Identification and characterization of uterine microorganisms and selection of treatment strategy in repeat breeding cows to increase conception rate in dairy farms in Chattogram

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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JUNE 2021

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Author

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%	Percent
µg	Microgram
AI	Artificial Insemination
ABST	Antibiotic Sensitivity Test
BA	Blood Agar
BCS	Body Condition Score
bp	Base pairs
CI	Confidence Interval
CL	Corpus Luteum
CR	Conception Rate
CVASU	Chattogram Veterinary and Animal Sciences University
CVM	Cervico- Vaginal Mucus
DNA	Deoxyribonucleic Acid
E.coli	*Escherichia coli*
EMB	Eosin Methylene Blue Agar
et al.	et alia (and others)
FAO	Food and Agriculture Organization
FSH	Follicular Stimulating Hormone
GDP	Gross Domestic Product
GnRH	Gonadotrophin Releasing Hormone
IGF-I	Insulin Like Growth Factor
LH	Luteinizing Hormone
LVF	Low Volume Flushing

MCA	Mac-conkey Agar
mm	Millimeter
MR	Methyl Red
MSA	Mannitol Salt Agar
NA	Nutrient Agar
NB	Nutrient Broth
0R	Odds Ratio
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PMN	Polymorphonuclear Neutrophlis
RB	Repeat Breeding
V-P reagent	Voges-Proskauer reagent

Abstract

Repeat breeding is one of the major constraints in profitable dairy farming due to repeated conception failure. The present study was conducted with an objective to calculate the prevalence of repeat breeding in cows, to isolate, identify, and confirm predominant bacteria from uterine samples and also to perform antibiotic sensitivity test for selection of appropriate antibiotic. The data were collected by interviewing the owner of the cows from 20 farms having 515 breedable cows located at different areas of Chattogram Metropolitan region. Uterine lavage samples were collected from 50 repeat breeder and 50 fertile healthy crossbred cows.

The overall prevalence of repeat breeding cows was 12.23%. The breed, BCS, age and parity of cows influenced the prevalence of repeat breeding in cows. It was observed that, 64% repeat breeders examined were found to be positive for bacterial isolation. Of the 50 repeat breeders, 32 samples (64%) yielded 75 bacterial isolates; where Staphylococcus was predominant 27 (36%), followed by Bacillus 22 (29.33%), *E. coli* 17 (22.66%), Pseudomonas 9 (12%). The most common species identified from the uterine samples were *Staphylococcus aureus* and *Bacillus subtilis*.

The ABST in the present study revealed that the maximum number of bacterial isolates were sensitive to gentamicin (85.33%) and ceftriaxone (81.33%) followed by ciprofloxacin (80%) and tetracycline (49.33). The lowest sensitivity was observed for ampicillin (24%) and penicillin (14.66 %). The repeat breeding cows treated with injection GnRH on the day of AI increased conception rate (60%) compared with other treatment given in this study. Whereas, intrauterine infusion with Gentamicin 10% improved conception rate (50%).

It is concluded that, overall prevalence of repeat breeding is influenced by breed, age, BCS and parity of cows. The predominant bacterial isolated from repeat breeder cows are Staphylococcus, Bacillus and *E.coli*. GnRH injection on the day of AI may improve the conception rate in non-infectious causes while, gentamicin may be effective for infectious repeat breeder cows.

Key words: Repeat breeding, Prevalence, Microorganisms, Antibiotic

Sensitivity test and GnRH

Chapter-1

Introduction

Bangladesh has about 24.08 million cattle population (FAO, 2018). Statistics show that about 2.9% of national GDP is covered by the livestock sector, and its annual rate of growth is 5.5%. About 20% of the population of Bangladesh earns their livelihood by raising cattle. The main reproductive objectives of dairy farm is one calf by cow per year. In the recent past, the incidence of infertility becomes relatively increased with consequent decline in productivity of farm animals.

Pregnancy rate after insemination measures the economy of dairy farm. But, one of the major constraints of profitable dairy farming is low conception rate. Reproduction is affected by many factors, but the pathological alteration caused by microbes in the genital tract of the animal appears to be the main factor for infertility (Prajapati, 2005; Patel et al., 2009). Among reproductive disorders, repeat breeding plays a vital role in delayed conception in Bangladesh resulting in increased inter calving interval. Repeat breeder cow is generally defined as any cow that has not conceived after three or more services, has normal estrus cycle, is free from palpable abnormalities, shows no abnormal vaginal discharges, has calved at least once before and is less than ten years old (Zemjanis, 1980).

Usually about 9-12% cows are expected to be repeat breeder in a herd with normal fertility and with 50-55% conception rate (Reneau and Conlin, 1984). The occurrence of repeat breeding in India was 5 to 32% in cows and 6 to 30% in buffaloes (Gupta and Deopurkar, 2005). In Sweden, the prevalence of repeat breeding in dairy heifers was 10% (Bage et al., 2002). The reported prevalence of repeat breeding cows in Bangladesh was 11% (Boettcher and Perera, 2007). Major causes of repeat breeding are fertilization failure and early embryonic death and influenced by ovulatory failure, uterine infection, genetics, error in estrus detection, improper timing of service. When artificial insemination is used, some of the animals might have been inseminated at improper time (Shamsuddin et al., 2001) leading to increased proportion of repeat breeding in Bangladesh.

Bovine genital infection either specific or nonspecific in nature, accounts for large number of pregnancy failure in cows as mentioned by Leblanc et al. (2002) and El-Khadrawy et al. (2011). Generally, nonspecific uterine microorganisms of the genitalia is considered to be the main cause of repeated conception failure. Pathogenic organisms isolated from an infected uterus are generally present in environments and are capable of infecting other organs (Azawi, 2008).

Number of bacteria in a variety of combinations, *Actinomyces spp, E. coli, Fusobacterium spp, Pasteurella spp, Pseudomonas spp, and Staphylococcus spp* have been recovered from infected uterus. Among them, *Archanobacterium pyogenes* and *E. coli* are usually associated with uterine infection in cattle (Usmani et al., 2001 and Seals et al., 2002). These bacteria alter the uterine environment, affecting the fertility by impairment of sperm transport, sperm death and hostile environment to the subsequent development and maintenance of conceptus, leading to their death. Microorganisms alter the pH of uterine environment causing inflammation and denudation of uterine mucosa and thereby interfere with the implantation (Singh et al., 1996). Early embryonic death (< 42 days) is a crucial factors in reproduction failure resulting economic loss to the dairy farm (Rahman, 1996).

Repeat breeders are mainly treated with administration of gonadotrophin releasing hormone (GnRH) and intrauterine infusion with Lugol's Iodine (Taponen et al., 1999). GnRH has been used at the time of AI in repeat breeding cows to increase conception rate (Hossain, 2002). It is likely that double AI in a single heat may improve the conception rate in repeat breeding cows. It is also reported that increased in fertility of dairy cows by injecting GnRH after 5 days of AI (Singh and Singh, 2014).

Intrauterine infusion of antibiotic during AI is the rational treatment for repeat breeding cows (Huber, 1982). Antibiotic sensitivity need to be studied for monitoring the exact response to specific antibiotic. The inappropriate use of antibiotics cause chronic endometritis and subsequent repeat breeding problems. Excessive uses of new antimicrobials underscore the importance of in-vitro testing for specific antibiotic sensitivity test. Hence, proper antibiotic selection is important to prevent the antimicrobial resistance and elimination of infection (Chandrakar et al., 2002). For this isolation and identification of microorganism of reproductive tract infections is

very essential, so that, specific antibiotics could be used to control reproductive tract infections. Antibiotic sensitivity test showed moderate to high sensitivity to amoxicillin, oxytetracycline and ciprofloxacin in repeat breeding cows (Gani et al., 2008). Unlike many parts of Bangladesh, Chattogram characterizes as a dense dairy zone and repeat breeding is one of the major constraints in profitable dairy farming but very few research works were done on RB. Based on above ground and in response to local service demand, the present study was undertaken with the following objectives:

a. Prevalence of repeat breeder cows at different dairy farms at Chattogram Metropolitan area.
b. Identification and molecular characterization of uterine microflora responsible for repeat breeding.
c. Evaluation of treatment strategy during and post (day 6 and 12) AI on conception rate of repeat breeder dairy cows.

Chapter-2

Review of literature

2.1. Definition of Repeat Breeder

Repeat breeding (RB) is a substantial problem in cattle farming leading to huge economic loss for the dairy farmers due to more inseminations, increased calving interval and increased culling rates (Lafi et al., 1992). A repeat breeder is generally defined as any cow that has not conceived after three or more services, has normal estrus cycle, is free from palpable abnormalities, shows no abnormal vaginal discharges, has calved at least once before and is less than ten years old (Zemjanis, 1980). The clinical examination of the animal may fail to reveal any definite lesion or condition to explain the failure of conception (Roberts, 1986). Perez-Marin and Espana (2007) defined RB cows as a heterogeneous group of sub fertile cows with no anatomical abnormalities or infections that exhibit a variety of reproductive disturbances in a consistent pattern over three or more consecutive heat cycles of normal duration 17–25 days. Repeat breeder cows are those cows with healthy clinical and functional genital tracts that do not get pregnant after three or more artificial inseminations (Ferreira et al., 2016).

2.2. Prevalence of Repeat Breeding

The problem of repeat breeding is recognized world-wide but the prevalence rate varies from one area to another e.g. 10% in Sweden (Hewett, 1968), 5% in Israel (Francos, 1974), 22% in Bangladesh (Rahman et al., 1996), 20% in Scandinavia (Roine and Saloniemi, 1978), 22% in India (Singh et al., 1981). In Bangladesh, 13.0 to 22.0% cows had been identified as repeat breeders, of which 81.8% repeat breeding occurs due to infectious agents (Samad, 1996). Karwani and Sharma (2003) observed that the overall incidence of repeat breeding in cattle and buffaloes was 19.61% and 15.6%, respectively. Sarder et al. (2010) found 20.2% prevalence of RB in relation to age, breed, parity, body weight and BCS of cows in 10 different upazillas of Rajshahi district. Higher incidence of repeat breeding due to combination of multiple etiologies than other single causes was observed by Singh et al. (2008).

Kaikini et al. (1981) found 32.11% prevalence of reproductive disorders in cattle and also reported prevalence of repeat breeding as 21.9% in crossbred cows. Whereas, Singh et al. (1983**)** reported prevalence of repeat breeding in crossbred cows between 7.4 -18.6%. Saxena (2005) reported prevalence of repeat breeding in cattle 5.5 -33.3% and in buffaloes 6 -30.6%. Bhat et al. (2012) reported the prevalence of repeat breeding as 28.31% in cattle. They conducted study on 1074 cattle, out of which 304 cattle were found to suffer from repeat breeding. They observed that the repeat breeding was due to breed, season, ovulatory disturbances and ovarian cyst. Khan et al. (2016) reported the overall prevalence of repeat breeding as 27.33% (21.67% cattle and 33.04% buffalo).

2.3. Etiology of Repeat Breeding

El-Khadrawy et al. (2011) divided the causes of Repeat Breeding into two major categories i.e. Non-infectious and Infectious. The author mentioned the non-infectious causes as poor management, chromosomal aberrations, hormonal imbalance, anatomical defects of reproductive tract and infectious causes are metritis, endometritis, salpingitis, cervicitis and vaginitis.

2.3.1. Noninfectious causes

2.3.1.1 Nutrition

The qualitative and quantitative differences in the ration in dairy cattle may cause reproductive disorders (Pedroso and Roller, 1996; Dovensky et al., 1996). Poor nutrition can result in weak estrus expression and reduced ovulations, low conception and fertilization rates (Imakawa et al., 1986). Cows in negative energy balance (NEB) have lower blood concentrations of insulin and insulin like growth factor (IGF-I). These endocrine hormones can influence GnRH secretion by acting on GnRH neurons, or their neuronal pathways or on the pituitary gonadotrophins (Williams et al., 2002). Repeat breeder dairy cows had lower levels of serum Zn, Cu, P and I as compared to normal animal (Ceylon, 2008). This was confirmed after the improvement in recovery and conception rates in RB buffaloes and cattles following supplementation with mineral mixture (Ahmed et al., 2010; Das et al., 2009; Sah and Nakao, 2006).

2.3.1.2. Management

Wolfensen and Meidan (2000) studied that heat stress turns the buffaloes into repeat breeders or anoestrus during summer season. Cows under heat stress have reduced duration and intensity of estrus, altered follicular development and impaired embryonic development. (Jordan, 2003) due to the stress, increase in the level of circulating corticosteroids and by reducing the concentration of progesterone altering the follicular activity and ovulatory mechanism, leading to inferior oocytes and embryo quality and modified uterine environment which reduce the likelihood of embryo implantation.

2.3.1.3. Hormonal factors

An abnormal endocrine status during folliculogenesis and ovulation failure has been major causes of RB in cows (Maurer and Echtemkamp, 1985). Early embryonic mortality leading to repeat breeding syndrome may be due to diminished response to circulating LH (Shelton et al., 1990). It was reported that ovulation of very small follicles in lactating cows resulted in smaller corpus luteum, lower serum progesterone concentrations and lower conception rates compared to ovulation of larger follicles (Vasoncelos et al., 2001).

2.3.1.4. Genetic factors

Chromosomal aberrations as well as autosomal recessive genes are the major causes of early pregnancy failure in animals and may be account for 20.0% of total embryonic and fetal death (King, 1990). A study regarding Robertsonian translocation 1/29 in cattle (Rodriguez et al., 2010) informed that cows diagnosed with this chromosomal defect were classified as Repeat Breeder Cow (RB).

2.3.1.5. Anatomical defects of the reproductive tract

Anatomical or functional changes of reproductive tract can drive to gestational failure and infertility. Traumatic injuries of the vagina can lead to the formation of complete adhesions between the vaginal wall and development of pyometra (Tibary and Anouassi, 1997). Singh et al. (2009) conducted a study in 183 cattles with the history of repeat breeding and found 7.1 % had acquired anatomical abnormalities. Oviductal abnormalities, that complicate and frequently inhibit the reproduction, are present in

6.0 to 15.0% of adult cows and can reach up to 80.0% in those with a history of infertility or repeat breeding. Acquired uterine alterations, as metritis, are critical to the resumption of the normal cyclicity during postpartum period, provoking repeat breeding (Shresta et al., 2004). Cervical traumatic stenosis and obstruction prolapse of cervical rings, adhesions or functional incompetence can be detected associated with repeat breeding (Tibary and Anouassi, 1997)

2.3.2. Infectious Causes of Repeat Breeding

It was reported that an increased incidence of RB may be due to subclinical endometritis which decreases reproductive performance by its negative impact on first service conception and pregnancy rates in cows (Kasimanickam et al., 2004). There is a correlation between repeat breeders and endometrial abnormalities (Francos, 1979; Santana et al., 1998). Francos (1979) observed that from 3.5 to 5.7% of cows with metritis had repeat estrus. Uterine infections (specific and nonspecific) will adversely affect the reproductive indexes by enlargement of the uterine and cervical postpartum involution, by alteration of follicular development (Lewis, 1997) and by increased embryo mortality and repeat estrus rates (Santana et al., 1998). Various species of bacteria inhabit reproductive tracts of cows. Symbionts bacteria become pathogenic when the animal is stressed while others are immediately pathogenic. E'duvie et al. (1985) and El-Azab et al. (1988) isolated *Staphylococcus aureus*, *Escherichia coli*, *Aracnobacterium pyogenes*, *Proteus mirabilis*, *streptococcus spp.*, *Pasteurella multocida*, *Proteus vulgaris*, *Klebsiella spp*. and several anaerobic microorganisms from the uteri of cows with a history of repeat breeding.

2.4. Risk factor of Repeat Breeding

2.4.1. Age

Many researchers have shown that the incidence of repeat breeders is higher in older cows than the younger ones (Hewett, 1968; Dekruif, 1978 & Bartlett et al., 1986). Erb et al. (1985) reported that increasing age was associated directly with an increased risk of veterinarian assisted dystocia, retained placenta and metritis. There was a higher risk for a first calver of becoming a RB animal than for a multiparous cow (Gustafsson and Emanuelson, 2002).

2.4.2. Season

The efficiency of reproduction is not uniform throughout the year, and different views are expressed in the literature as regards seasonal effects. Hewett (1968) observed higher incidence of the repeat breeder syndrome in cows calving during the autumn and winter. Photoperiod length and temperature variations are associated to the season, and could affect the endocrine regulation of the estrous cycle. Karwani and Sharma (2003) stated that Seasonal incidence of Repeat Breeding in cattle was highest (21.9%) in humid hot summer followed by autumn (20.5%) whereas in buffaloes incidence (20.6%) was found in dry hot summer followed by humid hot summer (18.5%).

2.4.3. Herd size

Hewett (1968) showed that repeat breeders are more common in large herds than in small herds. He reported that in small herds 8.5% of the animals failed to become pregnant after four or more inseminations, whereas a 13.1% was reported in the larger herds. De Kruif (1978) found that the interval between parturition and conception was shorter as the size of the herd increased, but that the calving rate after first service decreased as the size of herd increased.

2.4.4. Heat detection

The poor heat detection is one of the main causes of RB and infertility in cattle (Lafi and Kaneene, 1988; Perez-Marin and Espana, 2007). O'Farrell (1975) recommended that checks conducted 5 times daily were adequate to detect at least 80% of cows in heat. Bailie (1982) concluded that increasing the frequency of heat detection checks decreased the number of inseminations per conception and decreased the number of days open in dairy cattle. With good detection of oestrus the optimal time to inseminate, can be found by the am-pm rule Foote (1979). Cows first seen in oestrus in the morning are best inseminated in the afternoon of the same day while cows first seen in oestrus in the evening should be inseminated next morning.

2.5. Pathogenesis of reproductive tract diseases

The non-specific and specific pathogens of the reproductive tract affect the fertility of cow and results in major number of pregnancy failure in animals. With the invasion of these pathogens female reproductive tract become more harmful for sperm viability also affect implantation of fertilized ovum (Raghavan et al., 1971). These pathogens cause uterine mucosa denudation and uterine pH alteration that is harmful for sperm survival in uterine lumen (Raghavan et al., 1971).

Barman et al. (2013) reported that, the facultative anaerobic organisms normally present in reproductive tract create inflammatory condition when uterine defense mechanism becomes weak. Samatha et al. (2013) observed that surface epithelium gets severally damaged in chronic endometritis with lacking of many cilia on cells and microvilli destruction of glandular structures. Pathogenic organism penetrate epithelial cell of the uterus and releases bacterial toxin which leads to disturbance in normal functioning of tissue. The bacteria and its products induce uterine inflammation and reduce pituitary FSH, suppress LH release. As a result ovarian follicular growth and function is disturbed and the ovulation is delayed in the dairy cow which leads to failure of fertilization (Ahmed and El-sheikh, 2014).

2.6. Uterine defense mechanism

The uterine defense mechanism maintained in several ways; anatomically, by the columnar epithelium tissues covering to endometrium; immunologically, through the action of poly morphonuclear inflammatory cells and humoral antibodies; chemically, by mucus secretions from endometrial glands (Dhaliwal et al., 2001). Under normal conditions, there are various anatomical and functional barriers, which prevent pathogenic microorganisms from colonizing in the uterus (Foldi et al., 2006).

Impairment or weakening in the defense mechanisms of the reproductive tract which include the normality of the endometrium, uterine involution, mucous secretion, antibodies and phagocytosis may lead to bacterial colonization in the uterus and subsequently result in uterine infection (Fredriksson et al., 1985; Ali et al., 2010). In the uterus, the cellular defence against bacterial contaminants is provided by uterine leukocytes (Romaniukowa, 1984; Vandeplassche, 1984). After experimental intrauterine infection, the PMN population within the uterine lumen usually increases (Watson et al., 1990; Butt et al., 1993). Polymorphonuclear (PMN) cells derived both from blood and uterine exudates are engaged in uterine defense mechanisms (Hussain, 1990). The phagocytic activity of blood neutrophils could be greater than that of uterine exudate neutrophils (Romaniukowa, 1984). In contrast, Anderson et al. (1985) found no significant difference between the phagocytic ability of blood neutrophils and uterine exudate neutrophils. However, the phagocytic ability of the PMN cells in the uterine fluid is seemed to be lower than in the blood plasma.

2.7. Microflora isolated from reproductive tract

Murthy et al. (1974) reported the presence of *E.coli* (27.63%), *Staphylococcus* (18.59%) and *Pseudomonas* (12.06%) in higher frequency followed by *Bacillus* (9.54%), *Proteus* (6.53%) and *Corynebacterium* (4.02%) in the CVM samples of repeat breeder cows and heifers. Panangala et al. (1978) reported that common organisms isolated from the CVM samples of normal cows were *Staphylococcus, Escherichia, Bacillus and Corynebacterium but Corynebacterium and Enterobacteria* were more common in repeat breeders. Deka et al. (1979) reported that 20 percent samples taken from normal cows were positive for bacteria against 51.4 per cent from repeat breeder cows. Dholakia et al. (1987) investigated 520 samples of cervical mucus from repeat breeder cows and reported that 76.92 per cent samples gave the

bacterial isolates, viz., *Staphylococci, Streptococci, Corynebacterium, Bacillus* and gram positive bacilli in different proportion. Their antibiotic sensitivity test reported that gentamicin was the most effective antibiotic followed by chloramphenicol and neomycin. Gupta and Deopurkar (1993) isolated *E.coli* in higher frequency, followed by *Staphylococcus aureus, S. typhi* and *Pseudomonas aeruginosa* from repeat breeder cows. Ramaswamy *et al*. (1998) isolated *E.coli, Staphylococcus aureus, Bacillus spp., Proteus spp.* and *Pseudomonas spp.* in their study on non-specific microflora of cervical mucus samples from repeat breeder cows during estrous. Arora et al. (2000) recovered *Escherichia coli, Staphylococcus aureus, Proteus spp., Streptococci, Bacillus spp., Staphylococcus epidermis, Klebsiella pneumonia, Pseudomonas aeruginosa* and also *Corynebacterium pyogenes* from repeat breeding cows and buffaloes. The bacteriological study was done on the uterine discharge samples of 55 normal estrus stage fertile and 97 repeat breeding cows by Kamal et al. (2001). Qualitative studies revealed bacteria from uterine discharge of 51 (52.5%) repeat breeding cows in contrast to only 14 (25.4%) normal estrus fertile cows. The most commonly isolated organisms were *Escherichia coli* (22.68%), followed by *Staphylococcus spp.* (20.6%), *Bacillus spp* (16.4%), *Streptococus spp* (12.3%), *Corynebacterium spp* (8.2%), *Proteus* (7.2%), *Pseudomonas spp*. (6.2%), *Micrococcus spp.* (4.1%) and *Klebsiella spp*. (3.1%). Chandrakar et al. (2002) recovered different bacterial isolates from repeat breeder cows which are *Staphylococcus aureus* (29.16%), *E.coli* (20.83%), *P. aeruginosa* (16.66%), *Streptococcus* (12.50%) and *Proteus* (4.16%). Gani et al. (2008) investigated that *Staphylococcus spp* was highest in frequency (37.8%), followed by *E. coli* (29.7%), *Pseudomonas* (18.9%) while, Gram negative minute rod shaped bacteria was found in 24.3% cases in repeat breeder cows. Azizunnesa et al. (2011) conducted a study on 109 uterine samples, from diseased and healthy animals. Most prevalent bacteria in diseased animal were *Staphylococcus spp* and *Streptococcus spp* found in 33% and 27.3% of the cases respectively. In non-diseased cases, the highest number of samples was positive for *Bacteroied spp* (13.2%) followed by *Streptococcus spp* (10.4%). Ahuja et al. (2017) concluded that higher isolates of *Staphylococcous* were found in Normal cyclic cows while Bacillus in repeat breeding cows.

2.8. Antibiotic sensitivity test

Antibiotic sensitivity test by Bauer and Kirby's disc diffusion method is an age old method of microbiological investigation. The disc diffusion method provides a simple and reproducible means of testing antibiogram, i.e. bacterial susceptibility to different antibiotics. Shah and Dholakia, (1983) in their study of antibiotic sensitivity patterns of bacterial isolates from cervico vaginal mucus of endometritis cows revealed that the isolates were more sensitive to gentamicin (89.87%), ampicillin (75.95%) and neomycin (74.68%), followed by streptomycin (59.49%), tetracycline (43.04%), penicillin (29.12%) and furadantin (17.72%).

Sharda et al. (1991) observed in their study that gentamicin was highly effective (73.68%) against the bacteria isolated from RB cows and buffaloes followed by penicillin (68.12%), while ampicillin (15.79%) was found to be the least effective. Singh et al. (1996) observed that the isolates which were obtained from CVM samples of repeat breeder cows and buffaloes were found sensitive to Gentamicin (88.9%) and chloramphenicol (77.8%) and also reported that most of the isolates were resistant to penicillin (81.48%). Kamal et al. (2001) was conducted an antibiotic sensitivity test of uterine discharge from 97 repeat breeding and 55 normal fertile cows. The antibiotic spectra of isolated bacteria revealed Gentamicin (92.8%) to be the most effective antibiotic, followed by chloramphenicol (86.7%) and Neomycin (79.5%). Kanamycin and streptomycin had limited effect whereas ampicillin, tetracycline and Penicillin G were least effective. Rao et al. (2001) reported that drug sensitivity pattern of the microbial agents involved in endometritis, showed maximum sensitivity to gentamicin (80.98%), and followed by co-trimoxazole (51.75%), chloramphenicol (50.73%), kanamycin (45.85%), ampicillin (28.29%) and oxytetracycline (24.88%). Ingawale et al. (2003) studied antibiogram pattern of bacterial isolates from the metritis cows and revealed that most of the isolates were sensitive to ciprofloxacin (83.33%), followed by enrofloxacin(79.16%), gentamicin (77.08%) and chloramphenicol (62.5%); but most of them were resistant to ampicillin and sulphamethaxozole. Mane et al. (2009) performed antibiotic sensitivity pattern of the bacterial isolates from repeat breeder cattle and buffaloes and revealed that ciprofloxacin (93.75%) was most effective drug followed by gentamicin (84.37%) and chloramphenicol (78.12%).

2.9. Molecular technique for bacterial confirmation: Polymerase Chain Reaction (PCR)

Polymerase chain reaction have allowed for more reliable microbial identification and surveillance. PCR is a powerful technique for the culture-independent detection and characterization of microorganisms (Guo et al., 2012) and some attempts have been made to identify bacteria from uterine discharges by PCR (Liu et al., 2009; Bicalho et al., 2012; Guo et al., 2012). It represents a major advance in terms of the speed, sensitivity and specificity of diagnostic methods and has been increasingly used to identify several bacterial species from food and clinical samples (Stone et al., 1994).

2.9.1. Detection rpoB gene in *Staphylococcus spp*.

The PCR protocol for rpoB gene was standardized in the present study as per the method described by Euzeby, 1997. The habitat of *Staphylococcus* is skin, skin glands and mucous membranes of humans and many animals. The genus *Staphylococcus* includes both pathogenic and saprophytic strains that have been isolated from animal products such as meat and milk, and from environmental sources (Kloos et al., 1991). Coagulase-positive species like *Staphylococcus aureus* are the cause of many types of infection (Forbes et al., 1998). During the last two decades, coagulase negative *staphylococci* (CNS) have also emerged as pathogens causing medical device- related infections (von Eiff et al., 2002).

2.10. Treatment of repeat breeding

Treatment of repeat breeding depends on diagnostic classification of clinical cases in to infectious and non–infectious categories. The line of treatment differs in both the categories and also with diagnosis of particular cause of the repeat breeding.

2.10. 1. Noninfectious category

The treatment with GnRH during oestrus may be affecting time of ovulation, fertilization rate, corpus luteum development, progesterone secretion and embryonic survival, whereas GnRH administration on day 5th of the estrus cycle in crossbred cows causes ovulation of the first wave dominant follicle with formation of accessory corpus luteum; synchronized emergence of follicular wave by two days after GnRH administration and two wave cycle were altered to three wave cycles. Rao and Naidu. (1987) administered 100µg GnRH (Buserelin acetate) in cows at the time of insemination and found 54.14 per cent conceptions against 36.00 per cent in control cows, where the difference was statistically significant. Sankar et al. (1989) studied exogenous administration of synthetic analogue of 250 µg GnRH at the time of AI and found the first service conception rate 60.00 per cent in post-partum crossbred cows as against 40.00 per cent in control group. Ryan et al. (1991) treated cows with 10 µg GnRH (Buserelin acetate) at the time of AI and after 12 days of AI in two groups and third group of cows was kept as untreated control, where they found the conception rate as 48.80, 51.50 and 42.40 percent, respectively. Roy et al. (1995) concluded that the conception rate of inseminated animals in control group was 33.30 per cent whereas it was 73.60 percent in GnRH treatment group at the time of onset of heat. Jayakumar and Vahida (2000) administered 10µg of Buserelin in 15 repeat breeder cows on day 12th after AI in cows and 2.5 ml saline in 15 repeat breeder cows, where the conception rate was observed as 40.00 and 33.33 per cent, respectively. Sonwane (2000) studied the efficacy of GnRH on conceptions and observed as 65.00 and 40.00 per cent in treatment and control groups, respectively.

Ingwale et al. (2001) concluded that GnRH administration has beneficial effect in improvement of conception rate in case of induced estrus as 66% against 50% in the control group of cows. Rangnekar et al. (2002) reported 70.00 per cent conception rate in repeat breed HF cows with GnRH treatment at the time of AI. Anjum et al. (2010) treated cows with Dalmarelin (GnRH analogue) at the time of AI and observed 68.75% conceptions as against 37.50% in control group. Patel et al. (2010) studied efficacy of Buserelin acetate in 18 repeat breeder crossbred cows and concluded that Buserelin acetate treatment at the time of insemination may be used for increasing conception rate up to 66.66%. Kumar et al. (2010) studied efficacy of Buserelin acetate in 19 repeat breeder cows and concluded that Buserelin acetate treatment at

the time of insemination may be used for increasing conception rate up to 73.68 per cent. More et al. (2012) proved that exogenous administration of Buserelin acetate @ 10 mcg at the time of AI resulted in conception rate of 75.00 per cent in repeat breeder cows whereas progesterone and GnRH injection after 5 days of insemination resulted in 50.00 and 62.50 per cent conceptions, respectively. It can be concluded that the non-infectious treatment has high success with GnRH treatment at the time of AI than other hormonal treatment and schedules during estrus cycle.

3.10. 2. Treatment of infectious repeat breeding category

Nonpathogenic organisms present in external genitalia avail opportunities to multiply in endometrial environment on getting transferred through open cervix under stressful conditions. Infectious repeat breeding problem fails to interpret that whether the infection is causing fertilization failure, embryonic mortality or both. However, Dholakia et al. (1987) opined that non-specific bacterial infection plays a crucial role in repeat breeding animals by causing inflammation of endometrium resulting in to early embryonic mortality. Even after selection of most appropriate antibiotics (single or combination), it is necessary to consider route of administration, dose, stage of administration and even repeatability of administration for planning of AI and subsequent assured conception. Warriach et al. (2009) reported the overall pregnancy rates after three services of Gentamicin treated cows (80%) did not differ significantly ($P>0.05$) from control cows (93%). However overall pregnancy rates of Enrofloxacin treated cows (33%). As reported by Mane. (2010), Ciprofloxacin and Tinidazole may be used as the most effective drug for the treatment of repeat breeding in animals at field condition as compared to other intrauterine preparations.

Singh et al. (2015) observed that the pregnancy rate after three services of gentamicin treated cows (87.5%) did not differ significantly ($P>0.05$) from control cows (91.7%). However, overall pregnancy rates of Enrofloxacin treated cows (50%) were significantly lower than Gentamicin and control group. Asaduzzaman et al. (2016) reported 45.8% of cows recovered when intrauterine administration of antibiotics followed by AI in next heat than that of no treatment control group 30.4%.

Chapter 3

Materials and Methods

The present study was carried out at different private dairy farms at Chattogram region, Bangladesh during November, 2019 to March, 2020. Cows, reported to be repeat breeder, were investigated clinically at field level and diagnostic laboratory procedures were attempted in microbiology laboratory. The data were collected by interviewing the owner of the cows from 20 farms having 515 breedable cows located at different areas of Chattogram Metropolitan region. In this study uterine lavage samples from 50 repeat breeder and 50 fertile healthy crossbred cows, were examined for isolation and identification of predominant bacteria affecting genitalia. Treatment was given, on the basis of antibiotic culture sensitivity test. Pregnancy diagnosis of each cow was done by rectal palpation and transrectal ultrasonography using linear array probe.

3.1. Selection of farm and animals

The different private dairy farms were selected in different region of Chattogram. A total of 63 repeat breeder cows out of which 50 cows were selected for study. The cows were between 3 to 10 years old, with body condition scores (BCS) ranging from 1 to 3.5 and body weight (325-480 kg). Repeat breeding (n= 50) cows were selected on the basis of history of repeated conception failure, breeding records, nature of estrous cycle and per rectal examination of reproductive tract. The data were recorded in a ready questionnaire. A total of 50 repeat breeding cows were randomly distributed into 5 experimental groups for different treatment regimens.

3.2. Management of cows

The cattle were stall fed and kept under intensive housing system. Seasonally available green fodder with wheat and paddy straw and concentrate mixture were fed to all cattle. Approximately 5-10 kg roughages were given to each animal daily. Cows feed were also supplemented with 500-1000 gram concentrate daily. The concentrates were mainly rice polish, wheat bran, oil-cake and common salt. All cattle had free access to drinking water. The amount of daily milk yield per cow was determined by interviewing the farmers. The daily milk yield ranged from 10 to 20 L.

3.3. Clinical examination

To confirm the absence of clinical abnormalities, the uterine horns and ovaries of the cows were examined by rectal palpation. Both horns were then examined from the base to the tip for their size and symmetry, tone and thickness of the wall. In case of uterine asymmetry, the bigger horn was palpated to confirm pregnancy or pyometra. Acyclicity, possibly associated with chronic uterine infection was assessed by palpating thick uterine wall with or without intra luminal content along with the presence of palpable corpus luteum. The vaginal discharges of the cows were evaluated by a gloved hand and only the animals with clear mucus were chosen for this study.

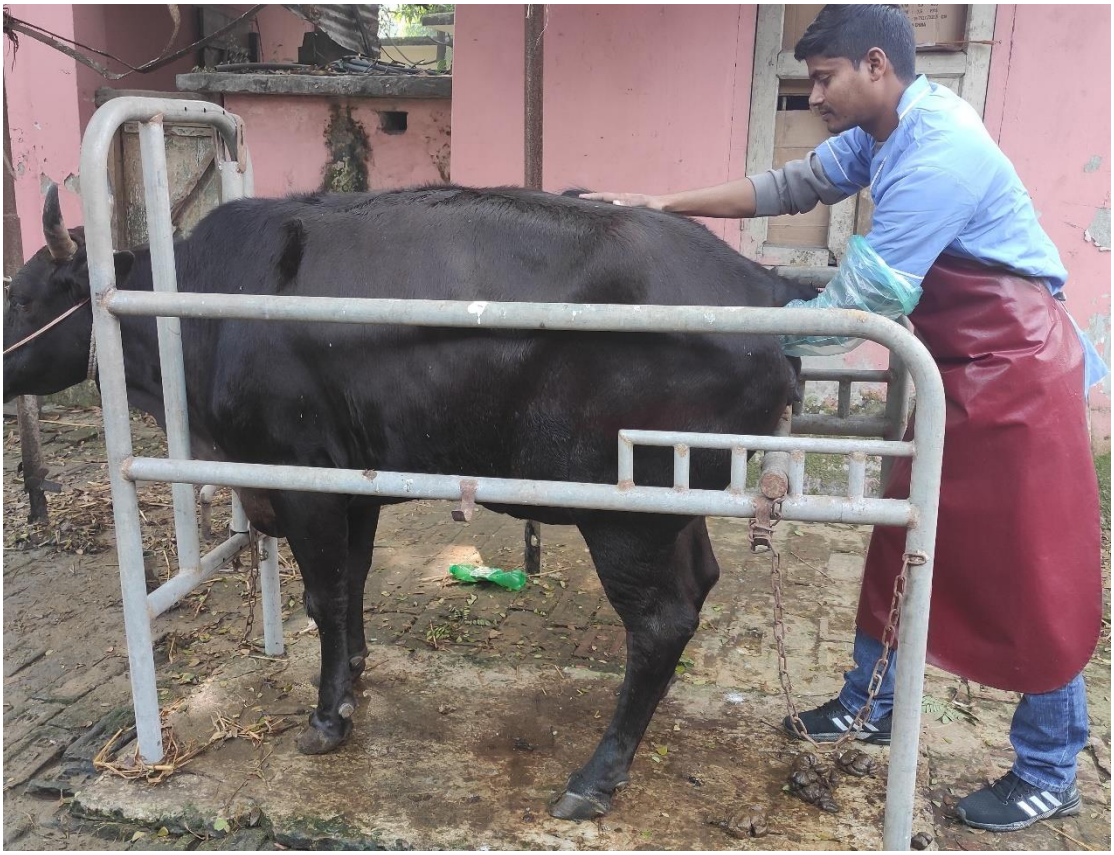


Fig. 1: Rectal palpation of repeat breeder cow to confirm the absence of clinical abnormalities

3.4. Questionnaire and data collection

A structured questionnaire was prepared to acquire farm and cow level management, demographic, health, production and reproduction data. The questionnaire was designed to comprise mostly closed and open ended (categorical) questions to ease data processing, minimize variation, and improve precision of responses (Thrusfield, 2005). The questionnaire was filled up by repeated questioning to the farmers and also farm manager and attendant and complemented with taking records from register book wherever applicable. Important farm and cow level intended data included stock inventory, demographic like breed, age, pregnancy status determined from birth records and dentition characteristics as described by Pace and Wakeman, (1983), Body Condition Score by Wildman et al., (1982), farm managemental information like types of feed supplied source of roughages (own formulated/collected/purchased), housing pattern, type of floor in the animal house (concrete, muddy), system of offering ration, history of deworming and vaccination, breeding method, history of reproductive disorders, farm owner socio-economic information, knowledge on repeat breeding were collected. A questionnaire model used in the study is provided in the Appendix-I

3.5. Sample collection

The uterine fluid samples were collected by using a flushing technique aided by a sterilized two way foley catheter when animals were in estrus. After restraining of animal in travis the vulvar and perineum region was washed with a mild antiseptic, ethyl alcohol (95%) solution and wiped properly with absorbable sterile cotton. The LVF method between 100 and 150 mL of sterile saline (0.9%) was infused into the uterus (Kasimanickam et al., 2005) using a two way foley catheter supported by an AI gun. After the instrument had been passed through the cervix into end the uterus, the AI gun was withdrawn from the female genital tract, leaving just the foley catheter. The catheter's cuff was inflated to prevent vaginal or cervix contamination. The other end of the catheter was connected to a rubber piece in order to facilitate infusion of the saline solution using a 50 mL syringe. After the infusion of 100 to 150 ml of sterile saline into the uterus, the uterus was massaged for 20 seconds and then retracted to recover the 10-15ml fluid by negative pressure aspiration into a syringe.

The recovered fluid was then transferred into a falcon tube containing PBS and placed in an ice box and transport to laboratory within an hour after collection.

Fig. 2: Collection of uterine sample from RB cow using foley catheter, flushing technique

3.6. Bacterial isolation and identification

The collected samples were cultured and evaluated for isolation and identification of non-specific microbes on the basis of cultural, morphological, colony characteristics, and biochemical reaction. The cultural examination of samples for bacteriological study was done according to the standard method as per suggested by Cruickshank et al. (1980).

3.7. Sterilization of glasswares and plasticwares

All the glasswares and plasticwares used during this study were procured from Hi media and they were autoclaved at 121°C temperature and 15 lb. pressure for 15 minutes before use.

3.8. Media, reagents and stains used for microbiological work

Solid media

The solid media used for bacteriological analysis were (i) Blood Agar (BA; Difco), (ii) Nutrient Agar (NA; Oxoid), (iii) Eosin- Methyline-Blue Agar (EMB; HiMedia), (iv) MacConkey Agar (MA; Merk), (V) Mannitol salt agar (MSA; Merk). Media were purchased from Ekushey Surgical Mart, Chattogram.

Liquid media (broth)

The liquid media used for the bacteriological analysis were (i) Nutrient Broth (NB; BBL), (ii) Methyl-red Voges-Proskauer broth (MR-VP Broth; HiMedia)

Reagents

The reagents used during the study were methyl red indicator, phenol red, reagents for Gram's staining like Methylene blue, Gram's iodine, safranin, 3% hydrogen peroxide solution, normal physiological saline solution and other common laboratory chemicals and reagents.

Preparation of media, nutrient broth and media plate

Nutrient agar, EMB, Mannitol Salt agar, Blood agar etc. were weighed as per instructions of the manufacturer and dissolved in distilled water in a sterilized flask. After proper plugging of the flask, these were autoclaved at 121°C temperature and 15 lb. pressure for 15 min.

After autoclaving, media was cooled up to 45°C. About 15-20 ml of particular media was poured in each sterilized petri plates. After pouring media, plates were kept for solidification. After solidification these plates were kept at 37°C for 24 hours for sterility testing.

3.9. Isolation of bacteria by culture method

Each sample of uterine fluid earlier kept into transport media was divided and inoculated separately in Nutrient agar (NA) and Blood agar (BA) to promote growth of bacteria. Each group of these media was incubated aerobically at 37°C. The colonies obtained after primary inoculation, were inoculated to selective media like Eosin Methylene Blue agar. Streak plate method was used for inoculating sample on nutrient agar plates or on the selective media plates. For primary isolation a loopful of broth culture was streaked on nutrient agar and selective media like EMB and Mannitol salt agar and incubated at 37°C for 24 hours. Plates were examined for bacterial growth after 24 hours of incubation. In this way single bacterial cells get isolated by streaking. Further selective media was used to identify specific bacteria after primary isolation. A tentative identification of bacteria was done based on colony morphology and Grams staining. Cultures were preserved by storing the culture tubes in refrigerator at 4 to 5°C to facilitate identification. Different bacteria produce different but characteristic colonies, allowing for early presumptive identification of mixed cultures. From these colonies, uniform colonies were selected. Further selective media was used to identify specific bacteria after primary isolation. The organisms were inoculated in 4 ml vial containing 1 ml NB at 37°C for 24 hours. After the growth of the organisms, 1 ml 50.0% glycerin was added and finally it was preserved at - 20°C for further studies

3.10. Examination of bacterial cultures

The cultural examination of uterine sample for bacteriological analysis was done according to the standard methods (Carter, 1979 and Cowan, 2004). The examination followed detailed study of colony characteristics, cellular morphology and biochemical properties. In order to find out different types of microorganisms in uterine discharge of different kinds of bacterial colonies were isolated in pure culture on the NA, SS, EMB, MSA and BA. The isolated organisms with supporting growth characteristics on various media were subjected to Gram's staining, motility test, and different biochemical tests, such as sugar fermentation test for acid and /or gas, hemolytic activity, indole production test, catalase test, coagulase test, Methyl-red and Voges-Proskauer (MR-VP) test.

3.10.1. Hemolytic activity

To examine the hemolytic activity of isolates of bacteria were inoculated separately on to blood agar (BA) and incubated at 37°C for 24 hours. The colony developed on the BA was examined for various types of hemolysis. Hemolytic patterns of the bacteria were categorized according to the types of hemolysis (Cowan, 2004) on blood agar and were listed as mentioned below:

i) α hemolysis: A zone of greenish discoloration around the colony manifested by partial hemolysis

ii) β hemolysis: Complete clear zone of hemolysis around the colony

iii) γ hemolysis: Non-detectable hemolysis.

3.10.2. Gram's staining

Gram's staining was performed for the morphological study of bacteria to provide information about the presumptive bacterial identification as per recommendation of Cruickshank et al. (1980). 24 hour old colony was mixed with a drop of sterile saline on a glass slide. The smear was dried, heat fixed and flooded with crystal violet and left for a minute. Then the slide was gently rinsed with tap water and flooded with Gram's iodine and left for a minute and again gently rinsed with tap water. After that

the smear was decolorized using 95% ethyl alcohol or acetone and immediately again rinsed with water. Finally, the smear was flooded with safranin to counter-stain and left for 45 seconds. Those bacteria which retain the violet stain are called Gram-positive and those, which are decolorized and stained red by the counter stains, are Gram negative. The staining characters were studied by microscopic examination under oil immersion. The isolates were identified as Gram positive or Gram negative bacteria. The morphology of bacteria that are observed in Gram stained smears are mentioned below.

Individual cellular morphology: Bacilli, Cocci (Streptococci, Staphylococci, Diplococci), vibrios, pirochaetes.

Bacilli: Different shapes and sizes particularly slender, large, Plumpy, Cocco-bacilli

Poles/edges of individual cell: Rounded, Club-shaped, Sharp-cut/square, Branched

Cellular arrangement: Discrete/scattered/singly arranged, Chain formed-short chain and long chain.

3.11. Biochemical tests

After presumptive identification of bacteria based on the microscopic growth characteristics, colony morphology and staining of the isolates and further their biochemical characteristics were determined. Different biochemical tests like Indole test, Methyl Red test, Vogues-Proskaur test, Citrate utilization test, Urease test, and Catalase test was performed to determine the enzymatic activity and metabolic pathways.

Coagulase test

The test was conducted as described by Cowan (2004) and Carter (1979). For the coagulase test, horse plasma was used. Diluted (0.5ml) rabbit plasma (1:5 with sterile physiological saline). A simple slide coagulase test (Carter, 1979) was performed as a presumptive test. 1-2 drop of diluted plasma was mixed with an equal volume of freshly cultured broth (or emulsified colony in a drop of water) of a particular organism on a microscopic slide. A positive result was indicated by macroscopically clumping of the bacterial cells within five seconds. Pathogenic Staphylococci showed

coagulase positive reaction whereas non-pathogenic Staphylococci showed negative reaction.

Catalase test

This test was used to differentiate bacteria, which produce the enzyme catalase, such as Staphylococci, from that non-catalase one such as, Streptococci (Cowan, 2004). A loopful of bacterial colonies mixed properly with a drop of 3% Hydrogen peroxide solution on a clean glass slide. The production of gas bubbles within few seconds was considered as positive test.

Indole test

Peptone water (2 ml) was inoculated with the 5 ml of bacterial culture and incubated at 37°C for 48 hours. Kovac's reagent (0.5 ml) was added, shake well and examined after 1 minute. A red or pink color in alcohol layer indicated a positive reaction.

Methyl Red test

The test was conducted by inoculating a colony of the organism in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37°C, a drop of methyl red solution was added. A red coloration was positive and indicates an acidic pH resulting from the fermentation of glucose. A yellow coloration indicated negative result (Cheesbrough, 1985).

Voges-Proskauer test

Sterile glucose phosphate peptone water (2 ml) was inoculated with the 5 ml of test organisms and incubated at 37°C for 48 hours. A very small amount of creatine added and mixed. Sodium hydroxide (3 ml) was added and shakes well. The bottle cap was removed and left for an hour at room temperature. Positive reaction was indicated by the slow development of pink color.

Citrate utilization test

The Simmon's citrate agar medium was inoculated with culture and incubated at 37º C for 96 hours and a positive reaction was seen when the medium turned blue and growth was observed on streaks. A negative test was indicated by the original green color.

Oxidase test

The test was used to classify negative aerobic rods that produce cytochrome oxidase (Cowan, 1985). To perform the test, a piece of filter paper was placed in a clean Petridis and 2-3 drops of freshly prepared oxidase reagent were added. A colony of the test organism was smeared on the filter paper. Development of a pink color within a minute that changed finally to black on standing was considered as positive.

3.12. Isolation and identification of *Staphylococcus spp*

Isolation of Staphylococcus was based on the morphology and cultural characteristics. The colonies of Staphylococci are round, glistening, convex, smooth and opaque on Mannitol Salt agar. They are Gram-positive cocci arranged in cluster. B-hemolysis was produced by most strains on BA. The coagulase test was performed for the identification of the pathogenic Staphylococcus aureus from non-pathogenic ones.

3.13. Isolation and identification of *Escherichia coli*

For isolation of *Escherichia coli*, the samples were first inoculated on MC agar and incubated at 37°C for 24 hours. Colonies from the MC agar were subculture on EMB agar. Colonies on EMB agar with metallic sheen were suspected as positive and were confirmed by biochemical test IMViC test, E. coli was positive to indole and Methyl red tests but negative to VP and citrate tests.

3.14. Isolation and identification of *Pseudomonas spp*

For isolation of Pseudomonas the samples were first inoculated into NB at 37°C for 48 hours. On NB it produced greenish pigment. Then the samples were inoculated into BA, where it produces grayish irregular, effuse, rough colonies. On Grams staining, Gram negative rod shaped with rounded end bacteria arranged in small bundles or chain form were found. It was then confirmed by biochemical test.

3.15. Isolation and identification of *Bacillus spp*

For the isolation and identification of Bacillus, the samples were diluted and inoculated on BA and then incubated at 37°C for 24-48 hours. On Gram's staining the Gram positive large sporulated rod-shaped bacteria in chain form indicated Bacillus.

3.16. Molecular identification of *nuc* gene for confirmation of *S. aureus*

Identification of bacteria was also performed by non-culture method (molecular technique). For this purpose amplification technique like Polymerase chain reaction (PCR) was used. All Staphylococcal isolates were sub-cultured on blood agar. Bacterial genomic DNA was extracted using crude boiling lysis method. Finally, coagulase positive *S. aureus* was confirmed by the PCR amplification of *nuc* gene (a characteristic thermonuclease gene of *S. aureus*).

The primer sequences used were: au-F3 (Forward) 5′ TCGCTTGCTATGATTGTGG 3′ and au-nucR (Reverse) 5′ GCCAATGTTCTACCATAGC 3′. The amplification condition was initial denaturation at 95°C for 2 minutes, followed by 30 cycles of final denaturation at 95°C for 30s, annealing at 56°C 35s, initial extension at 72°C for 60s and final extension at 72°C for 2 minutes. For positive and negative controls respectively *S. aureus* ATCC 29213 strain and nuclease free water was used.

Electrophoresis of PCR products

The amplified products were analyzed using horizontal submarine gel electrophoresis in 1.5% agarose gel. A separate well charged with 100 bp and 250 bp DNA ladder was allowed to run simultaneously. After sufficient migration, the amplified product was visualized and confirmed over gel documentation system. The relative molecular weight of the amplified product was calculated against 100 bp.

3.17. In-vitro antibiotic sensitivity test

In-vitro antibiotic sensitivity test was performed against different pure colonies at primary isolation from uterine samples of repeat breeder crossbred cows using six different antibiotic disc by standard disc diffusion technique. Each uterine sample was streaked on nutrient agar and the plate was incubated at 37°C for 24 hours. Further from mixed bacterial colonies, uniform colonies were selected and streaked on selective media. Specific bacterial colony from selective media was inoculated in nutrient broth and incubated for 6-8 hours until turbidity developed. Then the broth culture was spread over the surface of Muller Hilton agar plates with the help of a sterilized cotton swab. The inoculated plate was dried in the incubator at 37°C for 10

min. six antibiotic disc were used for antibiotic sensitivity test which were Ciprofloxacin (10µg), Ceftriaxone (10µg), Gentamicin (50µg), Tetracycline (10µ), Ampicillin (10µg) and Penicillin (10µg) placed over the inoculated plate at equal distances. The plates were incubated at 37° C in an inverted position and results were observed after 12 hours. The zone of inhibition was measured by scale in mm and sensitive antibiotic was determined on the basis of zone size interpretation chart provided by manufacturer. Most sensitive antibiotic was determined on the basis of maximum zone of inhibition.

3.18. Treatment of Repeat Breeders

Treatment of repeat breeding depends on diagnostic classification of clinical cases in to infectious and non-infectious categories. The line of treatment differs in both the categories and also with diagnosis of particular cause of the repeat breeding.

Serial no	Group	No. of animals	Treatment protocol
1	A	10	GnRH during AI
2	B	10	GnRH at day 6 of post AI
3	C	10	GnRH at day 12 of post AI
4	D	10	Intrauterine infusion, 1st dose on onset of estrus, 2nd dose 8 hours after AI
5	E (control)	10	Untreated with any hormonal or antibiotic drugs

3.18.1. Hormones and antibiotics

Gonadotrophin releasing hormone (GnRH)

Inj Ovurelin contains gonadorelin acetate, a decapeptide identical to the endogenous Gonadotrophin releasing hormone (GnRH). Each ml of the vial contains gonadorelin acetate equivalent to 100µg, in 20ml vial marketed by Renata Pvt. Limited.

Gentamicin

It belongs to aminoglycosides, acts on 30S ribosomes is most potent antimicrobial agent with broad spectrum activity. They are highly active against gram negative and

gram positive aerobes. Gentamicin is available as Inj. Genacyn vet in market was used for intrauterine infusion. Inj. Genacyn vet containing 10% concentration in 10ml vial is marketed by Square Pharmaceutical Ltd.

3.18.2. Treatment protocols

Treatment protocols of hormonal and non-hormonal therapies with control group were undertaken in repeat breeder crossbred cows for trial and the details are as under.

Group A

All the repeat breeder's cows in this protocol were administered on the day of estrus with inj. Ovurelin 2.5ml intramuscularly at the time of AI.

Group B

All the repeat breeder's cows in this protocol were administered on the 6^{th} day of post AI with inj. Ovurelin 2.5ml intramuscularly.

Group C

All the repeat cows in this protocol were administered on the 12^{th} day of post AI with inj. Ovurelin 2.5ml intramuscularly.

Group D

All the repeat breeder's cows in this protocol underwent intrauterine infusion. First dose on onset of estrus, 2^{nd} dose 8 hours after AI. Antibiotics Gentamicin 10%, 10ml diluted with equal volume of normal saline was infused into each uterine horn.

Group E (control)

No treatment was given to the repeat breeder's cows from control group throughout the period of study. However, those animals detected in estrus were inseminated at proper time.

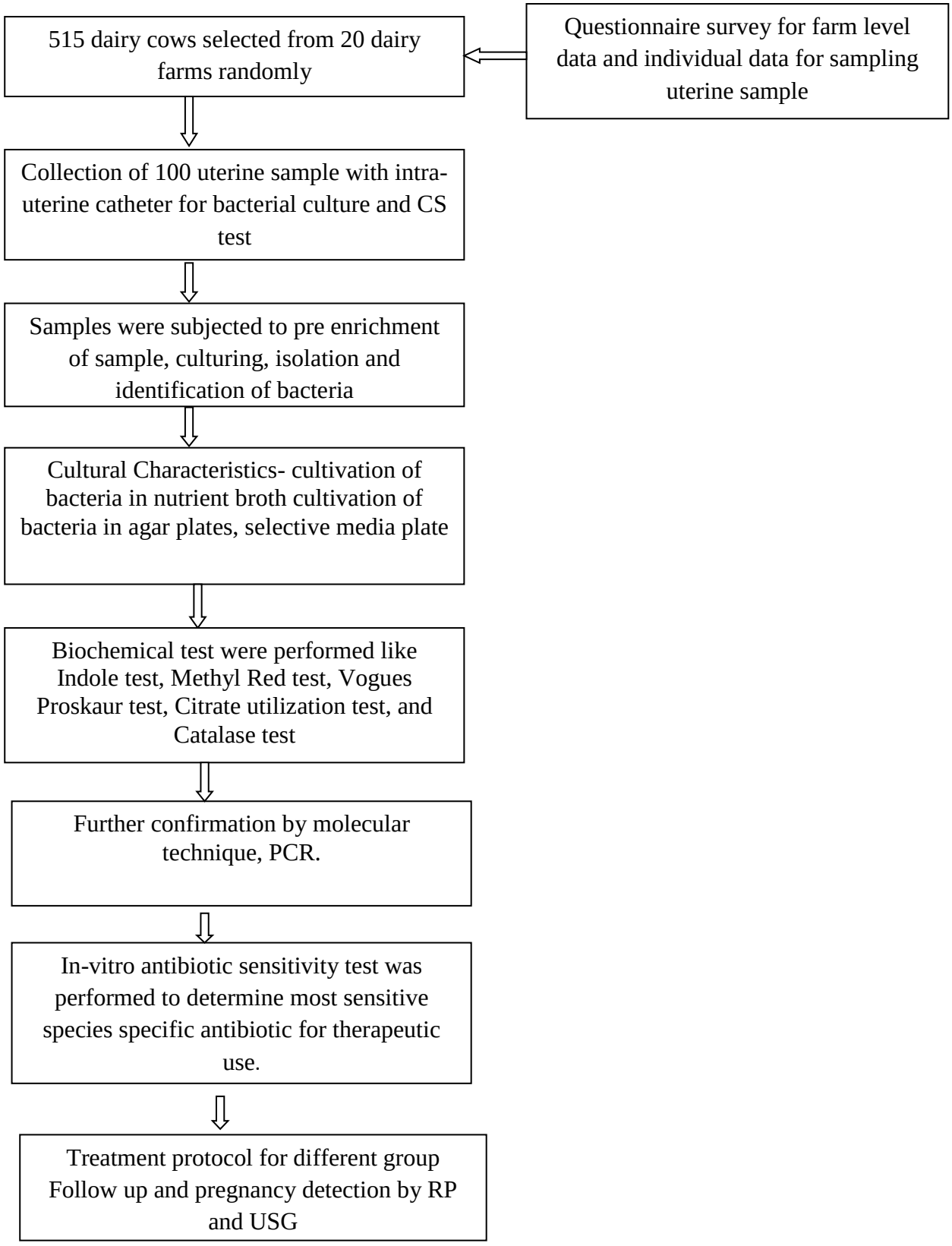
3.18.3. Follow up and pregnancy diagnosis

Cows from all five protocol groups were followed. Rectal palpation and transrectal ultrasonography were performed on each and every cow for detection and conformation of pregnancy within 2-3 months of AI. The pregnancy positive cows were considered as conceived. The reproductive data was properly recorded.

3.19. Statistical analysis

The data were stored in a Microsoft Excel spreadsheet (Microsoft Corporation) and analyzed using STATA version 13.0 for Windows (College Station, Texas, USA). For univariate analysis all explanatory variables were tested with outcome variable (1= Repeat breeder and 0=Normal) using 2x2 table and measures of association was determined using Chi-square test. The potential risk factors at cow level were screened through the univariate measures of association and exposures with P value of ≤0.05 were considered to recruit in multivariate analysis. Finally basic model of multivariate effect logistic regression was constructed to identify potential risk factors of Repeat Breeding in commercial dairying in Chittagong.

Flow chart for identification and characterization of uterine microorganisms and selection of treatment strategy in repeat breeding cows to increase conception rate in dairy farms in Chattogram



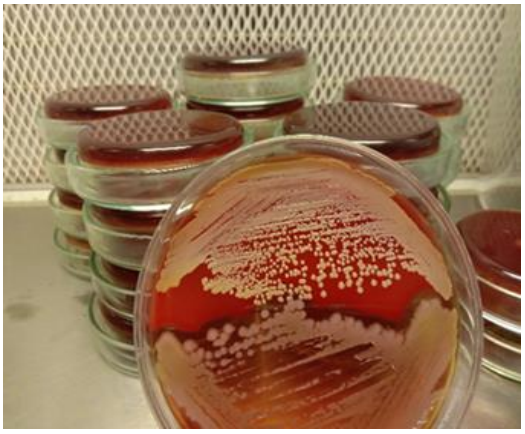


Fig. 3: *Staphylococcus* in Blood agar

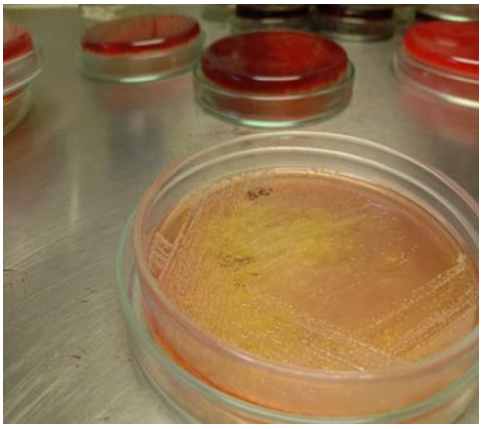


Fig.4: *Staphylococcus* in Mannitol Salt agar

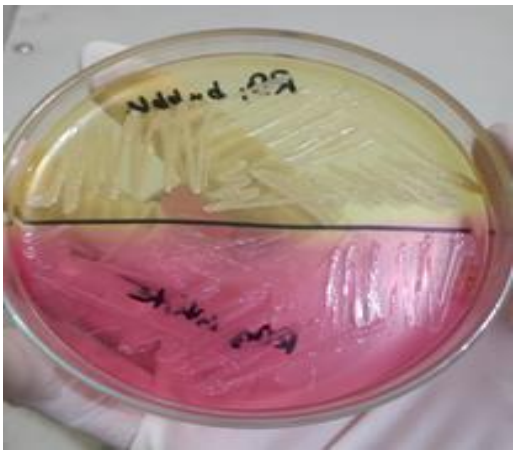


Fig. 5: *S. aureus* and *S. epidermidis*

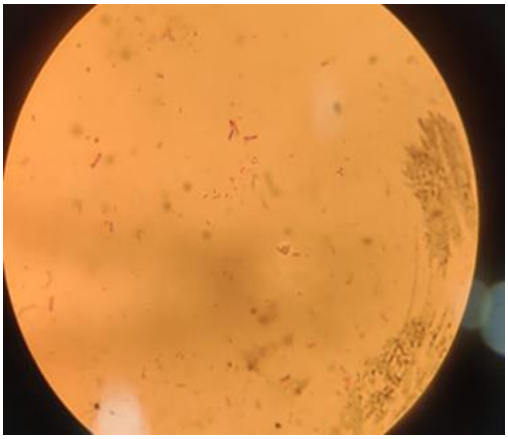


Fig.6: *Bacillus spp.* (Gram's stain)

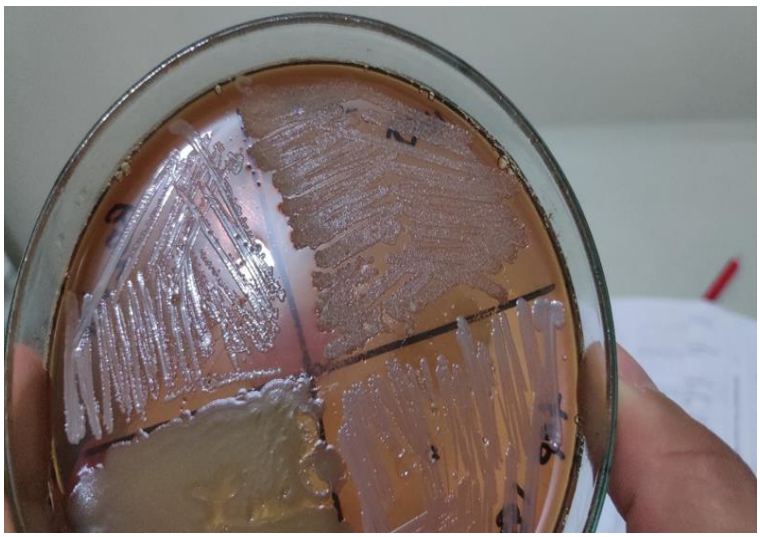


Fig. 7: *Staphylococcus aureus* on top left and bottom right, *Bacillus subtilis* on top right and bottom left on Blood agar

Fig. 8: *E.coli* in Blood agar

Fig. 9: *E.coli* in MacConkey Agar

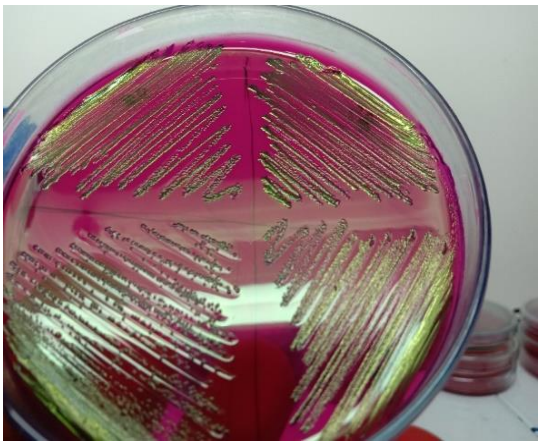


Fig. 10: *E.coli* in EMB agar

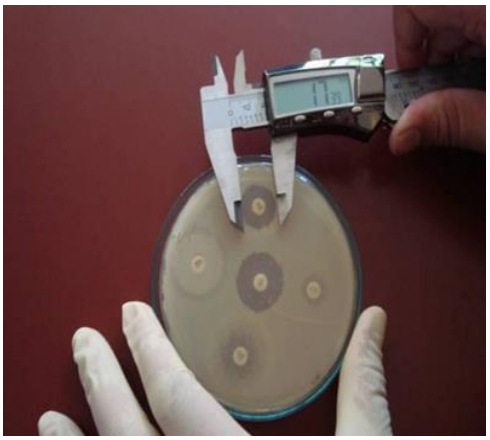


Fig 11: Culture Sensitivity Disc

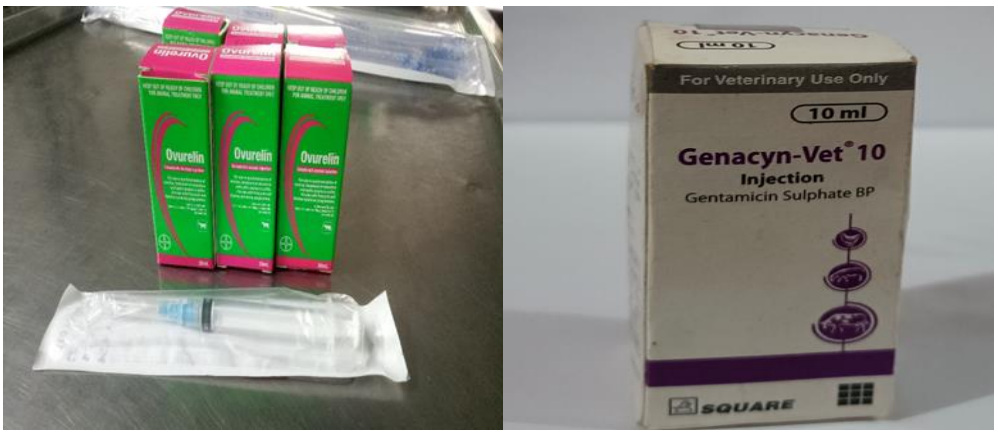


Fig.12: GnRH and Gentamycin drugs

Indole test positive

Catalase test positive

MR test (+ -)

VP test (+ -)

Fig 13: Biochemical Test

Chapter 4

Results and Discussion

The overall prevalence of repeat breeding cows in Chattogram area was 12.23%. It was observed that, 64% repeat breeders examined were found to be positive for bacterial isolation. Out of the 50 RB cows, 32 samples (64%) yielded 75 bacterial isolates; where Staphylococcus was predominant 27 (36%), followed by Bacillus 22 (29.33%), E. coli 17 (22.66%), Pseudomonas 9 (12%). The most common species identified from the uterine samples were Staphylococcus aureus and Bacillus subtilis. The ABST in the present study revealed that the maximum number of bacterial isolates were sensitive to gentamicin (85.33%) and ceftriaxone (81.33%) followed by ciprofloxacin (80%) and tetracycline (49.33). The lowest sensitivity was observed for ampicillin (24%) and penicillin (14.66 %). GnRH on the day of AI increased conception rate (60%) compared with other treatment given in this study. Whereas, intrauterine infusion with Gentamicin 10% improved conception rate (50%).

4.1. Overall prevalence study of RB cows

Table 1. Prevalence of RB in cows with respect to farm

SN.	Name of the farm	Total breedable cows	No. of RB cows	No. of sample collection		Prevalence of repeat breeding cows (%)
				Normal fertile cows	Repeat breeder cows	
1	Furken Dairy Farm	23	3	2	2	13.04
2	Osman Dairy Farm	25	5	3	3	20.00
3	Ma Dairy Farm	17	2	2	2	11.76
4	Sahagad Dairy Farm	22	3	2	2	13.64
5	AM. Dairy Farm	19	2	2	2	10.53
6	RN. Dairy Farm	21	3	3	3	14.29
7	Faizal Dairy Farm	26	3	2	2	11.54
8	Amin Dairy Farm	31	4	3	3	12.90
9	Liza Dairy Farm	35	6	3	3	17.14
10	Super Dairy Farm	40	2	2	2	9.09
11	Tajmir Dairy Farm	27	3	3	3	11.11
12	Bhuiya Dairy Farm	34	4	3	3	11.76
13	Samia Dairy Farm	25	3	3	3	12.00
14	Pusti Dairy Farm	22	2	2	2	9.09
15	J.N. Dairy Farm	30	3	3	3	10.00
16	Green Dairy Farm	35	5	3	3	14.29
17	Khaja Dairy Farm	22	2	2	2	9.09
18	Chowdhury Farm	30	3	2	2	10.00
19	Jahangir Dairy Farm	23	3	3	3	13.04
20	Saara Agro Farm	19	2	2	2	10.53
	Total	515	63	50	50	12.23

Total 20 registered dairy farms were selected for the survey in Chattogram regions. A total of 515 breedable cows were in the source population. Among the 515 cows 63 (12.23%) cows were repeat breeders. Sample were collected from 50 fertile cows and 50 repeat breeder cows. The prevalence of repeat breeding in cows with respect to different farm is presented in Table 1. The overall prevalence of repeat breeding in cows was 12.23% and numerically the lowest prevalence was observed as 9.09% and the highest prevalence was observed in Osman Dairy Farm (17.86%). Moreover, the prevalence of repeat breeding in cows was almost similar in different small holding dairy farms in the present study.

The present study recorded the overall prevalence of repeat breeding cows to be found 12.23% (Table 1), which in close agreement with the earlier findings of Asaduzzaman et al. (2016) who reported 11.5%. In Sweden, the prevalence of repeat breeding in dairy heifers was 10% (Bage et al., 2002). Similar to present study, the reported prevalence of repeat breeding in cows in Bangladesh was 11% (Boettcher and Perera, 2007). Gustafsson and Emanuelson (2002) also reported the overall prevalence of repeat breeding 10.1% in cattle of Swedish dairy herds. Karwani and Sharma (2003) observed that the overall incidence of repeat breeding in cattle and buffaloes was 19.61% and 15.6%, respectively which is slightly higher than the current study. Higher prevalence rate of RB cows were reported by Bhat et al. (2012) and Sarder et al. (2001) as 28.31% and 20.2%, respectively. However, lower prevalence of RB 4.2% was recorded by Narladkar et al. (1994).

The variations might be due to study design, difference in geographical location, agro climatic zones individual variations and study size. According to previous report in our country, 13.0 to 22.0% cows had been identified as repeat breeders, of which 81.9% repeat breeding occurs due to infectious agents (Samad, 1996). Karwani and Sharma, (2003) found that seasonal incidence of repeat breeding in cattle was highest (21.9%) in humid hot summer followed by autumn (20.6%). But, the seasonal incidence of repeat breeding was not included as a variable in the present study. The present study however, has a reason to argue with earlier report, as RB cows supposed to be healthy cyclic cows and does not necessarily resulted from any infectious cause. Therefore, the prevalence of RB as recorded in the present study was more acceptable and indicates the overall RB problem in commercial dairying in Chattogram.

4.2. Occurrence of RB cows on the basis of breed, age, parity and BCS

Table 2. Univariate logistic regression analysis of Repeat Breeder cows associated with animal factors

Variable	Category	RB, n (%)	95% CI	P value ($\chi 2$ test)	OR	95% CI	P value (LR)
Breed (n=515)	Local (33)	3(9.09)	1.91-24.33	0.794	Ref		
	Sahiwal (55)	6(10.91)	4.11-22.24		1.22	0.28-5.26	0.785
	HF cross (427)	54(12.65)	9.64-16.17		1.44	0.42-4.90	0.552
Age (n=515)	3-4(191)	19(9.95)	6.09-15.09	0.36	Ref		
	5-6(156)	19(12.18)	7.49-18.36		1.25	0.63-2.46	0.508
	7-10(168)	25(14.88)	9.86-21.17		1.58	0.83-2.99	0.157
Parity (n=515)	1(120)	17(14.17)	8.47-21.71	0.019	Ref		
	2-3(168)	11(6.55)	3.31-11.41		0.42	0.19-0.94	0.035
	4-6(134)	17(12.69)	7.56-19.53		0.88	0.42-1.81	0.730
	7-10(93)	18(19.35)	11.89-28.85		1.45	0.70-3.00	0.313
BCS (n=515)	3-3.5(223)	13(5.83)	3.14-9.76	<0.001	Ref		
	2.5(208)	20(9.62)	5.97-14.45		1.71	0.83-3.54	0.144
	1-2(84)	30(35.71)	25.55-46.91		8.97	4.38-18.3	<0.001

Univariate logistic regression analysis revealed that, the prevalence of occurring RB was significantly varied with parity and BCS at CI (95%). No significant effect was observed for breed and age of animal.

Table 3. Multivariable logistic regression model to evaluate the effect of different animal factors on repeat breeding

Variables	Categories	Multiple logistic regression		
		OR	95% CI	P Value
BCS (n=515)	3-3.5(223)	Ref		
	2.5(208)	1.33	0.58-3.02	0.495
	1-2(84)	7.49	3.46-16.21	<0.001
Parity (n=515)	1(120)	Ref		
	2-3(168)	0.62	0.26-1.47	0.285
	4-6(134)	1.25	0.53-2.89	0.603
	7-10(93)	1.12	0.49-2.54	0.776

OR= Odds Ratio, CI = Confidence Interval

Final multivariate logistic regression model showed that cows having poor BCS(1-2) possess more than 7.49 times higher probability (risk) of being repeat breeder than cows having fair body condition score. While, cow having fair BCS (2.5) is only 1.33 times at the risk of being repeat breeder. Above table shows that as the parity of cow increases, the changes of being repeat breeder also increases. Cows with parity (7-10) are more prone (1.12) times than cows with first parity (95% CI).

4.2.1. Occurrence of repeat breeding in cows with respect to breed.

The prevalence of repeat breeding in cows with respect to breed is presented in (Table 2). The lowest occurrence of repeat breeding was observed in local cows (9.09 %) and the highest occurrence was observed in Friesian cross cows (12.65%). This study failed to identify significant effect among the breed group with the prevalence of repeat breeder (P>0.05). On contrary to the findings, Singh et al. (1983) reported that Holstein, Sahiwal cows and their crosses are very much prone to be a repeat breeder.

Higher prevalence of repeat breeding has also been reported in cross breed cows than those of local breed counterpart (Mandefro and Negash, 2014).

Kaikini et al. (1981) found 32.11% incidence of reproductive disorders in cattle and also reported incidence of repeat breeding as 21.9% in crossbred cows which is less than our finding. Whereas, Singh et al. (1983) reported incidence of repeat breeding in crossbred cows between 7.4-18.6%. On contrary to the findings, Sahiwal breeds has highest (21.7%) incidence of Repeat Breeding (Karwani and Sharma, 2003) and Saxena et al. (2004) found the incidence of Repeat Breeding to be 20.4% in Sahiwal breeds, The reasons for lower occurrence of repeat breeding in local cows can be explained by the fact that local cows are more resistant to environment of Bangladesh than that by Friesian cross cows. The higher incidence may be due to use of Sahiwal in breeding programs using frozen semen which on thawing has lower motility (Karwani and Sharma, 2003).

4.2.2. Effects of age on occurrence of RB cows

Effects of age of cows on occurrence of repeat breeding are presented in (Table 2). The association of age with the repeat breeding was not significant ($P>0.05$). Brooks (1998) found no statistically significant difference in the incidence of repeat breeders occurring in first lactation heifers compared to older cows which favors this study.

However, among the affected cows, significantly higher proportion of cows (14.88%) was affected with repeat breeding at 7-10 years of age than that of 3-4 age (9.95%) and (12.18%) 5-6 years. It has been reported that age impacts negatively on fertility (Hodel et al., 1995) and higher repeat breeding rates have been reported in older cows (Hewett, 1968; Gustafsson and Emanuelson (2002)). This may be due to variations in hormonal levels or different ability of the ovarian response between different age groups (Bullman and Lamming, 1978).

4.2.3. Effects of parity on occurrence of RB cows

Effects of parity of cows on occurrence of repeat breeding are presented in Table 2. Among the affected cows, significantly lower proportion of cows (6.55%) were affected with repeat breeding at 2^{nd} - 3^{rd} parity than that of cows at 4^{th} - 6^{th} (12.69%) and 7^{th}-10^{th} (19.35%) parity ($P<0.05$). The result shows that the as parity of cow

increases, the chances of being repeat breeder also increases. Cows with parity (4-6) are more prone (1.25) times than cows with first parity (Table 3).

In contrast, Coleman et al. (1985) reported a higher conception rate in multiparous cows than that in primiparous cows. Boyd and Reed (1961) reported an increased conception rate with advancing parity from 2 to 6, and then declined at parities 7 and 8. This may be explained by the fact that cows with first parity may suffer from more negative energy balance than that of other parities resulting in high occurrence of repeat breeding. Because, first parity cows need more energy supplementation for milk production and its growth, and usually, the farmers supply equal quantity of feed supplement to all parous cows.

4.2.4. Effects of BCS on occurrence of RB cows

Effects of BCS of cows on occurrence of repeat breeding are presented in Table 2. Among the affected cows, significantly lower proportion of cows (5.83%) suffered from repeat breeding at BCS 3-3.5 than that of cows at BCS 2.5 (9.62%) and 1-2 BCS (35.71%) ($P<0.05$). The results from multivariate logistic regression model showed that cows having poor BCS (1-2) possess more than 7.49 times higher probability (risk) of being repeat breeder than cows having fair body condition score. While, cow having fair BCS (2.5) is only 1.33 times at the risk of being repeat breeder at CI (95%). Individual cows with low BCS suffer more from negative energy balance resulting in increased occurrence of repeat breeding. Negative effect of poor BCS resulting lower conception rate in cows has been documented (Shamsuddin et al., 2001).

4.3. Isolation, identification and molecular confirmation of predominant bacteria from uterine samples

Categorization of different bacteria isolated on the basis of selective media, morphology, Gram staining method, biochemical analysis and molecular techniques elaborated here under.

Staphylococcus spp.

On the basis of selective media i.e. Mannitol salt agar (MSA), Fig. 4 which was used in the present study, *Staphylococcus spp.* produced round, glistening, convex, smooth

and opaque. MSA helps to demonstrate the ability of a bacterium to grow in a 7.5% salt environment. Species of staphylococci are able to tolerate this salt concentration. Thus MSA selectively isolates *Staphylococcus* spp. On Gram staining, the colony from selective media showed the spherical shaped and cluster formed Gram positive cocci which appears as bunch of grapes. On performing biochemical tests, *Staphylococcus spp* showed Catalase positive, Methyl Red (MR) positive, Voges – Proskaure (VP) positive whereas, Indole negative, Citrate negative. Fig.13

Bacillus spp

On Gram's staining the Gram positive large sporulated rod-shaped bacteria in chain was found (Fig.6). It was late confirmed by biochemical test. *Bacillus spp*. showed Catalase positive, Citrate positive, Voges – Proskaure (VP) positive whereas, Indole negative, Methyl Red (MR) negative. Many rod shaped Bacilli produced β-hemolysis on blood agar.

Escherichia coli

On the basis of selective media i.e. Eosin Methylene Blue Agar (EMB Agar) *Escherichia coli* produced metallic sheen in EMB agar (Fig. 9) EMB agar is both selective and differential culture media. EMB media assist in visual distinction of *E. coli*. The combination of the two dyes eosin and methylene blue inhibits most Gram positive bacteria but allows many Gram negative organisms to grow. In EMB agar *E.coli* colonies have a characteristic green metallic sheen because it cause rapid fermentation of lactose and produces strong acids thus there is rapid reduction in the pH of the EMB agar which is the critical factor in formation of green metallic sheen with dark center observed in *E. coli*. On Gram staining, the colonies from selective media, they were Gram negative, rod shaped and arranged singly. On biochemical analysis E. coli showed reactions like Indole positive, Methyl Red (MR) positive, Catalase positive, whereas Voges-Proskaure (VP) negative and Citrate negative. The results revealed that most commonly present predominant genital bacteria were Gram-negative bacilli i.e. *E. coli.*

Pseudomonas spp.

On NB it produced greenish pigment. Then the samples were inoculated into BA, where it produces grayish irregular, effuse, rough colonies. On Grams staining, Gram

negative rod shaped with rounded end bacteria arranged in small bundles found. On biochemical test it was found that *Pseudomonas spp.* reacted as Catalase and Citrate positive whereas Indole, Methyl red (MR), Voges-Proskaure (VP) negative (Fig. 13).

4.3.1 Molecular identification of nuc gene for confirmation of S. aureus

All Staphylococcal isolates were sub-cultured on blood agar. Bacterial genomic DNA was extracted using crude boiling lysis method. Finally, coagulase positive S. aureus was confirmed by the PCR amplification of nuc gene (a characteristic thermonuclease gene of S. aureus). For PCR the primer sequences used were: au-F3 (Forward) 5′ TCGCTTGCTATGATTGTGG 3 and au-nucR (Reverse) 5′ GCCAATGTTCTACCATAGC 3′. The amplification condition was initial denaturation at 95°C for 2 minutes, followed by 30 cycles of final denaturation at 95°C for 30s, annealing at 56°C 35s, initial extension at 72°C for 60s and final extension at 72°C for 2 minutes (1). For positive and negative controls respectively S. aureus ATCC 29213 strain and nuclease free water was used.

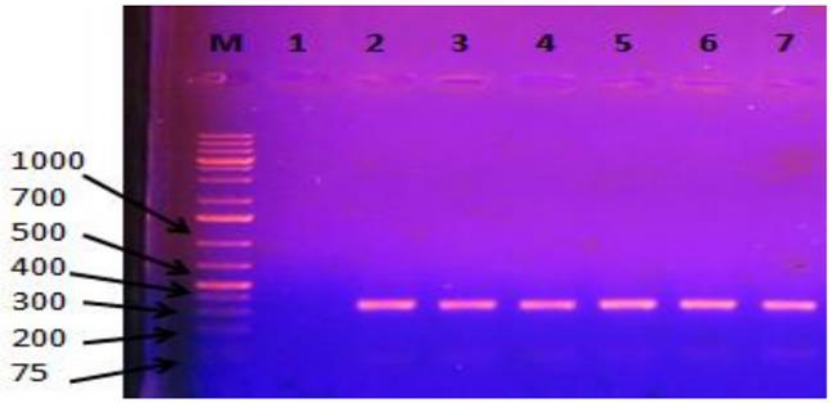


Fig.14: Electrophoresis image of PCR products of *Staphylococcus aureus* isolates showing specific amplified bands on 1% agarose gel. M = 1 kb plus DNA Marker. L2-L7 = Staphylococcus aureus Positive band L1 = Negative control.

4.4. Rate of isolation of bacteria per sample

In the present study, bacterial isolates were 28% from samples of normal fertile cows, whereas 64% from repeat breeder cows (i.e. 32 out of 50) (Table 4). This finding is in close agreement with (Gani et al., 2008) who reported (69.6%) bacteria isolated from the repeat breeder cows and (30.4%) from normal fertile cow's uterine samples. However, a higher percentage 76.9%, 81.9%, 87.2% of such bacterial infections of the repeat breeder cows were observed by Dholakia et al. (1987), Murthy et al. (1974) and Rahman et al. (1984), respectively. Similarly, Zahid (2004); Shukla and Sharma (2005) and Zaman *et al*. (2015**)** also reported the higher percentage (i.e. 89, 91.12 and 91.11%) of bacterial growth in repeat breeder animal. Therefore, our findings are in concurrence with all previous researchers.

In the present study the overall ratio of bacterial isolates per uterine sample obtained from normal fertile and repeat breeder cows are shown in the table 4. It is observed that in the repeat breeder cow, the overall isolate per sample ratio is 2.34 which is higher than the results observed in normal breedable cows i.e. 1.71. In the previous studies by (Decun and Rosu, 1973) and **(**Heist and Tanabe, 1974) the rate of bacterial isolation per sample from infected cows was reported as 1.30 and 1.90 respectively which is lower than our findings. Whereas Panangala et al. (1978) reported isolates per sample from normal fertile cows 2.89 which is slightly higher than our study.

Table 4. Number of bacterial isolates recovered per sample in normal fertile cows and RB cows

Group	Total number of Samples	No. of Positive sample	Total number of Isolates	Overall isolate/ sample ratio
Normal fertile cows	50	14 (28%)	24	1.71
Repeat Breeders Cow	50	32 (64%)	75	2.34

4.5. Different types of bacteria isolated from uterine samples

The predominant bacteria isolated from repeat breeder as well as normal animals were *Staphylococcus spp., Bacillus spp. Pseudomonas spp. and Escherichia coli* (Table 5) similar reports of isolation of bacteria from the genital tract of subfertile cows were reported by Murthy et al. (1974), Sirohi et al. (1989) and Singh et al. (1996). Out of 50 fertile cows only 14 cows exhibited bacterial growth and a total of 24 different kinds of isolates were obtained. Out of 24 isolates, 11 *Staphylococcus spp*, 10 *Bacillus spp* and 3 *E. coli* (Table 5).

Whereas out of 50 RB cows sample, 32 uterine samples showed bacterial growth and a total of 75 isolates were obtained. Out of 75 isolates, 27(36%) of *Staphylococcus spp*, 22(29.33%) of *Bacillus spp*. 17(22.66%) of *E. coli* and 9(12%) of *Pseudomonas spp* were obtained. Gani et al. (2008) reported the similar finding. We also found Bacillus spp 22.66% which is in agreement with Kamal et al. (2001). On the contrary Gani et al. (2008) found a higher percentage (35.1%) of *Bacillus spp* in RB cows. In this present study, it was found *Pseudomonas spp* 12% in RB cows which is slightly lower with the findings of Das et al. (1995) and Gani et al. (2008) who reported *Pseudomonas aeruginosa* at 17.3% and 18.9% respectively. Shwetha (2003), Bhat and Bhattacharyya (2012) and Udhayavel et al. (2013) also reported the isolation of these organisms from cervico vaginal sample of cows suffering from endometritis repeat breeding. Similar to the present findings, Panangala et al. (1978) and Ali et al. (2010) has reported that the most frequently isolated bacteria from fertile and infertile cows were *E. coli*, *Staphylococci*, *Proteus*, *Pseudomonas*, and *Bacillus spp*. Arora et al. (2000) reported isolation of *Escherichia coli, Staphylococcus aureus, Proteus spp., Streptococci, Bacillus spp., Staphylococcus epidermis, Klebsiella pneumonia, Pseudomonas aeruginosa and Corynebacterium pyogenes* from repeat breeder cows and buffaloes.

Nonspecific bacterial infections of genital tract of repeat breeder cows play a major role in causing inflammation of endometrium which results in early embryonic death. Therefore, frequently bacterial examination of cervico vaginal mucus has been suggested for effective reproductive health control in dairy cows (Dholakia et al., 1987).

It is reported that about 60% of the identified bacteria from normal uterus were also found in repeat breeders as well as from endometritis cows and the frequency of the isolates were much higher than that of normal cows (Panangala et al., 1978; Singh and Pant, 1999; Gani et al., 2008 and Azizunnesa et al., 2011).

The different bacterial isolates recovered from endometritic repeat breeder cows are widespread distributed in nature and have reported to be associated with a large variety of nonspecific infections of the genital tract of cows and buffaloes leading to reduced fertility (Chandrakar et al., 2002). All these bacteria which are responsible for non-specific infection of genitalia cause infertility in the form of repeat breeding or abortion.

Table 5. Different types of bacteria isolated from sample of normal fertile cows and RB cows

Type of bacteria		Number of Isolates from Normal fertile cows	Number of Isolates from Repeat Breeders Cow
Gram Positive	*Staphylococcus*	11	27
	Bacillus	10	22
Gram Negative	*E.coli*	3	17
	Pseudomonas	0	9
Total Isolate		24	75

4.6. Antibiotic sensitivity test (ABST)

Table 6. Percentage sensitivity of different bacterial isolates to different Antibiotic

	Number of isolates sensitive to different antibiotics						
Name of Organism	No. of Isolates	Gentamicin	Ceftriaxone	Ciprofloxacin	Tetracycline	Ampicillin	Penicillin
Staphylococcus	27	27	25	24	16	9	6
Bacillus	22	20	20	19	13	5	3
E.coli	17	15	14	15	8	4	2
Pseudomonas	9	2	2	2	0	0	0
Total	75	64	61	60	37	18	11
Sensitivity %		85.33	81.33	80.00	49.33	24	14.66

Antibacterial agents are widely used in treatment of infections of reproductive tract of bovines. However the efficacy of therapeutic agents needs to be evaluated from time to time due to continuous emergence of drugs resistance bacterial strains) and emergence of resistant mutants (Maness and Sparling, 1973) which presents a constantly changing pattern in susceptibility of microorganisms to the broad spectrum of antibiotics that are presently in use.

In the present study as indicated in the Table 6, the maximum numbers of bacterial isolates were sensitive to gentamicin (85.33%), ceftriaxone (81.33%), ciprofloxacin (80%) and tetracycline (49.33%). The lowest sensitivity was observed for Ampicillin (24%) and penicillin (14.66%). Individually, *Staphylococcus spp, Bacillus,* and *E. coli* were highly sensitive to gentamicin, ceftriaxone followed by ciprofloxacin and least sensitive to tetracycline followed by ampicillin and penicillin. While, *Pseudomonas spp* were resistant to tetracycline and ampicillin. Similar, finding was reported by Sadig et al. (2010) who observed that gentamicin was the highly sensitive antimicrobial drug against gram negative bacteria. In another study it has been observed that gentamicin and cephalexin were highly sensitive (93 and 77%, respectively) to aerobic bacteria, while oxytetracycline (42%) and amoxicillin (26%) were found to be resistant (Takamtha et al., 2013). In the earlier studies it has also been reported that, gentamicin are mostly effective in aerobic environment of the

postpartum uterus. Efficacy of sulfonamides and aminoglycosides may be decreased due to the presence of necrotic debris and purulent materials (Smith and Risco, 2002).

On the other hand, Singh et al. (1998) also observed maximum sensitivity of the isolates to gentamicin, tetracycline and chloramphenicol and recommended the antibiotics for the treatment of repeat breeding cows but in this investigation tetracycline was not encountered as effective drug and resistance to tetracycline was (49.33%). The variation in sensitivity may be related to geographical variation and injudicious use of antibiotics in a specific area. However, the result of sensitivity against the isolated bacteria flora indicated that this antimicrobial therapeutic use is more logical in treating sub-clinical uterine infection rather their questionable success for RB unless it's being generated of infectious origin.

The antibiotic susceptibility patterns needs to be evaluated time and again to decide the best available treatment for the uterine infections and to overcome the problem of drug resistance.

4.7. Different treatment protocol

Table 7. The efficacy of different regimens used for treatment of repeat breeding in cows

Group	Treatment regimens	No. of cows treated	No. of cows conceived	Conception rate (%)
A	2.5ml of GnRH at the time of AI	10	6	60
B	2.5ml of GnRH on the 6th day of post AI	10	2	20
C	2.5ml of GnRH on the 12th day of post AI	10	3	30
D	Intrauterine infusion with Gentamicin 10%	10	5	50
E	No treatment(control)	10	1	10

The present investigation clearly demonstrates that the repeat breeding cows can be managed by treating with single dose of GnRH, as the conception rate was (60%) (Table 7) at the time of AI. The improvement in conception rate after GnRH treatment in the present study is in agreement with the study of Patel et al. (2010) who studied efficacy of GnRH in 18 repeat breeder crossbred cows and found the conception rate up to 66.66%. Anjum et al. (2010) concluded that cows with Dalmarelin (GnRH analogue) at the time of AI improved 68.75% conceptions as against 37.50 % in control group which is nearly to our findings. Similarly, higher conception rate was reported by Rangnekar et al. (2002) 70.00%, Kumar et al. (2010) 73.68% and More et al. (2012) 75.00 per cent in repeat breeder cows. Whereas lower conception rate was reported by Jayakumar and Vahida. (2000) as 40%. In the present study GnRH doesn't proved to be effective in improvement of conception rate when administered on day 6th, and day 12th of insemination.

In the present study, conception rate in the repeat breeding cows was also improved (50%) after treating with intra uterine infusion of gentamicin. Mane et al. (2009) reported that repeat breeding animals treated with gentamicin sulphate, had 44.44 % conception rate which is nearby our findings. Whereas, Sarmah et al. (1993) and Gupta et al. (2005) recorded higher percentage of conception rate in gentamicin treated groups. Gentamicin when administered intrauterine attained maximum plasma concentration within 30 minutes (Al-Guedawy et al., 1983). These findings indicate that physiochemical characteristics of gentamycin are better compared to enrofloxacin in these repeat breeder cows (Warriach et al., 2008).

Protocol E or control group shows only 10% conception. This means few cases of repeat breeding cows can be recovered without treating with any antibiotics or hormones. This may be explained other way that sometimes cows might be inseminated either early or late leading RB.

It is concluded that GnRH and Gentamicin may be used as the most effective drug for the treatment of repeat breeding in animals at field condition as compared to other preparations.

Chapter 5

Conclusions

From the present study it can be concluded that, the overall prevalence of repeat breeding cows in Chattogram area was 12.23%. The prevalence of repeat breeding in cows was almost similar in different small holding dairy farms in the present study (12.3%). The breed of cows, herd BCS, age and parity influenced the prevalence of repeat breeding in cows. It was observed that, 64% repeat breeders examined were found to be positive for bacterial isolation. Of the 50 repeat breeders, 32 samples (64%) yielded 75 bacterial isolates; where Staphylococcus was predominant 27 (36%), followed by Bacillus 22 (29.33%), E. coli 17 (22.66%), Pseudomonas 9 (12%). The most common species identified from the uterine samples were *Staphylococcus aureus* and *Bacillus subtilis.*

The ABST in the present study revealed that the maximum number of bacterial isolates were sensitive to gentamicin (85.33%) and ceftriaxone (81.33%) followed by ciprofloxacin (80%) and tetracycline (49.33). The lowest sensitivity was observed for ampicillin (24%) and penicillin (14.66 %). The present investigation also clearly demonstrates that the repeat breeding cows can be treated with single dose of GnRH at the time of AI. The improvement in PR after GnRH treatment in the present study was 60%. In the present study, a good proportion of repeat breeding cows (50%) conceived after intrauterine infusion with Gentamicin 10%.

Chapter 6

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APPENDIX I

Identification and characterization of uterine microorganisms and selection of treatment strategy in repeat breeding cows to increase conception rate in dairy farms of Chattogram

Questionnaire

01. Name and address of the Farm: ……

02. Name of the Farm Owner: …… 03. Contact No: .....

04. Total No. of Cows in a Farm: .....

05. Presence of repeat breeder cow yes/ no; No. of repeat breeder: .....

06. Name/Tag no. of Cow: …..... 07. Breed: …….

08. Age: ..... 09. BCS: ..... 10. Body weight (kg): .....

11. Parity: ……

14. Nature of Feeding a. Roughage (Kg): ..... b. Concentrate (Kg): .....

15. History

a. Date of last calving..... b. Date of previous estrus: …

c. Date & Time of AI… d. No. of Previous (AI) done: …….

e. Source of semen: DLS /ACI /BRAC /ADL / Others: …….

f. Previous History of Diseases

Abortion /Still Birth /Retention of Placenta /Pyometra / Endometritis / Others

g. Previous History of Treatments: ……...

h. Vaccination: Yes / No

16. per rectal examination

a. Condition of uterus: gravid/ non gravid

b. Condition of ovary: Right ovary (smooth/ presence of Cl / follicles/ cyst)

Left ovary (smooth/ presence of Cl / follicles/ cyst)

17. Sample collection method: low volume lavage / cyto-brush / pipette method

APPENDIX II

Formula of Various Commercial Media

A. **Preparation of Media**

1. Nutrient Agar
   Lab-lemco Powder = 1.0 gm
   Yeast extract = 2.0 gm
   Peptone = 5.0 gm
   Sodium chloride = 5.0 gm
   Agar = 15.0 gm
   Distilled water = 1000 ml

2. Blood agar
   Blood agar base = 60 gm
   Distilled water = 1000 ml
   Bovine blood = 5 ml
Or
   Nutrient agar = 500 ml
   Sterile defibrinated blood = 25 ml

3. Eosin Methyline Blue Agar
   Peptone = 10 gm
   Sucrose = 5 gm
   Lactose =5 gm

Dipotassium phosphate = 2 gm
Agar =13.5 gm
Eosin = 0.4 gm
Methylene blue = 0.065 gm
Distilled water = 1000 ml

4. MacConkey Agar
   Peptone = 20.0 gm
   Lactose = 10.0 gm
   Bite salts = 5.0 gm
   Sodium chloride = 5.0 gm
   Neutral red = 0.075 gm
   Agar = 12.0 gm
   Distilled water = 1000 ml

5. Nutrient Broth
   Lab-lemco powder = 1.0 gm
   Yeast extract = 2.0 gm
   Sodium chloride = 5.0 gm
   Distilled water = 1000 ml

B. Preparation of Reagents:

1. Peptone water:
   Peptone -=I gm
   Distilled water = 1000 ml

2. 3 % Hydrogen Peroxide
   H_2O_2 - 3 ml
   Distilled water - 97 ml

3. V-P reagent-1
   5% alpha-naphthol in ethyl alcohol

4. Oxidase reagent:
   Tetramethyl-p
   Phenylenediamine = 0.1 ml
   Distilled water = 10 ml

5. Methyl red solution:
   Methyl red = 0.05 gm
   Ethanol (absolute) = 28 ml
   Distilled water = 22 ml

6. V-P reagent-2
   40% potassium hydroxide
   0.3% creatine

7. Phenol red solution
   0.2% aqueous solution

8. Bromthymol Blue Solution
   0.2% aqueous solution

9. Phosphate buffer saline (PBS):
   Sodium chloride = 8 gm
   Disodium hydrogen = 2.8 gm
   Potassium chloride = 0.2 gm
   Potassium dihydrogen = 0.2 gm
   Distilled water to make = 1000 ml

Gram Stain Solutions:
a. Stock Crystal violet:
   Crystal violet = 10 gm
   Ethyl alcohol (95%) = 1000 ml

b. Gram's Iodine solution:
Iodine crystals = 1 gm
Potassium iodide =2 gm

d. Decolorizer:
Ethyl alcohol (95%) = 250 ml

Acetone = 250 ml

Counterstain Safranin
Safranin = 250 ml
Ethyl alcohol (95%) = 250 ml

Biodata

The author of this manuscript, Dr. Chandra Jit Yadav S/o Mr. Jawahar Lal Yadav and Mrs. Indu Yadav, was born on 18 Sep, 1988 at Bishnu Rural Municipality, Sarlahi, Nepal. He passed his High school from Green Land secondary school, Malangwa and Intermediate from World Link Academy, Kathmandu in 2005 and 2007 respectively. Thereafter, he joined B.V.Sc. & A.H. degree in 2010 and passed in 2015 with the O.G.P.A of 7.1/10 from College of Veterinary & Animal Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India. He then joined the MS degree in Theriogenology 2019, in the Department of Medicine and Surgery, under Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University, Khulsi, Chittagong-4225, Bangladesh.

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