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Research Article

Prevalence of different bacterial species in the uterine fluid of repeat breeder cows

Md. Farhad Hossain Chowdhury, Mir Md. Iqbal Hasan[1], Moinul Hasan, Md. Siddiqur Rahman[2], Marzia Rahman[3] and Nasrin Sultana Juyena[*]

Department of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh, Bangladesh

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ABSTRACT

Among the diverse factors, bacterial infections in the reproductive tract potentially cause of repeat breeding in dairy cows. The present research was conducted to appraise the uterine organism in healthy fertile cows (HFC) and repeat breeder cows (RBC) and to establish a relationship with previous disease or disease conditions. A total of 43 uterine samples were collected from 10 healthy fertile and 33 RB cows for bacteriological study. Among RBC, 39.4% harbored bacterial isolates of *E. coli* (30.3%), *Staphylococcus aureus* (27.3%), *Staphylococcus epidermidis* (15.2%), and *Bacillus spp.* (18.2%). Total Viable Count (TVC) of RBC with isolates was higher (1.81 x 10^{10}CFU/mL) than in normal fertile cows (1.3x10^{10} CFU/mL). No bacterial isolates were recovered from 60.6% RBCs and 80.0% healthy fertile cows. A single, mixed infection of two, three and four bacterial species was observed in the uterine discharge of 12.1%, 9.1%, 12.1% and 6.1% RBC, respectively. From animal demographic data of RBC, the sub-clinical mastitis, acute mastitis, endometritis, dystocia, retention of placenta, and abortion were 42.4%, 39.4%, 39.4%, 12.1%, 9.1%, and 6.1%, respectively. In contrast, the presence of dystocia, endometritis, subclinical mastitis, retention of placenta and abortion was recorded in 50%, 30%, 30.8%, 23.1%, and 15.4% of healthy cows, respectively. This result could help the veterinarian to design the proper antibacterial therapy based on bacteria isolates in RBCs.

Introduction

Lactating cows with good reproductive performance is necessary for a profitable dairy farm (Ahuja et al., 2017). A good economic return depends on a one-year calving interval and maximum milk production by a cow per year (Gani et al., 2008). Among various reproductive diseases, repeat breeding syndrome reduces the conception rate and causes huge financial losses for Bangladeshi dairy farmers (Talukder et al., 2005; (Khair et al., 2013). Repeat breeders are defined as cows that have calved at least once, are less than 10 years of age, accounted to be healthy cyclic and continued as infertile even after three times of breeding and had no sign of any infection of reproductive diseases and no anomalous releases by reproductive tract (Yousuf et al., 2010; Regmi and Dhakal, 2020). Causes of the repeat breeding syndrome (RBS) are generally uncertain but possibly comprise dairy farm environment, management, estrus detection, breeding methods, and animal factors (Katagiri and Takahashi, 2004). The RBS is often associated with subclinical endometritis, late ovulation and ovarian dysfunction, and others which are the root cause of fertilization failure or early embryonic mortality (Parkinson, 2009). However, it is noticed that infections with diverse pathogens in the reproductive tract cause conception failure in cows (Bardos et al., 2020). Breeding heifers or cows with contaminated semen increases the uterus tract's

[*]Corresponding author: <nsjuyena@bau.edu.bd>

[1]*Department of Physiology, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet, Bangladesh*

[2]*Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh, Bangladesh*

[3]*Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh, Bangladesh*

microbial load (Cojkic et al., 2021). Moreover, bacterial infections play a significant role in infertility (Yoo, 2010). Therefore, the huge occurrence (20.48%) of RBS in cows in Bangladesh, the present research was conducted to assess the bacterial colonization in the uterine discharge of normal fertile and repeat breeder cows and study previous disease history associated with repeat breeder cows in dairy farms.

Materials and Methods

The study was conducted in the Department of Surgery and Obstetrics, Bangladesh Agricultural University, Bangladesh.

Study design

The research was divided into two parts. The first part included an investigation to categorize the repeat breeder cows with clinical abnormality. In the second part, an effort was made to estimate the prevalence of bacteria in the uterine discharge of repeat breeder cows and the HFC.

Collection of animal demographic data

A well-defined pre-structured questionnaire was prepared to record the animal data like age, breed, parity, date of last calving and their types, number of previous services, nutritional status (Body condition score), and nature of the genital discharge. Most of the data in this research was obtained through face-to-face interviews with the farm owners of the animal.

Selection of animal

Out of 172 Frisian, Jersey, and Sahiwal crossbreed cows, 33 repeat breeding cows and 10 randomly selected healthy fertile cows were selected from Khaja Dairy Farm, Chorlokkha, Karnaphuli, Chattogram in the present study during the estrous cycle. These animals were reared under an intensive farming system and fed by farmer's practice. Recorded cows were 30 to 110 months old with 1 to 6 parties. As regard breed quality, the animals belonged to cross-bred (n=43). We excluded the pregnant cow and the cows with anestrus or cystic ovaries detected by rectal palpation. Insemination before seven to eight days of sampling time and vaginal examination were avoided at the time of sampling.

Bacteriological media and broth

Bacteriological agar media used for the bacteriological study were Blood Agar (5% sheep blood, BA, Difco), Nutrient Agar (NA; Oxoid), Manitol Salt Agar (MSA), MacConkey Agar (MA; Merk), Peptone Agar (PA; HiMedia, USA), and Plate Count Agar (PCA). The liquid media (Broth), such as Nutrient Broth (NB; BBL™ Nutrient Broth, USA) was used for the bacteriological analysis.

Collection of specimens and culture of the bacteriological sample

The uterine discharge was collected at the time of estrus directly from the uterus of cows by a clean and disinfected intrauterine catheter (a 10 ml syringe and an AI gun) to perform bacteriological examination following the method described by (Singh et al., 1996). Aerobic (nutrient broth) transport media was used for the survival of bacteria during transportation. The strict aseptic precaution was maintained during the collection of uterine fluid. Initially, the NB was used for the collection and transportation of samples.

Examination of bacterial cultures

The cultural examination of the uterus fluid for bacteriological analysis was completed using standard bacteriological methods described by Hasan (2021) to find out the different species of bacteria.

The NB-containing samples were cultured again the NB overnight to enrich of bacteria. Then samples were divided and inoculated individually in Nutrient agar (NA), Blood agar (BA), Mannitol Salt Agar (MSA), and MacConkey Agar (MA) to promote bacterial growth. The pure culture with homogenous colonies was obtained in this study, as described by Cheesbrough (1985). The isolation and identification of bacteria were made from the examined bacterial cultures. Characterization into respective genera and species was performed on account of colony morphologic characters and reactions in the biochemical tests, including sugar fermentation test

for acid and/or gas formation, catalase test and coagulase test, described by Hasan (2021).

Sugar fermentation test

The sugar (carbohydrate) fermentation test in purple broth was performed by inoculating a loop-full of overnight NB culture of the organisms into 5 tubes containing 5 basic sugars (dextrose, sucrose, lactose, maltose, mannitol) separately. Then the mixtures containing tubes were incubated aerobically at 37°C for 24 hours. Acid production by *E. coli* was indicated by the color change from reddish to yellow in the medium, and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tube.

Catalase test

The catalase test was performed to differentiate the bacteria that produce the enzyme catalase (*Staphylococci spp.*) from the non-catalase-producing bacteria (*Streptococci spp.*). The slide catalase test was done by pouring 2-3 drops of 3% H_2O_2 solution on an over-bacterial culture. A positive reaction was indicated by a rapid effervescence.

Coagulase test

A simple slide coagulase test was performed as a presumptive test. In this case, 1-2 drop of diluted rabbit plasma was mixed with an equal volume of a particular organism's emulsified colony (in a drop of water) on a microscopic slide. A positive result was indicated by macroscopical clumping of the bacterial cells within 5 seconds due to coagulase production that converts fibrinogen to fibrin-like fibrin thread. Pathogenic *Staphylococci aureus* showed a positive reaction but non-pathogenic *Staphylococci epidermidis* showed a negative reaction.

Determination of total viable count (TVC)

Determination of TVC of bacteria was performed in this study from the collected fresh uterine fluid samples using PCA as described by ISO (1995) and Hasan (2021). The result of TVC was expressed as the number of CFU per ml of samples.

Statistical analysis

Both descriptive and statistical analyses were performed. Chi-square Test was done to determine a significant variation in the prevalence of bacterial isolates among the experimental groups. All the statistical analysis in the experiment was done by the use of computerized SPSS software version 20.

Results

Animal demographic findings

The result of the general investigation of the cows is presented in Table 1. Out of 172 crossbred cows, 33 repeat breeder cows and 10 randomly selected normal fertile cows were included in this study. Among 43 cross-bred animals (33 RBC and 10 HFC) about 46.5% were Holstein-Friesian crossbred, 23.3% were Jersey crossbred and 30.2% were Sahiwal crossbred cows. The maximum proportion of cows aged between 55 to 72 months (Table 1). About 32% of the animals had at least 3 parties; however, only 3 had as many as 7 parties. The breeding history of these cows is disclosed in Table 2.

Prevalence of different bacterial species in RBC uterine fluid

A total of 3 specific aerobic bacteria were isolated from 33 RBC and 10 HFC, which belong to 3 different genera such as *Staphylococcus* spp., *Escherichia coli,* and *Bacillus* spp. Among the isolated bacteria *E. coli* (Figure 1, Figure 2, Figure 3) was predominant in 30.3% of RBC, followed by *S. aureus* (Figure 4 and Figure 5) in 27.3%, *Bacillus* spp. (Figure 6 and Figure 7) in 18.2% and *S. epidermidis* (Figure 8 and Figure 9) in 15.2 % RBC. Figure 10 and Figure 11 present the identifying characteristic of *Staphylococcus* spp.

Table 1. Demographic data obtained in this study

Crossbred cows	Total population	Study group		Age group	Frequency (%)	Parity group	Frequency (%)
		RBC	HFC				
Holstein-Friesian	75	15	5	30-42 months	10	1	11
Jersey	48	8	2	43-54 months	8	2	9
				55-72 months	15	3	14
Sahiwal	49	10	3	73-90 months	5	4	6
				9-120 months	5	≥5	3

RBC= Repeat breeding cows; HFC= Healthy fertile cows

Table 2. Number of times the animals returned to estrus without completing the pregnancy

Parameter	Experimental animals							Total
	HFC	RBC						
Number of times repeated	0	3	4	5	6	7	8≥	43
sNumber of animals	10	7	10	6	5	3	2	

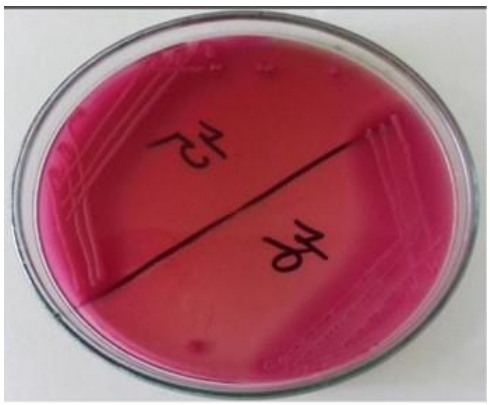

Fig. 1. Colonies of *Escherichia coli* in MacConkey agar (Aerobic culture; 48 hours)

The bacteriological study showed the presence of isolated bacteria in 39.4% of RBC. No isolates were present in 60.6% RBC and 80.0% HFC. Moreover, 12.1%, 9.1%, 12.1%, and 6.1% of RBC included a single species of bacteria and mixtures of two, three, and four species in uterine discharge, respectively. In comparison, 10% of HFC contained two isolates in uterine discharge.

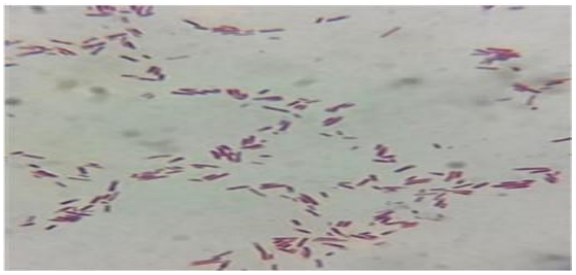

Fig. 2. Gram-negative bacilli of *Escherichia coli* (x1000; Gram's stain)

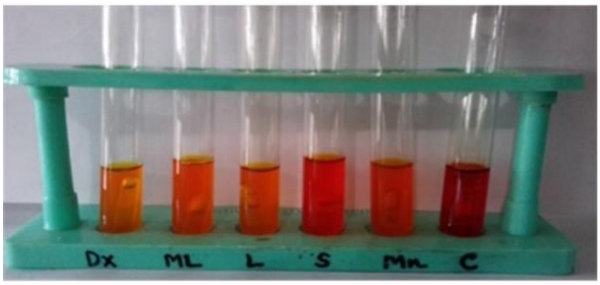

Fig. 3. Sugar fermentation test of *Escherichia coli*, producing acid and gas by fermenting 5 basic sugars (Positive, tube no. 1-5; Negative control, tube no. 6, from Left to the right)

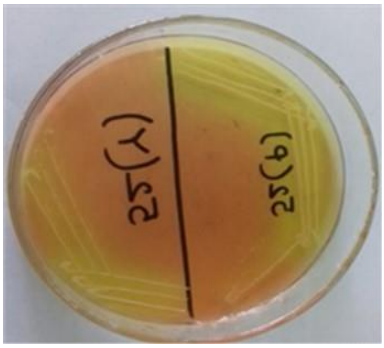

Fig. 4. Isolated colony agar plate-golden-yellow colonies of *Staphylococcus aureus* in Mannitol

Salt agar.

Fig. 5. Coagulase test showing positive reaction as an indication of *Staphylococcus aureus*

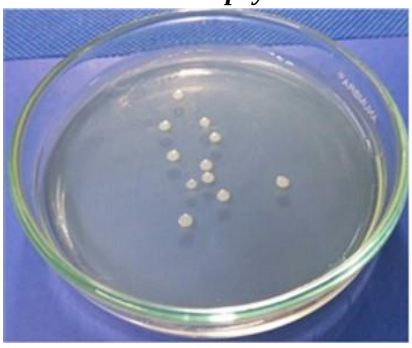

Fig. 6. Colonies of *Bacillus* spp., large, rough, flat, irregular with whip-like outgrowths on Nutrient agar (Aerobic cultures; 48 hours)

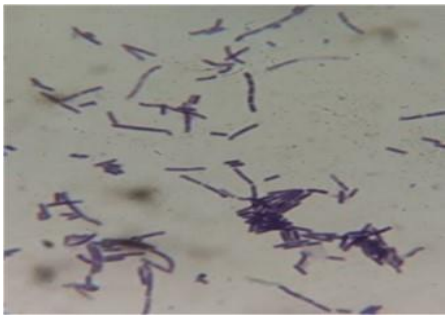

Fig. 7. *Bacillus* spp., gram-positive rods, sometimes in chains (×1000; Gram's stain).

Fig. 8. Round, smooth, shiny, opaque, pink colonies of *Staphylococcus epidermidis* in

Manitol salt agar.

Fig. 9. Coagulase test showing negative reaction as an indication of *Staphylococcus epidermidis*

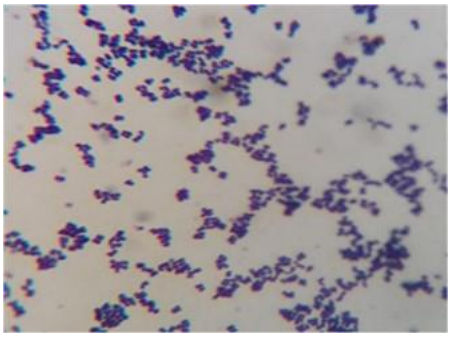

Fig. 10. *Staphylococcus* spp., Gram-positive, spherical shape, cellular arrangements in grape-like clusters (×1000; gram's stain)

Fig. 11. Catalase test showing gas bubbles as an indication of positive reaction for *Staphylococcus spp.*

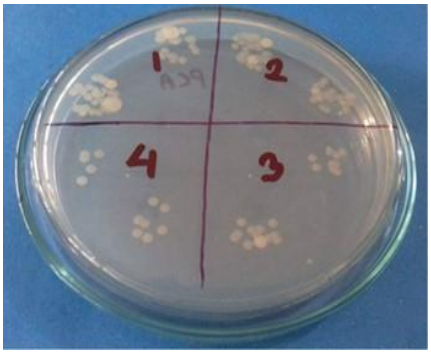

Fig. 12. Total Viable Count (TVC) on PCA.

Previous disease history of the selected cows

Table 3 shows different diseases recorded in selected cows. Most of the RBC was affected with more than one disease simultaneously, and multiple number of the bacterial isolate was found. Data were shown in Table 3, Table 4 and Table 5.

Table 3. Previous disease history in the selected cows

Name of the diseases	RBC (%) n=33	HFC (%) n=10
Sub-clinical mastitis	42.4*	20
Acute mastitis	39.4*	10
Endometritis	39.4	30
Dystocia	12.1	50
Retention of placenta	9.1	10
Abortion	6.1	10

*5% level of significance

Total viable count (TVC) of uterine samples

Figure 12 presents the TVC of samples. Results revealed that the average TVC was higher in RBC ($1.81x10^{10}$ CFU/mL) than in the HFC ($1.3x10^{10}$ CFU/mL). Among affected RBCs, the highest TVC was $2.05x10^{10}$ CFU/mL and the lowest TVC was $1.60x10^{10}$ CFU/mL (Table 4).

Table 4. Isolated bacteria and Total viable count (TVC) in the study population

Bacterial isolates		Cows with isolated bacteria			
Group	Isolates	RBC (n=33)		HFC (n=10)	
		Number	Percentage	Number	Percentage
Gram +ve cocci	*Staphylococcus aureus*	9	27.3	2	20
	Staphylococcus epidermidis	5	15.2	0	0.0
Gram +ve rods	*Bacillus* spp.	6	18.2	0	0.0
Gram –ve rods	*Escherichia coli*	10	30.3	1	10
TVC (CFU/mL)		1.81×10^{10} ($1.60x10^{10}$ - $2.05x10^{10}$)		1.3×10^{10} (1.2×10^{10} - 1.4×10^{10})	

Table 5. Presence of bacteria isolates in repeat breeder cows having diseases history

Previous diseases	RBC with no isolates (%); n=20	RBC with isolates; n=13				
		Single (%)	Two (%)	Three (%)	Four (%)	Total (%)
Endometritis	10	23.1	23.1	30.8	7.7	84.6
Acute mastitis	40	15.4	15.4	7.7	-	38.5
Sub-clinical mastitis	50	-	15.4	15.4	-	30.8
Dystocia	15	-	7.7	-	-	7.7
Retention of placenta	5	7.7	7.7	7.7	-	23.1
Abortion	0	-	-	-	15.4	15.4

Discussion

In most cases, the microbial study of the dam's uterus is ignored to identify the cause of RBCs. In the present study, 39.4%of repeat breeding cows were positive for bacterial infection. This finding coincides with several previous findings of bacterial isolation from the uterine fluid of infertile cows. Some previous studies opined that bacteria were found in the uterine fluid of 9-14% to 75-100% (Singh et al., 2000; Bhat et al., 2013; Bhat et al., 2014; Aghamiri et al., 2020) repeat breeder cows. However, different studies reported a higher percentage (73.91%) of bacterial infections in RBCs and in repeat breeding buffaloes (Khair et al., 2018; Kumar et al., 2004).

Results reveal that the major bacteria isolated from RBC and HFC were *E. coli, Staphylococcus* spp. and *Bacillus* spp., which resembles the report of Gani et al. (2008) and Patel et al. (2019). However, several previous studies also reported recovery of *Staphylococcus* spp., *Proteus* spp., *Streptococcus* spp., *Diplococcus* spp., *Peptococcus* spp., *Escherichia* spp., *Klebsiella* spp., *Pseudomonas* spp., *Bacteroides* spp., *Fusobacterium* spp., *Bacillus* spp., and *Corynebacterium* sp. from the cases of the cows of RBS (Singh *et al.,* 1998; Oliveria et al., 2011; Cakici and Akoz, 2019). These organisms were discriminated against as potential pathogens which render the female genital tract more harmful to the viability of the fertilized ovum to the uterine floor (Rosales and Ametaj, 2021). The qualitative study of the organisms from both groups of cows provides a means for comparison between the two sets of samples. Though most of the organisms isolated are categorized as non-specific agents of reproductive disorders, they can play a vital role as secondary invaders of the genital tract, changing the uterine and vaginal pH, interfering with the activity of spermatozoa and thereby resulting in infectious infertility (Dahiya et al., 2018).

The healthy uterine defense mechanisms (UDM) are important in inhibiting the immigration of invading bacteria (Rosales and Ametaj, 2021), which results from the damage of gamete and embryos leading to infertility (Sheldon et al., 2009). The presence of opportunistic bacteria in ($1.81x10^{10}$ CFU/ml) samples may reduce the resistance of endometrium and favor the access and immigration of pathogenic secondary bacteria, which in turn can make conditions unfavorable for successful fertility in cows.

The metabolic product of bacteria and endometritis exudates modify the uterus environment and hamper gestation (Osawa, 2021).

Moreover, we cannot ignore the relationship of age and body condition score to reproduction, production, and bacterial infections in the uterus.

It was found that most of the RBCs had a history of mastitis (both clinical and sub-clinical). Scientific studies on the negative effect of mastitis on fertility or reproduction are limited. The prevalence of subclinical mastitis and acute mastitis was significantly ($P<0.05$) higher in RBC compared to HFC, which resembles the findings of Chowdhury (2017) and Sikder (2018). Dairy cows that are milked tend to have weakened immune systems, making them more susceptible to illness, particularly infections that cause mastitis (Sordillo and Aitken, 2009). There is a theory that endotoxins, which cause the secretion of prostaglandins, mediate the adverse effect of mastitis on reproductive rates (Kumar et al., 2017). Besides these, the prevalence of endometritis was significantly ($P<0.05$) higher in RBC compared to HFC. Chowdhury (2017) reported a higher frequency of endometritis in RBC, parallel to the present findings. Endometritis causes early embryonic mortality (EEM) because of an inadequate uterine environment (Sheldon et al., 2009; Walsh et al., 2011). All the factors could affect the dairy cows' recurring estrus, which causes RBC. Hossain et al. (2016) found that 8-27% of cow reproductive failure occurred due to endometritis in Bangladesh. The endocrine, metabolic, and physiological condition of lactating cows during pregnancy is noticeably altered by prenatal stress and negative energy balance (Walsh et al., 2011). The

interaction of the above mentioned stresses and oxidative stress may compromise to the dairy cow's immun and inflammatory response (Sordillo and Aitken, 2009). Due to their impaired immune systems, high-yielding dairy cows are more susceptible to metabolic diseases, which act as a risk factor for utero-vaginal prolapse, dystocia, and retained placenta (Roche et al., 2009)

Conclusion

A bacteriological study of uterine samples of cows needs to be performed to identify the causes of repeat breeding cows. The higher pathogenic and opportunistic bacterial load may cause repeated conception failure in RBC. Infection of cows with endometritis may act as a predisposing factor for repeat breeders. A bacteriological study of uterine samples and previous disease history of cows could help identify the cause of RBS and proper treatment regimen, which in turn could help set proper strategies for preventing RBS in dairy cows.

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Conflict of interests

There is no conflict in interest to publishing the article.

Authors' contribution

MFHC and NSJ were designed for the study. MFHC was involved in sample collection and experimentation. MFHC, MMIH, MH, MR, and NSJ were involved in interpretating data and preparing the manuscript. MSR, MR, and NSJ were involved in the critical review of the manuscript.

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