphylococcus aureus* was resistant to Penicillin which agrees with our findings. Ampicillin and Gentamicin were among the highly sensitive antimicrobial agents against Gram negative bacteria (Sadig *et al.*, 2010). In a previous investigation, gentamicin and cephalexin were highly sensitive (93 and 77%, respectively) to gram aerobic bacteria, while oxytetracycline (42%), amoxicillin (26%) and streptomycin (25%) were resistant (Takamtha *et al.*, 2013).

In our study, Levofloxacin was the most sensitive and penicillin was the most resistant for both gram-positive and gram-negative pathogens. The treatment of bacterial infections is increasingly complicated by the ability of bacteria to develop resistance to antimicrobial agents. Generally, indiscriminate use of antibiotics leads to development of resistant strains due to bacterial mutation (Takamtha *et al.*, 2013). Chandrakar *et al.*, (2002) has reported the resistance to penicillin by Gram positive

bacteria. As reported in a previous investigation, aminoglycosides are mostly effective in aerobic environment of the postpartum uterus. Presence of necrotic debris and purulent materials has a negative impact on the efficacy of sulfonamides and aminoglycosides (Smith and Risco, 2002). Performance of β -lactam antibiotics and cephalosporins declines due to presence of organisms producing inactivating (β -lactamase) enzymes in postpartum uterus (Deori and Phookan, 2015). Emphasized on antimicrobial resistance, oxytetracycline was routinely used for uterine infection, especially after calving, via intrauterine infusion or intramuscular administration. This may be due to bacterial resistance to this antimicrobial agent and its requirement in high concentration to inhibit bacterial growth when tested in vitro (Risco *et al.*, 2007).

Endometrial cytology has been used as a diagnostic technique for identification of SCE (Sheldon and Dobson, 2004; Kasimanickam *et al.*, 2004). In our study, SCE was diagnosed in 6 dairy cows based on the presence of >10% PMNs (neutrophils) on day 43 postpartum. Kasimanickam *et al.*, (2004) reported subclinical endometritis upon the presence of >10% PMNs between day 34-47 postpartum in dairy cows.

Neutrophils form the first line of an increase in postpartum defense against the invading pathogenic organisms resulting in large neutrophil populations within the uterine lumen. Phagocytic activity of neutrophils helps in halting the propagation and establishment of bacterial infection in the postpartum uterus (Butt *et al.*, 1993). Due to intrauterine bacterial contamination and repair of the endometrium following parturition, uterine inflammation is a normal and necessary component of the postpartum uterine involution process (Ahmadi *et al.*, 2006). However, in some of the postpartum

cows, the inflammation exceeds the normal threshold and leads to SCE (Le Blanc, 2014).

Postpartum reproductive performance in our study indicated that cows having subclinical endometritis (SCE) have higher days to first AI, number of inseminations per conception and calving to conception interval as compared to cows not having SCE. There was a significant difference between infected and non-infected cows in terms of PMNs percentage and days to first AI ($P < 0.01$ and $P < 0.05$, respectively). Although cows with SCE do not have turbid uterine discharge but impaired reproductive performance has been associated with it (Chapwanya *et al.*, 2010; Kasimanickam *et al.*, 2005). However, SCE has a negative effect on reproductive performance because this increases the number of inseminations per conception, days to first artificial insemination and the calving to conception interval, thereby reducing the conception rate (Le Blanc 2008). SCE has been associated with an increase in days open and lower conception rate in cows (Sheldon and Dobson, 2004). This may be due to suboptimal conditions for sperm transportation and storage, oocyte maturation and ovulation, zygote development, implantation and embryonic and fetal growth (Gilbert, 2011). SCE is a highly prevalent disease having no clinical symptoms but significantly impairing the fertility of dairy cows (Sheldon *et al.*, 2006).

In conclusion, approximately 50% of dairy cattle develop uterine infections after parturition. In our study, the most efficient therapeutic approaches of the postpartum uterine infections were exposed. Uterine infections can be limited by systemic administration of Fluoroquinolone group of antibiotics during early postpartum phase. This information is useful for therapeutic treatment planning and antibiotic control usage. Subclinical endometritis (SCE) can

occur in postpartum cows which are presumed to be normal and results in poor reproductive performance. Thus, by using endometrial cytology, productivity losses can be minimized by timely diagnosis of SCE.

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