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BACTERIAL FLORA ASSOCIATED WITH REPEAT BREEDING AND UTERINE INFECTIONS IN DAIRY COWS

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ABSTRACT

The study was conducted on 51 uterine biopsy samples collected from 14 normal fertile and 37 repeat breeding cows for bacteriological examinations to find out the prevalence of bacterial flora and their role in repeat breeding syndrome. Presence of bacteria was detected in 23 samples (62.2%) repeat breeding cases in contrast to only 4 (28.6%) bacterial infections from normal fertile cows. About 60% of the microbial isolates were commonly recovered from repeat breeders as well as from normal fertile cows in varying proportions. Of the 37 suffered from repeat breeders, 23 samples (62.2%) yielded 54 bacterial isolates; where *Staphylococcus* was predominant 14 (37.8%), followed by *Bacillus* 13 (35.1%), *E. coli* 11 (29.7%), *Pseudomonas* 7 (18.9%) while Gram negative minute rod shaped bacteria was 9 (24.3%). The isolates of *Pseudomonas* and Gram negative minute rod shaped bacteria were obtained only from repeat breeder cows with mucopurulent uterine discharges. There was a positive correlation (r=0.94) between repeat breeders and bacterial infection of uterus. Antibiotic sensitivity in the present study showed that almost all types of bacterial isolates were found moderately and highly sensitive to amoxicillin, oxytetracycline and ciprofloxacin.

Key words: Bacteria, repeat breeding, uterine biopsy, histopathology and antibiotic sensitivity

INTRODUCTION

One of the major constraints of profitable dairy farming is low conception rate (Alam and Ghosh, 1994; Shamsuddin *et al.*, 2001). Economy of dairy farming largely depends on pregnancy rate after insemination. The twelve-month calving interval is advantageous for maximal milk yield per cow per year with good economic return (Opsomer *et al.,* 1996). It is accepted that bovine genital infections, either specific or non-specific in nature, account for large number of pregnancy failure in cows (Sirohi *et al.,* 1989). Generally, non-specific infection of the genitalia is considered to be the main cause of repeated conception failure (Sharma *et al.,* 1988; Singia *et al.,* 1993; Singh *et al.,* 1996) where there is an increase in the number of microorganisms and/or in their virulence. Bacterial infection is the most important among the various causes of the subfertility (Dholakia *et al*., 1987). Such a condition may cause cervicitis or endometritis of various degrees, which in turn may lead to embryonic death and repeat breeding problems (Elliott *et al.,* 1968). These infections affect fertility by altering the uterine environment resulting in impairment of sperm transport, sperm death and hostile environment to the subsequent development and maintenance of the conceptus leading to their death. Early embryonic death (< 42 days) is a major factor in reproduction failure, which in turn causes economic loss to the dairy industries (Rahman *et al.,* 1996). The indiscriminate use of broad spectrum antibiotics and corticosteroids for the treatment of reproductive disorders or the insemination of animals with contaminated semen may led to microbial infections of the uterine environment (Raghavan *et al.,* 1971; Garg *et al.,* 1982; Patgiri and Uppal, 1983). Considering these, the study was designed to assess the presence of bacteria in the uterine environment of fertile and subfertility cows with the aims to isolate and identify bacteria those are related with repeat breeding and finally investigate the antibiotic sensitivity to suggest the treatment for the control of such type of problem.

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MATERIALS AND METHODS

The study was conducted in the Department of Microbiology and Hygiene, Faculty of Veterinary Science, BAU, Mymensingh during the period from July 2006 to May 2007.

Selection of cows

A total of 51 cows of which 37 were repeat breeders and 14 normal fertile cows were selected from Dairy Department of Kazi and Kazi Tea Estate Ltd., Rawshonpur, Tetulia, Panchagarh. The cows were the Holstein-Friesian cross (n = 31), Sahiwal cross (n = 12) and Local (n = 8). Detail gynacological examination was carried out as described by Ball (1986), Settergren (1986). Cows reported to be normal cyclic and remained subfertile after three inseminations were classified as repeat breeders (Singh *et al.,* 1989; Singh and Pant, 1999). Pregnant cows and cows manifesting functional anoestrus or cystic ovaries upon rectal palpation were not included. No insemination was done at least seven to eight days prior to sampling. Vaginal examination with a speculum or any other manipulation of the reproductive tract via the vagina was avoided at sampling.

Collection and Culture of bacteriological specimens

Uterine biopsy samples were collected with sterilized metalic biopsy catheter designed by Dr. D. E. Noakes, Royal Veterinary College, London when animals were in oestrus. The perineal area was washed with soap and water. The sterilized biopsy catheter was inserted into the vagina and then passed though the cervix up to the body/horn of the uterus by holding the cervix through the rectum. The window of the biopsy catheter with sharpen edge was opened and with the help of the hand in the rectum, the medial uterine wall was pressed into the window. The tri-circular handle was smartly rotated to close the window inside the uterus in such away that pieces of endometrium (5 mm) along with tissue secretion was clipped off. Both aerobic (Nutrient broth; Difco Laboratories, Dertoit, Michigan) and anaerobic transport media (Liver extract broth) were used for dispatching the samples to the Microbiology Laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh for primary isolation. The anaerobic condition to these transport media was achieved by pouring 1.0 cm layer of autoclaved liquid paraffin over liver extract broths in the test tubes; the test tubes were heated 2-3 minutes to bubble up the residual oxygen before adding the paraffin. Specimens for bacterial cultures were transferred from the biopsied tissues into the transport media by aseptic swabbing.

Each sample of uterine biospsy from transport media was divided and inoculated separately in Nutrient agar (NA) and Blood agar (BA) media both aerobically and anaerobically at 37^{o}C to promote growth of bacteria. The colonies grown on primary cultures were repeatedly subcultured by streak-plate method on NA, BA, Eosin methylene blue (EMB) agar, MacConkey agar (MA) and Chocolate agar (CA) media according to the method described by Cheesbrough (1985). Specification of organisms was done based on the schemes presented in Burgey's Manual of Determinative Bacteriology and by standard bacteriological procedures described by Hillers and Moncla (1991). Identification of aerobic and facultative anaerobic bacteria was done based upon colonial morphology and pigment characteristics; the genus of bacteria was identified based upon reaction to Gram staining, cellular arrangement and reaction to bio-chemical test described by Farin (1989).

Processing of the tissues for histopathology

The tissues were processed following the standard technique for histology as per the method of Luna (1968).

In-vitro antibiotic sensitivity test

Different pure colonies at primary isolation were picked up aseptically with inoculating loop and diluted into the sterilized nutrient broth. These individual diluted samples were thereafter thickly poured on large sized NA, BA and EMB agar plates and allowed to spread gently over the entire surface with a glass spreader. After one-two minutes eight (8) different culture sensitivity antibiotic discs (Penicillin-G 10 µg, Amoxicillin 25 µg, Gentamicin 120 µg, Spiramycin 100 µg, Oxytetracycline 30 µg, Streptomycin 10 µg, Ciprofloxacin 5 µg, Sulphamethoxazole 25 µg) were placed circularly at each agar plates with the help of sterile forceps. The results were interpreted using zone size following 24 hours of incubation at 37^{o}C at aerobic condition. The following criteria were set to record the level of sensitivity according to the NCCLS (1984).

> Growth inhibition zone size <10 mm in diameter = Resistant (-), growth inhibition zone size >10 mm in diameter = Moderately sensitive (+) and growth inhibition zone size ≥15 mm in diameter = Highly sensitive (++).

Statistical analysis

To determine relationship between repeat breeding and uterine infections the correlation method were used using MS Excel 2000.

RESULTS AND DISCUSSION

Isolation and identification of bacteria

The types and frequency of bacteria isolated from the biopsied endometrial tissues are listed in Table 1. Five specific and non-specific genus of aerobic, facultatively anaerobic and strictly anaerobic bacteria were isolated from 14 normal fertile and 37 repeat breeding cows. About 60% of the bacterial isolates (3 of the 5 types) were identified and recorded in the present study which were commonly found in repeat breeders as well as from normal fertile animals and the frequency of the isolates were much higher than that of normal. These isolates belonged to the genera *Staphylococcus* spp*, Bacillus* spp and *Escherichia coli*, the isolates of *Pseudomonas* spp and Gram negative minute rod shaped bacteria in the repeat breeder cows were exceptions.

Table 1. Frequency and percentage distribution of bacterial isolates

Bacterial isolates		Frequency percentage of occurrence			
Family/ Group	Isolates	Normal fertile group		Repeat breeder group	
		Total no. of isolates	% of distribution	Total no. of isolates	% of distribution
Gram + ve cocci	*Staphylococcus* spp.	3	21.4	14	37.8
Gram+ ve rods	*Bacillus* spp.	3	21.4	13	35.1
Gram-ve rods	*Escheriachia coli*	1	7.1	11	29.7
	Pseudomonus spp.	0	0	7	18.9
Gram negative minute rod shaped bacteria		0	0	9	24.3

Staphylococcus spp. produced round, smooth, shiny, opaque, golden yellow colonies on NA (Fig. 1) that was spherical shaped and cluster formed Gram positive in Gram's staining (Fig. 2), that fermented five basic sugars producing acid with gas, and reacted as catalase (+ve) and coagulase (-ve). Bacillus spp produced Large, rough colonies on NA (Fig. 3) and in Gram's staining it was revealed as Gram positive, rod shaped and chain forming (Fig. 4) that fermented five basic sugars producing acid without gas and reacted as indole (-ve), MR (-ve) and VP (+ve). Escherichia coli produced metallic sheen in EMB agar (Fig. 5) and it was Gram negative, rod shaped, singly arranged in Gram's staining (Fig. 6), that fermented five basic sugars producing acid with gas (Fig. 7), and reacted as indole (+ve) (Fig. 8), Methyl Red (MR) (+ve), catalase (+ve), and Voges-Proskaure (VP) (-ve). *Pseudomonas* spp produced Large, rough colonies on NA and β hemolysis in BA. In Gram's staining it was revealed as Gram negative, rod shaped that fermented glucose and reacted as indole (-ve), MR (-ve) and VP (-ve). A striking shifting tendency of these pathogenic bacteria was evidenced when the gynaecological score changed from best (clear stringy mucus) towards worst (mucopurulent secretions). None of the pathogens (*Pseudomonas* and gram negative minute rod shaped bacteria) was isolated from animals having clear stringy mucus discharge. The frequency and percentage distribution of various isolates in relation to total number of cases of normal fertile and repeat breeders cow are presented in Table 1. Positive correlation (r = 0.94) was found between repeat breeders and percentage of bacterial infections in uterus. Out of the 37 repeat breeders, 62.2% yielded 54 bacterial isolates of which *Staphylococus* was predominant 37.8% followed by *Bacillus* 35.1% *Escherichia coli* 29.7% Gram negative minute rod shaped bacteria 24.3% and *Pseudomonas* 18.9%. The isolates of *Pseudornonas* and Gram negative minute rod shaped bacteria were obtained only from repeat breeder cows with mucopurulent vaginal discharge.

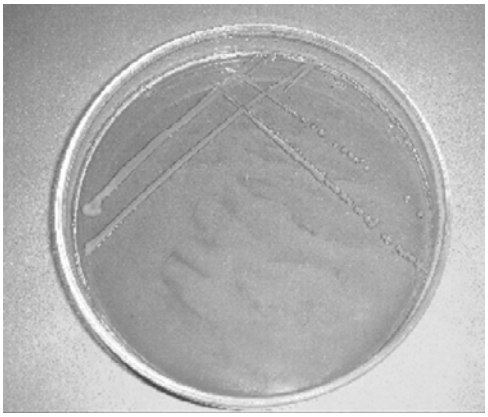

Fig. 1. Round, smooth, shiny, opaque, golden yellow colonies of *Staphylococcus* spp. (Nutrient agar; Aerobic culture; 24 hrs).

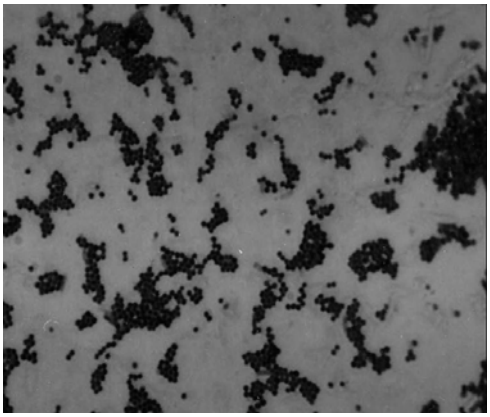

Fig. 2. *Staphylococcus* spp. Gram positive spherical cells arranged in grape like cluster (X 825; Gram's stain).

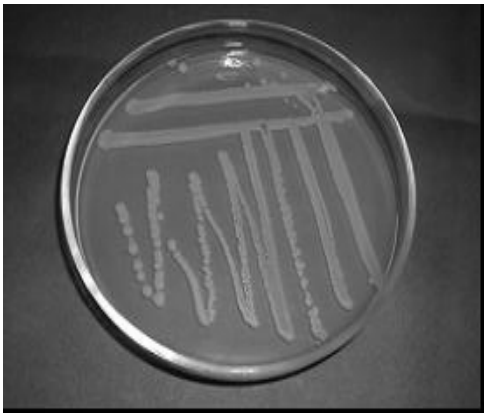

Fig. 3. Large, rough colonies of *Bacillus* spp. with whip like outgrowth on Nutrient agar (Aerobic culture; 24 hrs).

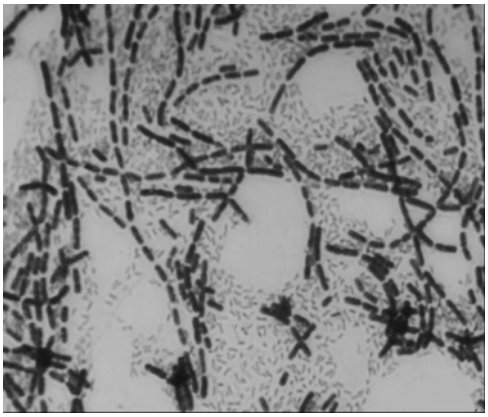

Fig. 4. *Bacillus* spp., Gram positive, rod shaped and chain forming (X 825; Gram's stain).

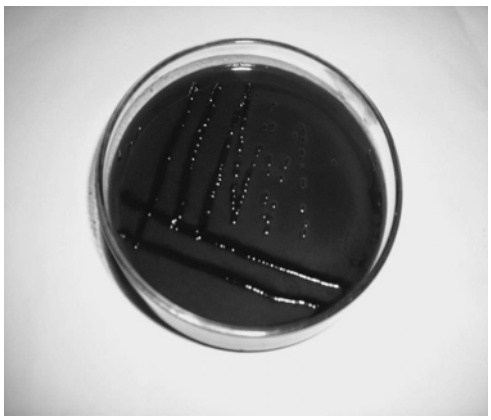

Fig. 5. Colonies of *Escherichia coli* producing metallic sheen in EMB agar (Aerobic culture; 24 hrs).

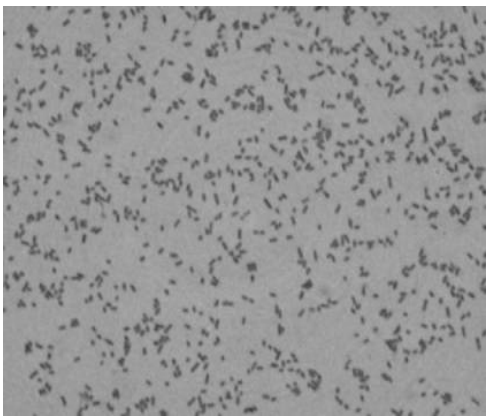

Fig. 6. Gram negative, rod shaped, singly arranged colonies of *Escherichia coli* (X 825; Grams stain).

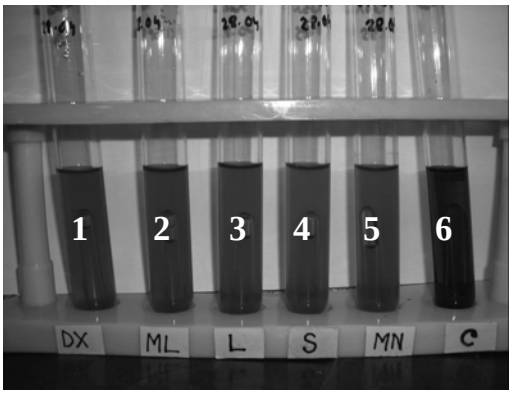

Fig. 7. Sugar fermentation test of *Escherichia coli*, producing acid and gas by fermenting 5 basic sugar (Positive, tube no. 1-5; Negative control, tube no. 6).

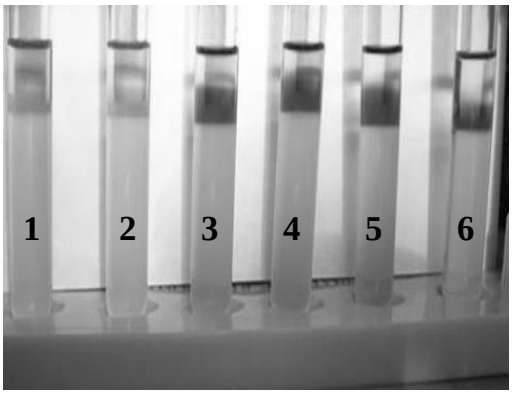

Fig. 8. *Escherichia coli* showing positive indole test indicating by red ring in the reagent layer (Positive, tube no. 3-6; Negative control, tube no. 1-2).

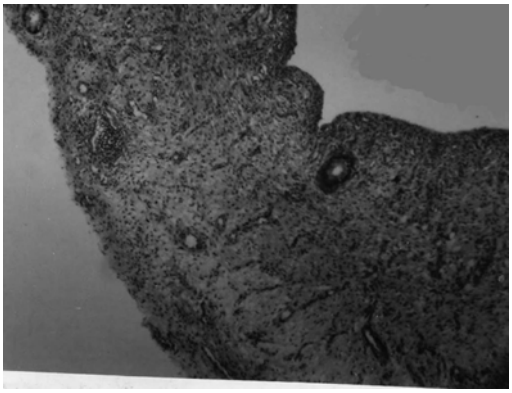

Fig. 9. Biopsied section of normal endometrium of cow (H and E stain X 82.5).

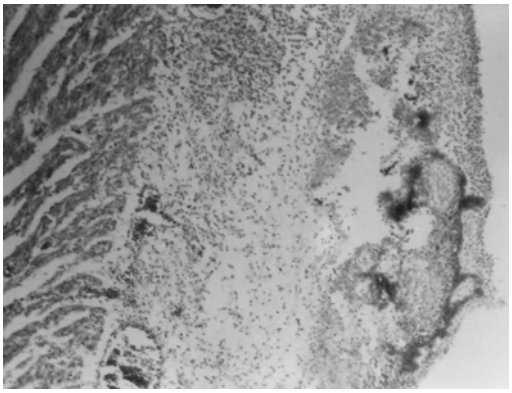

Fig. 10. Biopsied section of endometrium having moderate endometritis (H and E stain X 82.5).

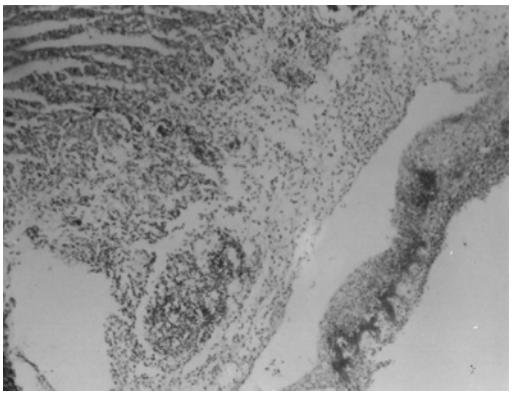

Fig. 11. Biopsied section of endometrium having chronic endometritis (H and E stain X 82.5).

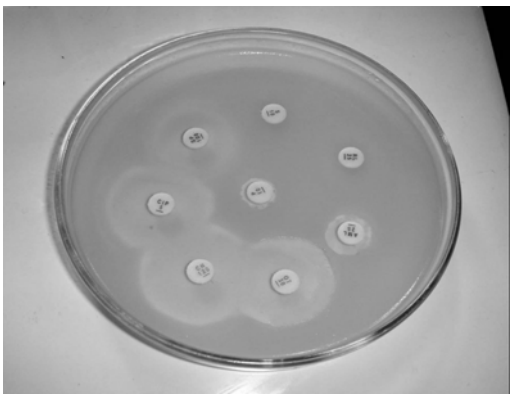

Fig. 12. Different types antibiotic sensitivity to *Staphylococcus* spp.

Generally, non-specific uterine infection was considered to be the main cause of repeated conception failure (Sharma *et al.,* 1988; Singly *et al.,* 1993; Singh *et al.,* 1996) where increased number of microorganisms and/or in their virulence causing cervicitis or endometritis of various degrees that may lead to embryonic death and repeat breeding problems (Elliott *et al.,* 1968).

Table 2. Total number of normal fertile cows and repeat breeders from which bacteria were isolated

Experimental cows	No. of cows examined	No. positive	% positive	No. negative	% negative	Total no. of isolates
Normal fertile cows	14	4	28.6	10	71.7	7
Repeat breeders	37	23	62.2	14	37.9	54

It was observed that, 62.2% repeat breeders examined were found to be positive for bacterial isolation (Table 2). This finding is in close agreement with several other reports from the subfertile cows (Singh *et al.,* 1998). However, a higher percentage 76.9%, 81.9% and 87.2% of such bacterial infections of the repeat breeder cows were reported by Dholakia *et al.* (1987), Murthy *et al.* (1974) and Rahman *et al.* (1984), respectively. It may be mentioned that bacterial organisms have been isolated from 9-14% (Eduvie *et al.,* 1985; Noakes *et al.,* 1991) to 94-100% (Panangala *et al.,* 1978; Malik *et al.,* 1987; Singh *et al.,* 1989; Sirohi *et al.,* 1989) of repeat breeders.

The predominant bacteria isolated from repeat breeder as well as normal animals were *Staphylococcus* spp., *Bacillus* spp. and *Escherichia coli* (Table 1). Similar reports of isolation of bacteria from the genital tract of subfertile cows were recorded by Murthy *et al.* (1974), Sirohi *et al.* (1989) and Singh *et al.* (1996). As regards bacterial isolation, Rahman *et al.* (1984), Dholakia *et al.* (1987), Farin *et al.* (1989), Singia *et al.* (1993) and Hariharan *et al.* (1994).

Bacteria were more frequently isolated from the mucopurulent vaginal discharge of repeat breeding cows (70%) compared to normal cyclic discharge (40.4%). Similar observation was made by Singh *et al.* (1998) who obtained bacteria from 76.9% repeat breeders with clinical abnormality of vaginal discharge and 45.8% repeat breeder cows having no clinical signs. Analysis of results of the present study consistent with the findings of Murthy *et al.* (1974) and Rahman (1996), indicated that the bacterial isolations were more frequent in cows, which repeated services of 5 times or more than in the groups returning only for 3 or 4 times, but no statistical analysis was done to comparative this study.

During the course of this study, the isolates of *Pseudomonas* and Gram negative minute rod shaped bacteria were recovered from repeat breeder cows with mucopurulent genital discharge that were in agreement with others (Fredriksson *et al.,* 1985; Farin *et al.,* 1989; Hariharan *et al.,* 1994; Rahman, 1996). The bacterial isolates recovered from the uterus of the repeat breeders as well as from normal cyclic cows have a positive correlation (r = 0.94), which agreed with the relationship between uterine infection and repeat breeders.

Uterine histopathology

Cows with genital discharge stringy transparent oestrus mucus did not show any pathological changes in the endometrium (Fig. 9). On the other hand, cloudy-condensed purulent discharges were associated with moderate to chronic endometritis (Fig. 10 & 11).

***In-vitro* antibiotic sensitivity test**

It was found that out of 4 aerobic bacteria *Bacillus* spp. and *Escherichia coli* are more or less effective against 7 antibiotic agents (Fig. 12). On the other hand, amoxicillin, oxytetracycline and ciprofloxacin are moderate to highly sensitive to the all of isolates. In contrast, penicillin and sulphamethoxazole are only sensitive to the Gram positive and Gram negative bacteria, respectively (Table 3). Similar, observations were recorded earlier by Rahman *et al.* (2002b). Antibiotic sensitivity test was performed only in aerobic condition. The sensitivity response to different antibiotics in aerobic environment studied, though the bacteria present in the uterus were of anaerobic environment.

It's sensitivity response need to be studied in anaerobic condition for assessing the exact response to specific antibiotics. It is suggested that penicillin and oxytetracyclin are the choice of drugs that can be used in treating uterine infections as because these antibiotics are effective in anaerobic environment of the uterus in presence of organic debris (Laing *et al.,* 1988).

Table 3. Antibiotic sensitivity pattern of isolated bacteria

Name of isolates	Name of the antibiotic diseases							
	P	AML	GN	SP	OT	S	CIP	RL
Staphylococcus spp.	+	+	+	++	++	-	++	-
Bacillus spp.	++	++	++	++	++	+	++	-
Escherichia coli	-	++	+	+	++	++	++	++
Pseudomonas spp.	-	++	+	+	++	++	++	++

- = Resistant, + = Moderately sensitive, ++ = Highly sensitive, P = Penicillin-G, OT = Oxytetracycline, AML = Amoxicillin, S = Streptomycin, GN = Gentamicin, CIP = Ciprofloxacin, SP= Spiramycin, RL = Sulphamethoxazole.

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