

**IDENTIFICATION, MOLECULAR CONFIRMATION AND
ANTIBIOTIC SENSITIVITY OF BACTERIA ISOLATED FROM
REPEAT BREEDER COWS IN RANGPUR DIVISION**

**A THESIS
BY**

**MST. MOUSUMI AFROJ
REGISTRATION NO.: 1504025
SEMESTER: JULY-DECEMBER, 2023
SESSION: 2022-2023**

**MASTER OF SCIENCE (MS)
IN
THERIOGENOLOGY**



**DEPARTMENT OF MEDICINE, SURGERY AND OBSTETRICS
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY
UNIVERSITY, DINAJPUR-5200**

DECEMBER 2023

**IDENTIFICATION, MOLECULAR CONFIRMATION AND
ANTIBIOTIC SENSITIVITY OF BACTERIA ISOLATED FROM
REPEAT BREEDER COWS IN RANGPUR DIVISION**

**A THESIS
BY**

**MST. MOUSUMI AFROJ
REGISTRATION NO.: 1504025
SEMESTER: JULY-DECEMBER, 2023
SESSION: 2022-2023**

Submitted to the
Department of Medicine, Surgery and Obstetrics
Faculty of Veterinary and Animal Science
Hajee Mohammad Danesh Science and Technology University, Dinajpur
In partial fulfillment of the requirements for the degree of

**MASTER OF SCIENCE (MS)
IN
THERIOGENOLOGY**



**DEPARTMENT OF MEDICINE, SURGERY AND OBSTETRICS
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY
UNIVERSITY, DINAJPUR-5200**

DECEMBER 2023

**IDENTIFICATION, MOLECULAR CONFIRMATION AND
ANTIBIOTIC SENSITIVITY OF BACTERIA ISOLATED FROM
REPEAT BREEDER COWS IN RANGPUR DIVISION**

**A THESIS
BY**

**MST. MOUSUMI AFROJ
REGISTRATION NO.: 1504025
SEMESTER: JULY-DECEMBER, 2023
SESSION: 2022-2023**

Approved as to style and content by

.....
Professor Dr. Begum Fatema Zohara
Supervisor

.....
Dr. Md Mominul Islam
Associate Professor
Co-Supervisor

.....
Professor Dr. Begum Fatema Zohara
Chairman
Examination Committee
And
Department of Medicine, Surgery and Obstetrics

**DEPARTMENT OF MEDICINE, SURGERY AND OBSTETRICS
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY
UNIVERSITY, DINAJPUR-5200**

DECEMBER 2023

**Dedicated
To
My
Beloved Parents**

ACKNOWLEDGEMENT

Firstly, I am highly obliged to almighty ALLAH for giving me support, strength, endurance and showered his blessing during the entire piece of work.

I take this opportunity to express extreme veneration and profound gratitude to my honorable Supervisor Professor Dr. Begum Fatema Zohara, Chairman, Department of Medicine, Surgery and Obstetrics, HSTU. I feel myself fortunate and greatly privileged to have worked under the supervision and guidance of Professor Dr. Begum Fatema Zohara. I am greatly indebted to her for her continuous optimism concerning this work, encouragement, sound advice and cooperation.

I would express my heartiest gratitude to my Co-Supervisor Dr. Md Mominul Islam, Associate Professor, Department of Pathology and Parasitology, HSTU to support during molecular part of my studies.

I am also grateful to Professor Dr. Md. Faruk Islam, Department of Medicine, Surgery and Obstetrics, HSTU for his valuable advice, encouragement, cooperation and support.

I feel enormously privileged to express my deep sense of gratefulness to the Chairman of Department of Microbiology and Department of Pathology & Parasitology to give me the opportunity for doing laboratory works in their Lab.

It was good opportunity for me to pay special thanks to all respected teachers, laboratory technicians and staffs of the Department of Medicine, Surgery and Obstetrics, Hajee Mohammad Danesh Science and Technology University, Dinajpur.

I would like to express my heartiest thanks to my friend Muhammad Mamunur Roshid for his moral support, valuable suggestions and kind hearted help. Special thanks to Sharif Md. Masud, Field man, Veterinary Teaching Hospital, HSTU for his help.

I cordially thanked the Ministry of Science and Technology for selecting me as one of their NST Fellow and University Grant Commission (UGC) for providing the financial support to complete this research work.

This thesis would not have been possible without the support and sacrifices of my beloved parents and husband. With deep emotions I acknowledge their love and affection.

Author

ABSTRACT

Recently repeat breeding syndrome becomes a significant challenge for dairy producer due to large economic loss in Bangladesh. The present study was conducted from January to June 2023 to observe the prevalence of repeat breeding syndrome, to isolate and identify the bacteria with their molecular confirmation and antibiotic sensitivity causing repeat breeding syndrome in cows in the northern region of Bangladesh. By using a questionnaire and history-taking techniques (more than 10 years old, one calf in life, three subsequent Artificial Insemination (AI) failures to conceive, having no perceptible anomalies of reproductive tract) to interview dairy owners, repeat breeding syndrome was identified. Bacteria were isolated and identified from the cervical mucus of affected cows. For confirmation of the detected bacteria, PCR was used. The agar disc diffusion method was utilized to investigate the antibiotic sensitivity of the detected isolates against widely used antibiotics in vitro. The prevalence of repeat breeding syndrome was 41.33%. The prevalence of *E. coli* was 40%, *Styphylococcus aureus* 40% and *Klebsiella spp.* 10%. Molecular detection of *E. coli* and *Styphylococcus aureus* was confirmed by using Eco 223 (F), Eco 455 (R), Sau 234(F) and Sau 1501(R) primers. The target genes were 16S and 23S rRNA and the size of the product amplified at 232 bp and 1267 bp respectively. According to the antibiogram profile, *E. coli* was resistant to ciprofloxacin, penicillin, tetracycline, amoxicillin, and erythromycin, but sensitive to ceftriaxone and gentamicin. While *Styphylococcus aureus* was resistant to ampicillin and erythromycin, it was sensitive to ciprofloxacin, ceftriaxone, and levofloxacin. It was also intermediately resistant to vancomycin and amoxicillin. *Klebsiella spp.* showed resistance to ceftriaxone, penicillin, and amoxicillin, while remaining sensitive to ciprofloxacin, tetracycline, and intermediate resistance to erythromycin and gentamicin. It should be concluded that *E. coli*, *Styphylococcus aureus* and *Klebsiella spp.* are the most common bacteria causing repeat breeding syndrome in cows. Treatment for repeat breeding syndrome should be done after performing antibiotic sensitivity test.

CONTENTS

CHAPTER	TITLE	PAGE NO.
	ACKNOWLEDGEMENT	i
	ABSTRACT	ii
	CONTENTS	iii-vi
	LIST OF TABLES	vii
	LIST OF FIGURES	viii-ix
CHAPTER I	INTRODUCTION	1-3
CHAPTER II	REVIEW OF LITERATURE	4-25
2.1	Reproductive physiology of cow	4
2.1.1	Hormonal Regulation of the Female Reproductive Tract	4
2.1.2	The Estrous Cycle	6
2.2	Problems associate with reproductive failure	9
2.2.1	Definition of Repeat Breeder	9
2.2.2	Prevalence of Repeat Breeding	10
2.2.3	Economic Impact of Repeat Breeding	11
2.3	Repeat Breeding and Its' Associated Risk Factors	11
2.3.1	Female factor	12
2.3.2	Male factors	12
2.3.3	Nutrition	13
2.3.4	Management	14
2.3.5	Hormonal factors	14
2.3.6	Genetic factors	14
2.3.7	Anatomical Defects of Genital Tract	14
2.3.8	Age	15
2.3.9	Parity	16
2.3.10	Peripartum abnormalities	16
2.3.11	Body condition	16
2.3.12	Milk yield	17
2.3.13	Season	17
2.3.14	Herd size	17
2.3.15	Heat detection	18

CONTENTS (Contd.)

CHAPTER	TITLE	PAGE NO.
2. 4	Causes of repeat breeding	18
2.4.1	Infectious Causes of Repeat Breeding	18
2.5	Pathogenesis of reproductive tract diseases	19
2.6	Uterine defense mechanism	19
2.7	Microflora isolated from reproductive tract	20
2.8	Molecular technique for bacterial confirmation: Polymerase Chain Reaction (PCR)	22
2.9	Antibiotic sensitivity test	22
2.10	Treatment of repeat breeding	23
2.10.1	Treatment of noninfectious repeat breeding category	23
2.10.2	Treatment of infectious repeat breeding category	24
CHAPTER III	MATERIALS AND METHODS	26-46
3.1	Study area	26
3.2	Data collection for prevalence study	26
3.3	Selection of cows for sample collection	27
3.4	Clinical examination	27
3.5	Collection of samples	28
3.6	Procedure of isolation and identification of bacteria	28
3.6.1	Laboratory preparation	28
3.6.2	Instruments and appliances	29
3.6.3	Media for culture	29
3.6.3.1	Liquid media (Broth)	29
3.6.3.2	Solid media	29
3.6.3	Chemicals, reagents, and solutions	30
3.6.4	Antibiotic discs	31
3.7	Preparation of culture media, broth, and media plate	32
3.7.1	Nutrient agar	32
3.7.2	MacConkey agar (MAC)	33
3.7.3	Eosin Methylene Blue agar	33
3.7.4	Mannitol Salt agar	33
3.7.5	Blood agar	33

CONTENTS (Contd.)

CHAPTER	TITLE	PAGE NO.
3.7.6	Muller-Hinton agar (MHA)	34
3.8	Isolation and identification of bacteria	34
3.8.1	Culture of bacteria	34
3.8.2	Morphology study by gram's staining method under microscope	35
3.8.2.1	The Procedure of gram's staining method	35
3.8.3	Biochemical test	36
3.8.3.1	Catalase test	36
3.8.3.2	Indole production test	36
3.8.3.3	Triple Sugar Iron (TSI) test	37
3.8.3.4	Methyl Red test	38
3.8.3.5	Voges-Proskauer (VP) test	38
3.8.3.6	Citrate utilization test	38
3.9	Antibiotic sensitivity tests	39
3.9.1	Antimicrobial susceptibility testing of isolated bacteria	40
3.10	DNA extraction	41
3.10.1	Materials used for isolation of bacterial genomic DNA	41
3.10.2	Procedure of DNA Extraction	42
3.11	Polymerase Chain Reaction (PCR)	43
3.11.1	Selection of primer for detecting <i>E. coli</i> and <i>Staphylococcus aureus</i>	43
3.11.2	Amplification of extracted DNA for confirmation <i>E. coli</i> and <i>Staphylococcus aureus</i>	43
3.11.3	Preparation of gel and gel-electrophoresis	45
3.12.	Statistical analysis	46
CHAPTER IV	RESULTS	47-63
4.1	Prevalence of major reproductive disorders in dairy cattle in Rangpur Division	47
4.2	Prevalence of Different Types of Bacteria Found in Repeat Breeding	48

CONTENTS (Contd.)

CHAPTER	TITLE	PAGE NO.
4.3	Characterization of bacteria by using different biochemical tests.	53
4.4	Results of morphological properties by Gram's Staining Technique	58
4.5	Result of antibiotic sensitivity test of isolated bacteria	60
4.6	Molecular confirmation of <i>E. coli</i> and <i>Staphylococcus aureus</i> by PCR technique	62
CHAPTER V	DISCUSSION	64-67
CHAPTER VI	CONCLUSION	68
	REFERENCES	69-90
	APPENDIX	91

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
1	Antibiotics with their disc concentration	31
2	Indication of TSI test.	37
3	Antimicrobial agents with their disc concentration and diameter of zone of inhibition	39
4	Properties of primer used in this study	43
5	Preparation of PCR product for Eco 223, Eco 455, Sau 234, Sau 1501 primer	44
6	Condition of PCR for <i>Staphylococcus aureus</i>	45
7	Condition of PCR for <i>E. coli</i>	45
8	Prevalence of major reproductive disorders in dairy cattle in Rangpur Division	47
9	Prevalence of Different Types of Bacteria Found in Repeat Breeding	48
10	The cultural characteristics of <i>E. coli</i> , <i>Staphylococcus aureus</i> , and <i>Klebsiella spp.</i> on various culture media	49
11	Characterization of <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> and <i>klebsiella spp.</i> using different biochemical tests.	54
12	Zone diameter interpretative standards for <i>E. coli</i>	60
13	Zone diameter interpretative standards for <i>Staphylococcus aureus</i>	61
14	Zone diameter interpretative standards for <i>Klebsiella spp.</i>	61

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE NO.
1	Diagram of a 21-day estrous cycle in which pregnancy does not occur. (Adapted from Deutscher, 1980)	6
2	Graph of the anatomical and hormonal changes that occur during a typical 21-day estrous cycle. (Adapted from Deutscher, 1980)	8
3	Selection of animal for sample collection	27
4	Collection of samples (a) sample collection, (b) sample in ice box, (c) sample in refrigerator.	28
5	Different kinds of Media	30
6	Different types of chemical reagents.	30
7	Antibiotics discs	32
8	Culture of Bacteria	35
9	Gram's staining	36
10	Antibiotic sensitivity test.	41
11	QIAamp [®] DNA mini kit (QIAGEN) used in DNA extraction	41
12	DNA extraction	42
13	PCR procedure	44
14	Gel-electrophoresis	46
15	Growth of <i>E. coli</i> on (a) nutrient agar (b) negative control	50
16	(a) Growth of <i>E. coli</i> on MacConkey agar (b) negative control	50
17	Growth of <i>E. coli</i> on (a) Eosin Methylene Blue agar (b) negative control	50
18	Growth of <i>Staphylococcus aureus</i> on (a) nutrient agar (b) negative control	51
19	Growth of <i>Staphylococcus aureus</i> on (a) blood agar (b) negative control	51
20	Growth of <i>Staphylococcus aureus</i> on (a) Mannitol salt agar (b) negative control	51

LIST OF FIGURES (Contd.)

FIGURE NO.	TITLE	PAGE NO.
21	Growth of <i>Klebsiella spp.</i> on (a) nutrient agar (b) negative control	52
22	Growth of <i>Klebsiella spp.</i> on (a) MacConkey agar (b) negative control	52
23	Growth of <i>Klebsiella spp.</i> on (a) Eosin Methylene Blue agar (b) negative control	52
24	Catalase test	55
25	Methyl Red test	55
26	Indole test	56
27	Voges-Proskauer test	56
28	Citrate Utilization test	57
29	Tripple Sugar Iron test	57
30	<i>Escherichia coli</i> under microscope	58
31	<i>Staphylococcus aureus</i> under microscope	59
32	<i>Klebsiella spp.</i> under microscope	59
33	Representative picture of antibiotic sensitivity test of <i>E. coli</i>	61
34	Representative picture of antibiotic sensitivity test of <i>S. aureus</i>	62
35	Representative picture of antibiotic sensitivity test of <i>Klebsiella spp.</i>	62
36	PCR amplification of <i>E. coli</i> .	63
37	PCR amplification of <i>Staphylococcus aureus</i> .	63

CHAPTER I

INTRODUCTION

Bangladesh is home to 24.856 million cattle, according to estimates. The cattle industry, which contributes roughly 1.85% of the nation's GDP and is expanding at a rate of 3.23% annually, is cited in statistics. 20% of jobs directly and 50% indirectly are provided by the livestock industry (Livestock economy, 2022-2023). One calf per cow per year is the primary reproductive goal of a dairy farm. After-insemination pregnancy rates are used to gauge a dairy farm's economic health (Jit Yadav, 2020). Repeat breeding (RB) is a substantial problem in cattle breeding leading to large economic loss for the dairy producer due to more inseminations, increased calving interval and increased culling (Lafi *et al.*, 1992; Bartlett *et al.*, 1986). Repeat breeding has been defined as failure to conceive from 3 or more regularly spaced services in the absence of detectable abnormalities (Zemjanis, 1980). Several studies on repetitive breeding syndrome have been carried out over the past 50 years. (Albihn, 1991; Gustafsson, 1985; Linares and King, 1980; Ayalon, 1978). Different scientists reported different opinions about the cause and treatment of repeat breeding. Many studies have reported that various factors lead to repeat breeder syndrome such as infectious agents by affecting the sperm or embryo, hormones deficiency and luteal problems, immunologic problem and anti-sperm antibodies, oocytes quality, nutrition, feeding, weather and season (Ferreira *et al.*, 2016; Yusuf *et al.*, 2010; Amiridis *et al.*, 2009). Many specific and non-specific uterine infections are associated with either fertilization failure or early embryonic mortality in repeat breeder cattle. Nonspecific infections of the genital tract invariably need some predisposing factor and generally involve an individual animal (Singh, 1998). In repeat breeder cattle, clinical endometritis and sub-clinical endometritis had an incidence of 54.15% and 1.40%, respectively, in Himachal Pradesh (Singh and Pant, 1998; Thakur *et al.*, 2006a). From the cervical mucus of repeat breeder cattle in Himachal Pradesh, the pure bacterial isolations reported were *Escherichia coli*, *Staphylococcus aureus*, *Bacillus spp.*, *Proteus spp.*, *Enterobacter spp.*, *Corynebacterium spp.* and *Pseudomonas aeruginosa*. Moreover, mixed cultures of *Escherichia coli* and *Bacillus spp.* *Escherichia coli* and *Staphylococcus aureus* as well as *Staphylococcus aureus* and *Pseudomonas spp* were also obtained (Sharma *et al.*, 2009). Numerous studies have led to the conclusion that in cattle with normal fertility the incidence of fertilization failure is approximately 10% and early embryonic death within 3 weeks

following fertilization accounts for approx. 30% leading to a total early pregnancy loss of close to 40% during the first 21 days post AI (Roche *et al.*, 1981). This means that on average 40% females will return to oestrus after each AI or mating. A number of experiments have reported a higher proportion of embryos with deviated morphology collected from RB animals compared to control animals suggesting an increased embryonic death rate as the cause of RB (Gustafsson 1985; Linares *et al.*, 1980; Ayalon, 1978). Recently, higher progesterone levels during oestrus in RB heifers compared to control animals have been reported, indicating hormonal deviations as one possible cause of RB (Gustafsson *et al.* 1986). There are only a few studies have been conducted to detect and molecular characterization of causal bacteria of RB. There are various proposed protocols for treatment of repeat breeder cows such as injection of gonadotropin-releasing hormone or its analogue around artificial insemination (Boettcher and Perera, 2007, Gustafsson and Emanuelson, 2002, Hewett, 1968), post-insemination supplementation with progesterone (Hossain, 2002; Hodel *et al.*, 1995), treatment of human chorionic gonadotropin 5 days after artificial insemination (Islam, 2011), intrauterine antibiotics (Perez- Marin *et al.*, 2012; Jainudeen and Hafez, 2000; Jeffrey *et al.*, 1990) and antiseptics (Perez-Marín *et al.*, 2012), uterine lavage by saline (Jainudeen and Hafez 2000) or even acupuncture and moxibustion (Perez-Marín *et al.*, 2012). Also, the effect of dimethyl sulfoxide was suggested on clearing the uterus from bacterial biofilm as well as improving the fertility of repeat breeder after antibiotic treatment (Jeffrey *et al.*, 1990). GnRH has been used at the time of AI in repeat breeding cows for improvement of conception in Bangladesh (Hossain, 2002). Moreover, intra-uterine infusion of antibiotic may be beneficial for treatment of repeat breeding cows with subclinical uterine infection. Accordingly, intra uterine infusion of penicillin improved the recovery rate in repeat breeding cows with uterine infection (Khair *et al.*, 2005). The treatment of repeat breeder bovines suffering from uterine infections need proper selection of antibiotics to prevent development of resistant strains of microbes and to eliminate infection as quickly as possible (Singh *et al.*, 2004). Recently RB becomes a significant challenge for dairy producer due to large economic loss. During the last 50 years, many studies have reported that many factors lead to repeat breeder syndrome such as infectious agents by affecting the sperm or embryo, hormones deficiency and luteal problems (Ferreira *et al.*, 2016; Yusuf *et al.*, 2010; Amiridis *et al.*, 2009). In Bangladesh, some experiments have also been conducted to investigate the causes of

repeat breeding (Ali *et al.*, 2020; Padder *et al.*, 2018). However, there are very few investigations on the identification and molecular characterization of infectious agents isolated from repeat breeder cows and their antibiotic resistance in our country. Therefore, considering the above points, the major objectives of the research was:

- To observe the prevalence of repeat breeding syndrome in Rangpur division of Bangladesh
- To isolate and identify the bacteria with their molecular confirmation causing repeat breeding syndrome
- To determine the antimicrobial sensitivity of the bacteria isolated from repeat breeder cows

CHAPTER II

REVIEW OF LITERATURE

One of a cow's most important traits in a dairy herd is her ability to breed. If a dairy herd produces one calf crop annually, the farmers will earn the most. The profitability of the dairy industry is greatly influenced by robust reproduction and production. The beginning of a cow's productive life cycle is parturition. Cows therefore require regular cycles of conception and calving in order to initiate subsequent lactation, which is an essential part of their productive life. For this reason, having a strong reproductive rhythm is crucial for a herd. Therefore, it stands to reason that the dairy farm may be adversely affected by any issues with herd reproduction. Repeat breeder cows are a huge source of financial loss and the bane of every dairy operation. For the sake of the enterprise and the herd, these problems need to be identified and fixed as soon as possible.

2.1 Reproductive physiology of cow

2.1.1 Hormonal Regulation of the Female Reproductive Tract

Hormones are unique chemical molecules produced by specialized glands called endocrine glands that are essential for normal female reproduction. These secretions go to different regions of the body via the blood and lymphatic fluids, where they have a variety of distinct effects. The uterus, placenta, gonads, hypothalamus, or pituitary can all be the source of reproductive hormones. The brain region known as the hypothalamus, which regulates reproductive hormones, is where hypothalamic hormones are made. Since many hypothalamus hormones are also known as releasing hormones, their function is to control the release of other hormones from the pituitary gland. Gonadotropin-releasing hormone (GnRH) is the main hormone released during reproduction. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are released by the pituitary gland, which is situated near the base of the brain, under the direction of GnRH. The ovary's ability to produce progesterone and estrogen is controlled by these pituitary hormones. While LH induces follicular rupture and the creation of the corpus luteum, FSH promotes the growth, development, and function of the follicle. The Graafian follicle on the ovary produces the feminine hormone estrogen.

Progesterone is another hormone that comes from the ovary and is generated by the corpus luteum. Every one of them is crucial to the female reproductive process.

The growth and operation of the secondary sex organs, the onset of heat, or estrus (the period of sexual receptivity), the type and rate of body tissue growth, particularly fat deposition, and the priming of the prepubertal heifer and postpartum cow for the onset of sexual activity are all regulated by estrogen. The pregnancy hormone is progesterone. It inhibits the release of estrogen and the continued growth of follicles. The female does not show signs of estrus while progesterone is being generated. In order to maintain the ideal uterine environment for pregnancy and to prepare the uterus to receive the fertilized egg, progesterone is required. Because both are required for the full development of several significant organs, the actions of estrogen and progesterone are not entirely distinct from one another. Progesterone completes uterine development, which is started by estrogen. If the uterine tissue is not adequately prepared by the action of progesterone and estrogen, the fertilized egg will not attach and live there. The uterus contracts rhythmically when estrogen is present. Progesterone, on the other hand, quiets the uterus, preventing any contractions that could interfere with pregnancy.

Both hormones are necessary for complete development of mammary gland. Progesterone is required for the formation of the clusters of milk-secreting alveoli on the ducts, whereas estrogen encourages the duct system's expansion. Progesterone often calms things down, while estrogen generally causes them to happen. The reproductive system of cows is known to be significantly regulated by a number of other hormones and hormone-like substances. A wide range of bodily tissues secrete prostaglandins, which have multiple purposes. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the main prostaglandin that affects the cow's estrous cycle and is produced by the uterus. A new estrous cycle can be launched in the nonpregnant female by causing the corpus luteum to regress late in the estrous cycle due to the natural luteolytic substance $PGF_{2\alpha}$. The corpus luteum remains in place throughout pregnancy because the growing embryo in a pregnant cow sends a signal to the uterus that inhibits $PGF_{2\alpha}$ release. Days 16 and 17 following fertilization are thought to be when a mother recognizes she is pregnant. Premature regression of the corpus luteum will occur in cows or heifers injected with $PGF_{2\alpha}$ between days 6 and 16 of the estrous cycle; females injected between days 10 and 16 will exhibit the best outcomes. Luteinization will usually not result from $PGF_{2\alpha}$ injections administered in the first five (1–5) and last five (17–21) days of the estrous cycle. The luteolytic response

triggers abortion in feedlot heifers and permits the use of PGF2 α in estrus synchronization programs in cow herds. Effective estrus synchronization programs have made use of a variety of combinations of estrogens, progestins, prostaglandins, and GnRH.

2.1.2 The Estrous Cycle

Over the course of several days, a set sequence of activities take place as part of the reproductive cycle of cows. In cows, the estrous cycle lasts 21 days on average (range: 17–24 days). The reproductive system is getting ready for ovulation, or the release of eggs, and estrus, often known as heat, the phase of sexual receptivity. The sequence of anatomical and hormonal changes that take place during a normal 21-day cycle, in which pregnancy does not occur, is depicted in Figures 1 and 2.

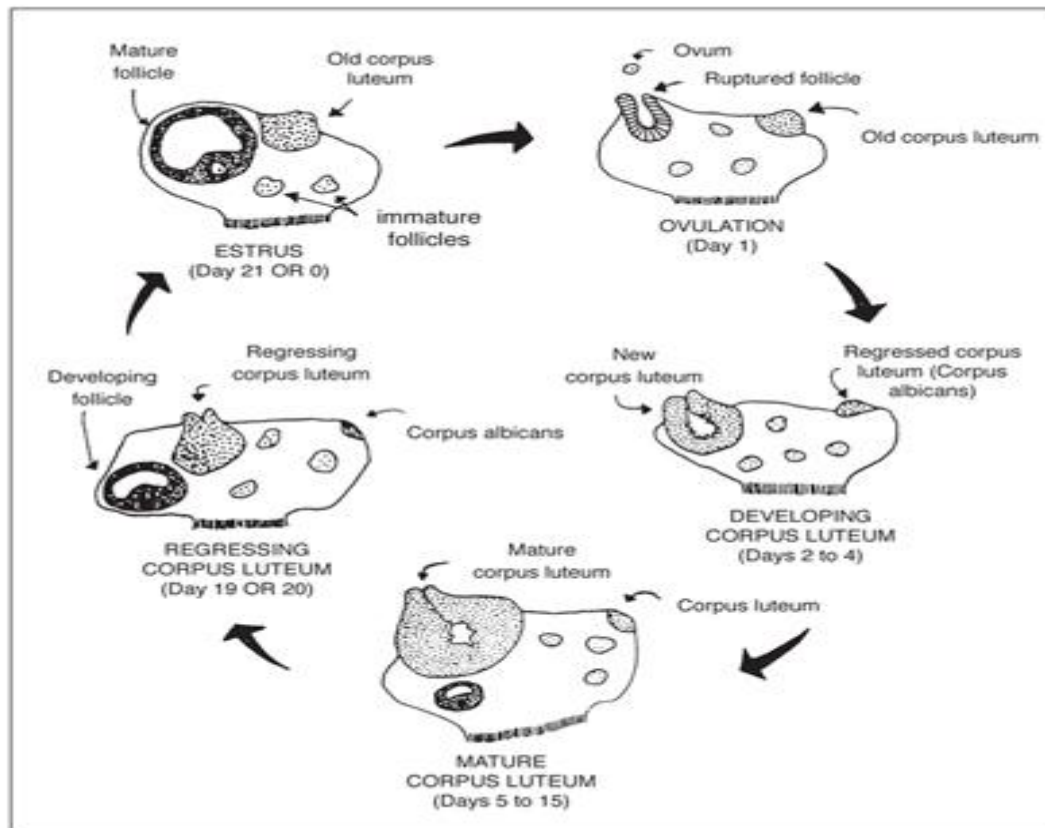


Figure 1. Diagram of a 21-day estrous cycle in which pregnancy does not occur. (Adapted from Deutscher, 1980)

Day 0: Because of a rise in estrogen levels, the cow is in estrus, or standing heat. The pituitary releases an excess of LH when the level of estrogen reaches a certain

threshold. The mature Graafian follicle ovulates (ruptures) in response to this LH surge at the end of standing heat.

Days 1—2: To become the luteal cells of the corpus luteum, the cells that once lined the follicle undergo a transformation. Hormonal action, especially that of LH, is responsible for this alteration in cell shape.

Days 2—5: To become the luteal cells of the corpus luteum, the cells that once lined the follicle undergo a transformation. Hormonal action, especially that of LH, is responsible for this alteration in cell shape.

Days 5—16: The corpus luteum keeps growing until day 15 or 16, when it usually reaches its maximal size and functionality. It secretes progesterone, a hormone that prevents the pituitary gland from releasing LH. The corpus luteum, which is still functioning, is the only functional component of the ovaries during this time. High progesterone concentrations prevent any follicles from maturing and/or ovulating.

Days 16—18: The ovary's increased follicular growth and subsequent secretion of estrogen trigger the uterus's PGF2 α secretion, leading to the corpus luteum's rapid regression.

Days 18—19: Progesterone's ability to inhibit LH and FSH is eliminated when progesterone secretion is reduced and the corpus luteum is nearly nonfunctional. Out of the several follicles that are first attracted, one experiences a spike in growth and activity that leads to its dominance. Larger Graafian follicles emit more estrogen as they mature, while smaller follicles recede.

Days 19—20: Estrus or heat will develop when the Graafian follicle releases more estrogen and the regressing corpus luteum releases less progesterone (cycle has now returned to day 0). Shortly after the onset of heat, a release of LH is triggered by the high levels of estrogen in the blood. The mature follicle bursts to release the egg after this spike in LH blood concentrations, and the cellular tissue that remains luteinizes to create a new corpus luteum (cycle has now reverted to days 1-2). Once again, progesterone becomes the dominant hormone.

Based on a 21-day average, the timing provided for these occurrences is simply approximative and varies for varying cycle durations (range of 17—24 days).

This explanation of the estrous cycle's events is predicated on a complete cycle without pregnancy. The corpus luteum does not regress; rather, it continues to function and release progesterone in the event that the egg is fertilized and begins to develop in the uterus. Heat does not generally occur during pregnancy and no follicles mature to the point of maturity. Progesterone levels that are higher encourage uterine quiescence, which offers the best circumstances for the growing fetus.

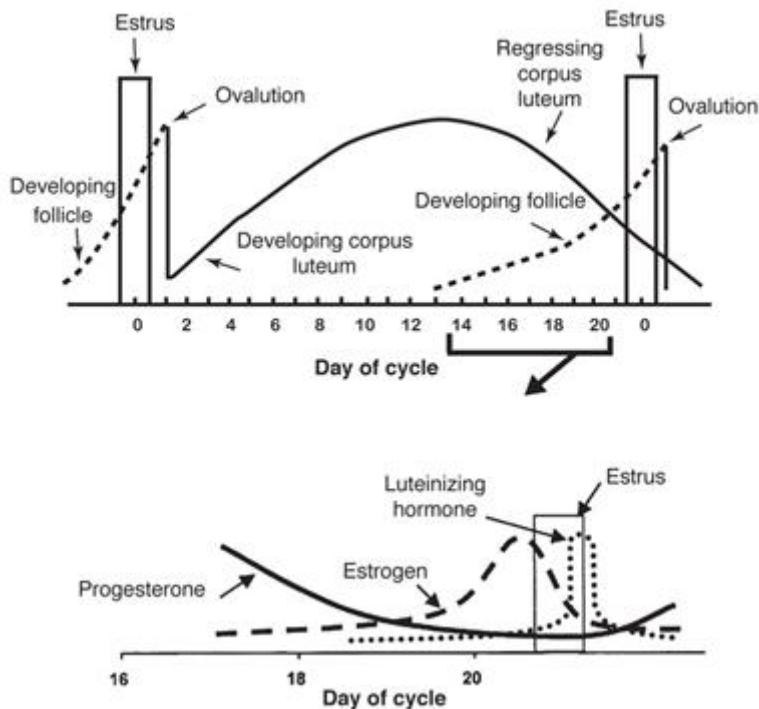


Figure 2. Graph of the anatomical and hormonal changes that occur during a typical 21-day estrous cycle. (Adapted from Deutscher, 1980)

The female will not go into estrus if there is a situation that extends the amount of time that blood progesterone concentrations stay high (e.g., implanting, injecting, or feeding progestins for estrus synchronization). Periodically, an animal may exhibit pseudopregnancy, in which case the corpus luteum does not retreat normally despite the absence of pregnancy. Occasionally, abnormally short estrous cycles (7—11 days) occur. The issue seems to arise from either the non-formation of a corpus luteum or its dysfunction, and the progesterone concentration not being high enough to inhibit the anterior pituitary's production of gonadotropins (FSH and/or LH).

Moreover, cows experience anestrus (noncycling) phases. For instance, after calving, cows often have an anestrus phase. The length of anestrus can be influenced by low nutrition, particularly in young cows nursing calves.

Ovulation does not always coincide with estrus, nor does estrus coincide with ovulation. Even if the female reproduces, heat without ovulation, or anovulatory heat, will not result in pregnancy. In cows, ovulation without the outward manifestation of heat (also known as silent or quiet heats) is not unusual, particularly in the initial weeks following calving. In most cases, these females won't "stand" to breed with a bull.

This talk, which covers the hormone regulation of the estrous cycle and pregnancy, is a very simplified version of the entire procedure. The reproductive process is actually a highly intricate process that involves numerous hormone interactions and occurrences.

2.2 Problems associate with reproductive failure

A number of basic reproductive problems, such as repeat breeder behavior (RB), abortion, anestrus behavior, dystocia, retained fetal membrane (RFM), metritis, and prolapse (both vaginal and uterine), directly impact the reproductive performance of dairy cows. (Khan *et al.*, 2016; Haile *et al.*, 2014; Shiferaw *et al.*, 2005). Reproductive problems affect dairy cow fertility and productivity by decreasing milk output, lengthening open days and calving intervals, failed uterine involution, and early culling of valuable cows (Gizaw *et al.*, 2007; Lobago *et al.*, 2006).

2.2.1 Definition of Repeat Breeder

Repeat breeders (RB) are cows who have given birth at least once previously, have not given birth after three or more services, have a normal estrus cycle, are under the age of 10, have no perceptible abnormalities, have no abnormal vaginal discharges, and have not given birth after three or more services. (Zemjanis, 1980). During the animal's clinical evaluation, it is possible that no obvious lesion or illness was discovered that would explain why conception failed. (Roberts, 1986). The term "RB cows" refers to a wide range of sub-fertile cows who exhibit a variety of reproductive problems across three or more subsequent heat cycles that normally last between 17 and 25 days and are free of any diseases or anatomical abnormalities. (Perez-Marin and Espana, 2007). Repeat breeder cows are those that have functional and healthy genitalia but have had

artificial insemination three or more times. (Ferreira *et al.*, 2016). Subclinical endometritis is one of the many variables that have been linked to the onset of RBC syndrome. (Salasel *et al.*,) nutritional deficiencies (Talukdar *et al.*, 2016) abnormal heat behavior or improper heat detection (Sood *et al.*, 2015; Cummins *et al.*, 2012), mismanagement in artificial insemination (AI) (Walsh *et al.*, 2011), or endocrine dysfunctions (Kafi *et al.*, 2017; Sood *et al.*, 2015). Subfertility in repeat breeder heifers has been linked to changes in hormone levels, including increased progesterone levels, aberrant follicular dynamics, delayed ovulation, and decreased oocyte quality (Bage, R *et al.*, 2002). A number of risk factors, including age, parity, body condition, milk yield, environmental factors, and peripartum and postpartum imbalances, also have an impact on these causes and increase the likelihood of imbalances and the incidence of RBC syndrome. Cows must have healthy ovarian dynamics, estrus behavior, uterine processes, and mating or AI in order for their reproductive systems to work properly (Bage, R *et al.*, 2002). Repeat breeding in dairy cows can be influenced by a number of endogenous factors that function at the ovary, oviduct, or uterus. These factors can impact the quality of oocytes and embryos. The analysis of the primary players who contribute to the development of recurring estrus in cattle is the major goal of the current review on RBC syndrome. Evidence is available for a hormonal asynchrony around estrus in repeat-breeder heifers (RBH). This asynchrony seems to be the underlying cause for a series of dysfunctions such as prolonged standing estrus and delayed ovulation, leading to fertilization failure (Bage *et al.*, 2002).

2.2.2 Prevalence of Repeat Breeding

Repeat breeding is a problem that is acknowledged globally, however the prevalence rate varies by region, for example, 10% in Sweden. (Hewett, 1968), in Israel, 5% (Francos, 1974), in Bangladesh 22% (Rahman *et al.*, 1996), in Scandinavia 20% (Roine and Saloniemi, 1978), in India 22% (Singh *et al.*, 1981). In Bangladesh, repeat breeders ranged from 13.0 to 22.0% of cows, with infectious agents accounting for 81.8% of those instances. (Samad, 1996). Repeat breeding was prevalent generally in both cattle and buffaloes, at 19.61% and 15.6%, respectively. (Karwani and Sharma 2003). In 10 distinct upazillas of the Rajshahi district, the prevalence of RB was 20.2%. This was correlated with age, breed, parity, body weight, and BCS of cows (Sarder *et al.*, 2010). Multiple

etiologies combined with repetitive breeding have a higher prevalence than other single etiologies do (Singh *et al.*, 2008).

The prevalence of reproductive problems in cattle was observed to be 32.11 percent, and crossbred cows also had a 21.9% prevalence of recurrent breeding. (Kaikini *et al.*, 1981). Repeat breeding is common in crossbred cows, ranging from 7.4 to 18.6% (Singh *et al.*, 1983). Repeat breeding is more common in cattle (prevalence: 5.5–33.3%) and buffaloes (prevalence: 6–30.6%) (Saxena 2005). Repeat breeding is common, with 28.31% of cattle (Bhat *et al.*, 2012). They studied 1074 cattle, of which 304 were found to have repeated breeding problems. They noticed that ovarian cysts, ovulatory abnormalities, breed, season, and these factors all contributed to the repetitive breeding. Repeat breeding is prevalent overall, at 27.33% (21.67% in cattle and 33.04% in buffalo). (Khan *et al.*, 2016).

2.2.3 Economic Impact of Repeat Breeding

Repeat breeding syndrome is considered one of the most significant reproductive issues on dairy farms, alongside other problems such as anestrus, retained placenta, dystocia, abortion, stillbirth, purulent vaginal discharge, and uterine prolapse. To estimate the cost of RBC syndrome, models take into account parameters such as veterinarian's fees, extra semen costs, expenses for therapeutic drugs, labor costs, decreases in milk yield, reduced calf production, losses due to culling and replacement, losses resulting from extended calving intervals, and on-farm deaths (Deka, R.P *et al.*, 2021; Liang, D. *et al.*, 2017; Levine, H.D. 1999). A comprehensive economic study on RBC syndrome was conducted in Michigan, noting that the highest economic losses or costs were associated with milk production, culling, and the cost of additional semen, among other factors (Lafi, S.Q. *et al.*, 1992). The authors suggested that proper nutrition should be provided during the dry period to prevent cases of milk fever or dystocia and reduce the incidence of RBC syndrome. According to studies, not only the postpartum and periovulatory periods are involved in RBC syndrome, but the dry period is also important in reducing estrus repetition in cows (Lafi, S.Q. *et al.*, 1992).

2.3 Repeat Breeding and Its' Associated Risk Factors

The two main categories of Repeat Breeding are infectious and non-infectious. The author included both infectious and non-infectious causes, including metritis,

endometritis, salpingitis, cervicitis, and vaginitis. Non-infectious causes included poor treatment, chromosomal abnormalities, hormonal imbalance, and anatomical malformations of the reproductive tract (El-Khadrawy *et al.*, 2011).

2.3.1 Female factor

Repeat breeding syndrome in cows has a variety of causes. The proportion of dairy cattle suffering from repeat breeding syndrome due to anatomical, functional, infectious and managerial reasons was 14.74%, 17.19%, 55, 66% and 12.41%, respectively (Thakur *et al.*, 2006; Singh, 2000; Singh and pant, 1998). A thorough gynecological investigation is required, taking into account not only each unique individual circumstance but also all potential circumstances that could affect the cows as a whole. (Perez-Marin and Espana, 2007). The most prominent findings for repeat breeding heifers (RBH) were prolonged duration of estrus, delayed LH peak, prolonged lifespan of the preovulatory follicle, and a late postovulatory rise in plasma progesterone. There was also a strong tendency for peri-ovulatory supra basal progesterone levels in RBH. It is suspected that these deviations cause changes in the microenvironment of the preovulatory follicle, negatively affecting the final maturation of the oocyte. The heterogeneity of the RBH group underlines the multifactorial cause of the repeat-breeder syndrome. Perioovulatory suprabasal progesterone levels in RBH also showed a considerable tendency. The preovulatory follicle's microenvironment may alter as a result of these variations, which could have an adverse effect on the oocyte's successful maturation. (Båge, R. *et al.*, 2002).

2.3.2 Male factors

Factors relating to the bull and semen can lead to repeated breeding. The subfertility seen in RBCs can be caused by a number of factors, including maternal origin as well as bull defects (such as subfertility, anatomical defects of the reproductive organs, low frozen-thawed semen quality, difficulties for mounting due to low libido or lameness, high hierarchy of infertile bulls) or management errors (improper technician placement or timing of semen deposition, insufficient heat detection (Pérez-Marín, C. C., & Quintela, L. A. 2023). To obtain a high pregnancy rate and a regular calving interval, bull fertility must be at its peak (either through natural breeding or AI). After freezing, sperm dosages for artificial insemination (AI) must contain at least 6 million motile sperm, and fertility

declines if sperm concentration is decreased. (Foote and Parks, 1993). Since around 50% of spermatozoa regain motility after thawing, frozen semen dosages are now packed with 15–25 million motile sperm prior to freezing. Although semen dosages may meet all requirements, there are donors with variable fertility. Low fertility males typically needed more sperm to accomplish their optimum conception than high fertility males (at any dosage). Males with limited fertility typically exhibited a higher percentage of aberrant cells in their seminal fluid. (Sullivan and Elliott, 1968). Aberrant sperm were produced as a result of spermatogenic abnormalities brought on by higher testicular temperature. High-fertility bull embryos progressed more quickly into the 2-cell and S phases of DNA synthesis, demonstrating high blastocyst viability (Acevedo *et al.*, 2001). (Eid *et al.*, 1994). When sperm DNA replication failed or there was damage to it during the S3 phase, embryos from low fertile bulls had a lengthier G2 phase. (Eid *et al.*, 1995).

2.3.3 Nutrition

Reproductive issues in dairy cattle could result from ration discrepancies in both quality and quantity. (Dovensky *et al.*, 1996). Low ovulation, weak estrus expression, and low sperm and egg counts can all be consequences of inadequate nutrition. (Imakawa *et al.*, 1986). In cows with negative energy balance (NEB), insulin and insulin-like growth factor (IGF-I) blood concentrations are lower. The GnRH neurons, their neural connections, or the pituitary gonadotrophins are all possible targets for these endocrine substances' ability to affect GnRH release. (Williams *et al.*, 2002). During the peripartum phase, which is usually the final week of pregnancy and the first two months following childbirth, negative energy balance, or NEB, takes place. Cows exhibit low serum insulin and glucose concentrations during this stage, which encourages the mobilization of adipose tissue. Non-esterified fatty acids (NEFA), which provide these animals with energy, rise as a result. However, due to their deleterious effects on immunity and postpartum health, prolonged high levels of NEFA and β hydroxybutyrate might negatively impair reproductive performance, resulting in anovulation, lower pregnancy rates, and increased embryo loss (Bisinotto, R.S. *et al.*, 2012; Santos, J.E.P. *et al.*, 2009). In comparison to normal animals, repeat breeder dairy cows had decreased serum levels of Zn, Cu, P, and I. (Ceylon, 2008). This was demonstrated after the RB buffaloes and cattle's recovery and conception rates improved after receiving a mineral supplement. (Ahmed *et al.*, 2010; Das *et al.*, 2009; Sah and Nakao, 2006).

2.3.4 Management

During the summer, heat stress makes buffaloes anestrus or repeat breeders. Reduced estrus duration and intensity, altered follicular development, and decreased embryonic development are all symptoms of heat stress in cows (Wolfensen and Meidan, 2000). Stress causes a rise in the level of circulating corticosteroids, which alters follicular activity and the ovulatory cycle. It also lowers progesterone levels, which changes the uterine environment and affects oocyte and embryo quality, decreasing the likelihood of embryo implantation. (Jordan, 2003). Deficient management such as poor estrus detection has been recognized as a main cause for a lower than normal pregnancy rate (Bage *et al.*, 2002).

2.3.5 Hormonal factors

Major contributing factors to RB in cows include ovulation failure and an altered endocrine state during folliculogenesis. (Maurer and Echtenkamp, 1985). Reduced responsiveness to circulating LH may cause early embryonic death that results in repeat breeding syndrome (Shelton *et al.*, 1990). According to a study, lactating cows with very small follicles ovulate less frequently than those with larger follicles, which led to smaller corpus luteums, lower serum progesterone levels, and reduced conception rates. (Vasconcelos *et al.*, 2001).

2.3.6 Genetic factors

Early pregnancy failure in animals is primarily caused by chromosomal abnormalities and autosomal recessive genes, which may account for 20.0% of all embryonic and fetal deaths (King, 1990). According to research on Robertsonian translocation 1/29 in cattle, cows having this chromosomal aberration were categorized as Repeat Breeder Cows (RB). (Rodriguez *et al.*, 2010).

2.3.7 Anatomical Defects of Genital Tract

Oviductal defects, such as tubal stenosis or occlusion, have been associated with the RBC syndrome (Garrido, M.R. et al; 2020). Due to these circumstances, spermatozoa and eggs cannot pass through the oviducts, and in the event that fertilization takes place, the embryo's entry into the uterus will be challenging. The estrous cycle in cows with oviductal changes is intact and exhibits a normal duration. These problems can

occasionally be rectified on their own. Although it can be difficult to confirm oviductal patency, studies have employed a variety of techniques, such as inspection at slaughterhouses (Kessy, B.M. and Noakes, D.E. 1979), ultrasonographic contrast liquid (Itoh, K. *et al.*, 2016; Kauffold, J. *et al.*, 2009), and dyes (phenol-sulphon-phthalein) (Garrido, M.R. *et al.*; 2020) or granules (starch) (Kessy, B.M. and Noakes, D.E. 1979), Inflammation of the tubules decreases sperm motility and interferes with sperm-oviduct contact; it is frequently linked to endometritis. Reduced ciliary beat frequency in the oviduct and deposition of mucus can also hinder the transfer of early embryos. Studies have revealed that approximately 20% of RBCs exhibit bilateral oviduct blockage or stenosis, whereas 24% of cows are found to have unilateral abnormalities. Research examines cervical consistency and patency in an effort to identify additional potential causes of RBC syndrome (Hartmann, D. *et al.*, 2016). It has been observed that cervicitis, which is distinct from endometritis, can be seen in RBCs with normal uterine findings. (Hartmann, D. *et al.*, 2016).

Changes in the reproductive system's anatomy or functionality can result in infertility and failed pregnancies. Complete adhesions between the vaginal wall and the development of pyometra can result from traumatic vaginal injuries (Tibary and Anouassi, 1997). Anatomical abnormalities were detected in 7.1% of the 183 cattle studied who had a history of repeat breeding. The presence of ovarian oviductal abnormalities, which usually complicate and prevent reproduction, 6.0 to 15.0% of adult cows, and it can go as high as 80.0% in those that have a history of infertility or repeat breeding (Singh *et al.*, 2009). The restoration of the postpartum period's normal cyclicity is dependent on acquired uterine changes, such as metritis, which leads to repeated breeding (Shrestha *et al.*, 2004). Repeat breeding is related with the development of cervical traumatic stenosis, obstruction from cervical ring prolapse, adhesions, or functional incompetence. (Tibary and Anouassi, 1997).

2.3.8 Age

Numerous studies have revealed that older cows are more likely than younger ones to be repeat breeders. (Bartlett *et al.*, 1986; Dekruif, 1978; Hewett, 1968). Age directly correlated with a higher incidence of metritis, retained placenta, and dystocia treated by a veterinarian. (Erb *et al.*, 1985). A first calver had a larger probability of developing RB than a multiparous cow did (Gustafsson and Emanuelson, 2002).

2.3.9 Parity

In cows with three or more parities, there is a higher incidence of RBC syndrome (Eshete, T. *et al.*, 2023). Every parturition is a risk period for the cow; postpartum illnesses (insufficient uterine involution, inflammatory affectation), or difficulties calving (dystocia, cesarean, retained fetal membranes) can affect reproductive activity and increase the likelihood of reproductive failures in subsequent estrous cycles in cows with more parities. One of the most important times for immunosuppression in cows is during periparturition. A recent research using beef RBCs found that, compared to cows with one or two recurrent cycles, low parity cows with repeated cycles are more likely to not conceive again in following parities. Moreover, it will be more difficult for cows that need four or more AIs to conceive in subsequent parities (Tanimura, K. *et al.*, 2022).

2.3.10 Peripartum abnormalities

In cows, parturition is a significant occurrence because it causes abrupt physiological changes that require adjustment to the new environment. The activity of immune cells is negatively impacted by periparturient hormone changes (Vlasova, A.N. and Saif, L.J. 2021). Negative energy balance (NEB) in lactating dairy cows causes hormonal and metabolic changes about three weeks prior to parturition. These alterations can impact neutrophil and lymphocyte function and lead to postpartum immunosuppression. Consequently, throughout the transition phase, cows are especially vulnerable to infections, both subclinical and clinical. Diseases that are commonly seen and linked to RBC syndrome include metritis, dystocia, retained fetal membranes, and metabolic abnormalities (Jeong, J.-K. *et al.*, 2022; Hasib, F.M.Y. *et al.*, 2020; Khair, A. *et al.*, 2018).

2.3.11 Body condition

It was shown that the prevalence of subfertility was higher in cows with poor (2.5) or medium (2.75–3.5) body condition scores than in those with higher scores (3.75) (Eshete, T.*et al.*, 2023). In cows with poorer body condition ratings, negative energy balance (NEB) lowers hormone release, apparent fertilization failures, and early embryonic deaths (Butler, W.R. 2003). Body weight loss is a sign of NEB and is linked to low protein, mineral, vitamin, and/or energy levels. (Westwood, C.T.*et al.*, 2002).

2.3.12 Milk yield

Dairy cows with high genetic merit have a higher chance of experiencing reproductive failure (Westwood, C.T.*et al.*, 2002). When cows produce a lot of milk, they put in a lot of nutritional demand and metabolic work, which has a negative impact on reproductive performance (Eshete, T.*et al.*, 2023). Primiparous cows with longer days to peak milk output are linked to RBC syndrome; this could be because insemination occurs at the same time as increased milk yield and increased metabolic needs, or it could be a result of stress related to nutrition, management, or diseases during their first lactation (Moss, N. *et al.*, 2002). Perhaps as a result of fat mobilization, there is a correlation between higher milk fat and protein content and a higher risk of conception failure. Estrus expression and the development of pregnancy are hampered in high-producing lactating cows due to their higher metabolism of progesterone and estradiol (Sangsritavong, S. *et al.*, 2002; Mann, G.E. *et al.*, 1995).

2.3.13 Season

Throughout the year, the effectiveness of reproduction varies, and the literature expresses a variety of opinions about the impact of the seasons. Cows calving during the fall and winter months were more likely to exhibit the repeat breeder syndrome (Hewett, 1968). Temperature and photoperiod fluctuations are correlated with the season and may have an impact on how the endocrine system controls the estrous cycle. In contrast to buffaloes, where the seasonal incidence of repeat breeding was found to be highest (20.6%) in dry hot summer and lowest (21.9%) in humid hot summer (18.5%), repeat breeding was most common in cattle during the humid hot summer and fell to a minimum (20.5%) during the autumn (Karwani and Sharma, 2003).

2.3.14 Herd size

Larger herds tend to have more repeat breeders than smaller herds do. In small herds, 8.5% of the animals failed to conceive after four or more inseminations, according to findings compared to a 13.1% in larger herds (Hewett, 1968). The time between conception and parturition was compressed as the herd's size grew, but the number of calves born after the first service dropped. (De Kruif, 1978).

2.3.15 Heat detection

One of the main reasons for RB and infertility in cattle is inadequate heat detection (Perez-Marin and Espana, 2007; Lafi and Kaneene, 1988). The majority of the cows in heat could be found with at least 80% accuracy with checks made five times per day (O'Farrell, 1975). The number of inseminations per conception and the amount of days open in dairy cattle reduced when the frequency of heat detection checks was increased. (Bailie, 1982). With good detection of oestrus the optimal time to inseminate, can be found by the am-pm rule (Foote, 1979). The ideal time to inseminate cows who enter oestrus for the first time in the morning is in the afternoon of that day, while the optimal time to do so for cows who enter oestrus for the first time in the evening is the following morning.

2. 4 Causes of repeat breeding

The two main categories of Repeat Breeding are infectious and non-infectious. Infectious causes included metritis, endometritis, salpingitis, cervicitis, vaginitis etc. Non-infectious causes included poor treatment, chromosomal abnormalities, hormonal imbalance, and anatomical malformations of the reproductive tract etc. (El-Khadrawy *et al.*, 2011).

2.4.1 Infectious Causes of Repeat Breeding

According to a survey, there may be a link between rising rates of RB and subclinical endometritis, which has a detrimental effect on first-service conception and pregnancy rates in cows and lowers reproductive performance. (Kasimanickam *et al.*, 2004). Repetition of breeding and abnormal endometrium are associated. (Santana *et al.*, 1998; Francos, 1979;). Recurrent estrus was experienced by 3.5–5.7% of cows with metritis. (Francos, 1979). Specific and generic uterine infections can change follicular growth, expand the uterine and cervical postpartum involution, and have a negative impact on reproductive indices. (Lewis, 1997) and by increased embryo mortality and repeat estrus rates (Santana *et al.*, 1998). The reproductive systems of cows are home to numerous types of bacteria. When an animal is under stress, some symbiont bacteria turn harmful, while others do so right away (E'duvie, 1985). In addition, *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *Streptococcus spp.*, *Pasteurella multocida*, *Proteus vulgaris*, *Klebsiella spp.*, and a number of anaerobic bacteria were identified from the uteri of cows with a history of repeated breeding (El-Azab *et al.*, 1988). The possibility

for assessing the vaginal microbiome and developing therapeutic approaches for RBC syndrome has been made clear by research on bacterial communities in the vagina. Porphyromonas and Pasteurellaceae have been detected in large quantities in RBC syndrome, and proteobacteria have been linked to dysbiosis during the postpartum period in subfertile cows. (González Moreno, C. *et al.*, 2022).

2.5 Pathogenesis of reproductive tract diseases

The fertility of cows is impacted by both non-specific and specific diseases of the reproductive tract, which causes a significant percentage of animal pregnancy failures. With the invasion of these pathogens, the female reproductive tract becomes more dangerous for sperm viability, which also affects embryo implantation. These infections damage the uterine mucosa and alter the pH of the uterus, which is hazardous for the survival of sperm in the uterine lumen. (Raghavan *et al.*, 1971). When the uterine defense mechanism gets compromised, the facultative anaerobic microbes that are normally present in the reproductive canal produce an inflammatory state. Barman *et al.*, 2013). With the loss of many cilia on cells and the microvilli destruction of glandular structures, the surface epithelium suffers multiple injuries in chronic endometritis. (Samatha *et al.*, 2013). Pathogenic organisms enter the uterine epithelial cells and release bacterial toxins that disrupt the tissue's normal function. Pituitary FSH is decreased and LH secretion is suppressed by the bacteria and its products, which also cause uterine irritation. As a result, the dairy cow's ovarian follicular growth and function are disrupted, which delays ovulation and results in fertilization failure. (Ahmed and Elsheikh, 2014).

2.6 Uterine defense mechanism

The uterine defense system is maintained in numerous ways, including chemically by mucus discharges from endometrial glands, immunologically by polymorphonuclear inflammatory cells, and architecturally by the columnar epithelial tissues covering the endometrium. (Dhaliwal *et al.*, 2001). Various anatomical and physiological barriers work together to keep harmful germs from invading the uterus under normal circumstances. (Foldi *et al.*, 2006).

Bacterial colonization of the uterus and subsequent uterine infection can be caused by weakening or impairment of the reproductive tract's defense mechanisms, which include

the endometrium's normal function, uterine involution, mucus secretion, antibodies, and phagocytosis (Ali *et al.*, 2010; Fredriksson *et al.*, 1985). Uterine leukocytes serve as the cellular defense against bacterial pollutants in the uterus (Romaniukowa, 1984; Vandeplassche, 1984). The uterine lumen's PMN population often rises after an experimental intrauterine infection (Butt *et al.*, 1993; Watson *et al.*, 1990). Polymorphonuclear (PMN) cells derived both from blood and uterine exudates are engaged in uterine defense mechanisms (Hussain, 1990). Blood neutrophils may have higher phagocytic activity than uterine exudate neutrophils (Romaniukowa, 1984). There is no discernible difference between blood neutrophils' and uterine exudate neutrophils' phagocytic capacities. The phagocytic capacity of PMN cells in uterine fluid, however, appears to be less than in blood plasma. (Anderson *et al.*, 1985).

2.7 Microflora isolated from reproductive tract

In the CVM samples taken from repeat breeder cows and heifers, *E. coli* (27.63%), *Staphylococcus spp.* (18.59%), and *Pseudomonas spp.* (12.06%) were found more frequently than *Bacillus spp.* (9.54%), *Proteus spp.* (6.53%), and *Corynebacterium spp.* (4.02%) (Murthy *et al.*, 1974). *Staphylococcus spp.*, *Escherichia coli*, *Bacillus spp.* and *Corynebacterium spp.* were often recovered from CVM samples of normal cows, while *Corynebacterium spp.* and Enterobacteria were more prevalent in repeat breeders (Panangala *et al.*, 1978). Samples taken from normal cows had a bacterium positivity rate of 20% compared to repeat breeder cow samples, which had a positivity rate of 51.4% (Deka *et al.*, 1979). Examination of 520 cervical mucus samples from repeat breeder cows, 76.92% of the samples contained bacterial isolates, including *Staphylococci spp.*, *Streptococci spp.*, *Corynebacterium spp.*, *Bacillus spp.*, and gram-positive bacilli in varying proportions. According to the analysis of antibiotic sensitivity, gentamicin, chloramphenicol, and neomycin were the three most effective drugs (Dholakia *et al.*, 1987). *E. coli* is found in repeat breeder cows more frequently than *Staphylococcus aureus*, *S. typhi*, and *Pseudomonas aeruginosa* (Gupta and Deopurkar, 2005). In a study on the non-specific microbiota of cervical mucus samples from repeat breeder cows during estrous, *E. coli*, *Staphylococcus aureus*, *Bacillus spp.*, *Proteus spp.*, and *Pseudomonas spp.* (Ramaswamy *et al.*, 1998). *Proteus species*, *Streptococci*, *Bacillus species*, *Staphylococcus epidermis*, *Escherichia coli*, *Staphylococcus aureus* are also found. Cows and buffaloes that are repeatedly bred can harbor *Klebsiella*

pneumonia, *Pseudomonas aeruginosa*, and *Corynebacterium pyogenes*. (Arora *et al.*, 2000). The uterine discharge samples from 97 repeat breeding cows and 55 normal estrus stage fertile cows were used in the bacteriological analysis. (Kamal *et al.*, 2001). In contrast to just 14 (25.4%) normal estrus fertile cows, 51 (52.5%) repeat breeding cows had bacteria from uterine discharge, according to qualitative tests. *Escherichia coli* (22.68%) and *Staphylococcus spp.* (20.6%), *Bacillus spp.* (16.4%), *Streptococcus spp.* (12.3%), *Corynebacterium spp.* (8.2%), *Proteus spp.* (7.2%), *Pseudomonas spp.* (6.2%), *Micrococcus spp.* (4.1%), and *Klebsiella spp.* (3.1%). *Staphylococcus aureus* (29.16%), *E. coli* (20.83%), *P. aeruginosa* (16.66%), *Streptococcus spp.* (12.50%), and *Proteus spp.* (4.16%) were among the several bacterial isolates found in repeat breeder cows (Chandrakar *et al.*, 2002). The most prevalent bacteria were *Staphylococcus spp.* (37.8%), *E. coli* (29.7%), and *Pseudomonas spp.* (18.9%), while repeat breeder cows had a 24.3% prevalence of Gram-negative minute rod-shaped bacteria. (Gani *et al.*, 2008). Investigation of 109 samples of uterus from both sick and healthy animals the most common bacteria in sick animals were *Staphylococcus spp.* and *Streptococcus spp.*, which were present in 33% and 27.3% of cases, respectively. The majority of samples from non-diseased cases (13.2%) and 10.4%, respectively, tested positive for Bacteroid (Azizunnesa *et al.*, 2011). *Staphylococcus spp.* isolates were more prevalent in normal cyclic cows than *Bacillus spp.* isolates were in repeat breeding cows. (Ahuja *et al.*, 2017).

2.8 Molecular technique for bacterial confirmation: Polymerase Chain Reaction

(PCR)

More accurate microbiological surveillance and identification are now possible because to polymerase chain reaction. A potent method for the culture-independent detection and characterisation of bacteria is polymerase chain reaction (PCR) (Guo *et al.*, 2012). There have been several PCR attempts to identify bacteria from uterine secretions. (Bicalho *et al.*, 2012; Guo *et al.*, 2012; Liu *et al.*, 2009;). It provides a significant improvement in terms of the speed, sensitivity, and specificity of diagnostic techniques, and it is increasingly being utilized to distinguish between various bacterial species in clinical samples and food (Stone *et al.*, 1994).

2.9 Antibiotic sensitivity test

One of the oldest techniques used in microbiological research is the Bauer and Kirby's disc diffusion method for testing antibiotic sensitivity. Antibigram, or bacterial resistance to several antibiotics, can be tested easily and consistently using the disc diffusion method. According to the antibiotic sensitivity patterns of bacterial isolates from endometritis cows' cervicovaginal mucus, the isolates were most 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%) (Shah and Dholakia., 1983).

The results of the study indicate that ampicillin (15.79%) was the least effective drug, whereas gentamicin (73.68%) and penicillin (68.12%) were both extremely efficient against the bacteria recovered from RB cows and buffaloes. (Sharda *et al.*, 1991). Gentamicin (88.9%) and chloramphenicol (77.8%) were found to be sensitive to the isolates from recurrent breeder cows and buffaloes, however most of the isolates were reported to be resistant to penicillin (81.48%) (Singh *et al.*, 1996). Uterine discharge samples from 97 repeat breeders and 55 healthy viable cows were tested for antibiotic sensitivity. The antibiotic spectrum of isolated bacteria showed that Gentamicin (92.8%), chloramphenicol (86.7%), and neomycin (79.5%) were the most effective antibiotics. Streptomycin and Kanamycin were ineffective, whereas ampicillin, tetracycline, and Penicillin G were the least effective. (Kamal *et al.*, 2001). Gentamicin (80.98%) had the highest drug sensitivity among the microbiological agents responsible for endometritis,

followed by co-trimoxazole (51.75%), chloramphenicol (50.73%), kanamycin (45.85%), ampicillin (28.29%), and oxytetracycline (24.88%). (Rao *et al.*, 2001). Most of the isolates were responsive to ciprofloxacin (83.33%), followed by enrofloxacin (79.16%), gentamicin (77.08%), and chloramphenicol (62.5%); nevertheless, most of them were resistant to ampicillin and sulphamethaxazole. This information was obtained from the antibiogram pattern of bacterial isolates from the metritis cows. (Ingawale *et al.*, 2003). Bacterial isolates from repeat breeder calves and buffaloes were tested for antibiotic sensitivity, and the results showed that ciprofloxacin (93.75%) was the most effective medicine, followed by gentamicin (84.37%) and chloramphenicol (78.12%). (Mane *et al.*, 2009).

2.10 Treatment of repeat breeding

The diagnosis of clinical cases and their classification into infectious and non-infectious categories will determine how to treat repeat breeding. The course of treatment varies depending on the category as well as the identification of the specific cause of the recurring breeding.

2.10.1 Treatment of noninfectious repeat breeding category

The timing of ovulation, fertilization rate, corpus luteum development, progesterone secretion, and embryonic survival may all be impacted by GnRH medication during the oestrus. In contrast, GnRH administration on day 5 of the estrus cycle in crossbred cows results in the ovulation of the first wave dominant follicle and the formation of an accessory corpus luteum; however, synchronized follicular wave emergence by two days after GnRH administration and two wave cycle were changed to three wave cycles. When 100µg of GnRH (buserelin acetate) were administered to cows during insemination, 54.14 percent of conceptions occurred as opposed to 36.00 percent in control cows, with a statistically significant difference between the two. (Rao and Naidu., 1987). Exogenous delivery of a synthetic counterpart of 250µg GnRH at the time of AI led to a first-service conception rate of 60% in post-partum crossbred cows compared to a control group's 40%. (Sankar *et al.*, 1987). The conception rate was found to be 48.80, 51.50, and 42.40 percent, respectively, after treating cows with 10µg of GnRH (Buserelin acetate) at the time of AI and after 12 days of AI in two groups, with the third group of cows being kept as an untreated control. (Ryan *et al.*, 1991). At the

time of the commencement of heat, the inseminated animals' conception rate in the control group was 33.30%, whereas it was 73.60% in the GnRH treatment group (Roy *et al.*, 1995). On the 12th day following AI, 15 repeat breeder cows received 10µg of buserelin and 2.5 ml of saline; the corresponding conception rates were 40.00% and 33.33% (Jayakumar and Vahida, 2000). In the treatment and control groups, the effectiveness of GnRH on conceptions was found to be 65 and 40%, respectively (Sonwane, 2000). GnRH injection improves the chance of conception in cases of induced estrus, increasing it to 66% compared to 50% in the cows in the control group (Ingwale *et al.*, 2001). With GnRH therapy during AI, repeat breed HF cows had a 70 percent conception rate (Rangnekar *et al.*, 2002). At the time of artificial insemination, cows treated with the GnRH analogue Dalmarelin saw 68.75% pregnancies, compared to 37.50% in the control group (Anjum *et al.*, 2010). Buserelin acetate's effectiveness was studied in 18 repeat breeder crossbred cows, and the results showed that when given at the time of insemination, it can increase conception rates by up to 66.66% (Patel *et al.*, 2010). Buserelin acetate's effectiveness was tested on 19 repeat breeder cows, and the results showed that when given at the time of insemination, it can increase conception rates by up to 73.68 percent (Kumar *et al.*, 2010). Buserelin acetate @ 10 µg exogenously administered at the time of artificial insemination (AI) caused a 75.00% conception rate in repeat breeder cows, as opposed to 50.00% and 62.50% conception rates following progesterone and GnRH injections given after 5 days of insemination. Conclusion: GnRH medication given at the time of AI has a higher success rate with regard to non-infectious treatment than do other hormonal treatments and schedules given at the time of the estrus cycle (More *et al.*, 2012).

2.10.2 Treatment of infectious repeat breeding category

It is possible to assess the vaginal microbiome in order to develop therapeutic approaches for RBC syndrome, according to research on bacterial populations in the vagina (González Moreno, C. 2022). When transferred through an open cervix under stressful circumstances, nonpathogenic organisms from the external genitalia have the chance to thrive in the endometrial environment. The issue of infectious recurrent breeding fails to consider whether the illness is causing infertility, fetal death, or both. Non-specific bacterial infection, which causes endometrial inflammation and early embryonic death, is a critical factor in animals that reproduce repeatedly. For the

planning of AI and subsequent ensured conception, it is vital to take into account the route of administration, dose, stage of administration, and even repeatability of administration after choosing the most suitable antibiotics (singly or in combination) (Dholakia *et al.*, 1987). Gentamicin-treated cows had overall pregnancy rates of 80% after three treatments, which were not substantially different from control cows' (93%) rates ($P>0.05$). However, the overall percentage of pregnant cows receiving enrofloxacin was 33% (Warriach *et al.*, 2009). Comparing various intrauterine formulations, ciprofloxacin and tinidazole may be the most useful medication for treating repeat breeding in animals in field conditions (Mane, 2010). In comparison to control cows (91.7%), the pregnancy rate in gentamicin-treated cows after three treatments (87.5%) was not statistically different ($P>0.05$). Nevertheless, the total conception rates of cows treated with enrofloxacin (50%) were considerably lower than those of the Gentamicin and control groups. (Singh *et al.*, 2015). In contrast to the 30.4% of the no treatment control group, 45.8% of the cows recovered after intrauterine antibiotic injection and AI in the subsequent heat. Asaduzzaman *et al.* (2016)

CHAPTER III

MATERIALS AND METHODS

The research was aimed at the isolation and identification of causal agents of repeat breeding in dairy cows.

3.1 Study area

The study was conducted under the supervision of the Department of Medicine, Surgery, and Obstetrics, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200. Some laboratory work was performed at the Microbiology, Department of Pathology and Parasitology laboratory, HSTU.

3.2 Data collection for prevalence study

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. 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 farm managerial 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.

3.3 Selection of cows for sample collection

This study was conducted in different dairy farms, located in Rangpur division of Bangladesh. Total 300 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=124) cows were selected on the basis of owner complaint, history of repeated conception failure, breeding records, nature of estrous cycle and per rectal examination of reproductive tract.



Figure 3: Selection of animal for sample collection

3.4 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.

3.5. Collection of samples

The cervical mucus samples were collected by using a sterile cotton stick 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 recovered cervical mucus samples were transferred into tubes containing PBS and placed in ice box and then transport to laboratory as soon as possible after collection.



Figure 4: Collection of samples (a)sample collection, (b) sample in ice box, (c) sample in refrigerator.

3.6. Procedure of isolation and identification of bacteria

3.6.1 Laboratory preparation

Glasswares, such as test tubes, micropipettes, cylinders, conical flasks, petri dishes, glass plates, slides, and vials, should be immersed in a dishwashing detergent solution for at least an entire night before use. After brushing, thorough washing, and autoclaving the glassware for 15 minutes at 121°C under 15 lbs. of pressure per square inch, the glassware was properly cleaned and sterilized. Items that had been autoclaved were dried at 50 °C in a hot air oven. Autoclaving was used to sanitize plastic disposables (micropipette tips).

3.6.2 Instruments and appliances

All items of glassware and appliances used during the course of the experiment were test tubes, conical flasks, petri dishes, pipettes, slides, cover slips, glass rod spreader, water measuring cylinder, dropper, stop watch, distilled water, forceps, scissors, tray, experimental test tube, stopper, vortex mixture, labeling tape, micropipette, test tube racks, water bath, bacteriological incubator, freeze (-20°C), refrigerator (4°C), sterilizing instruments, hot air oven, centrifuge tubes and machine, ice boxes, electronic balance, syringe and needle, immersion oil, cotton, slide, compound microscope, spirit lamps, match lighter, bacteriological loop, inoculum loop, autoclave machine, laminar air flow casting tray, well combs, voltage source, gel box, uv light source, microwave, gel-electrophoresis, PCR machine, spinner, genetic analyzer etc.

3.6.3 Media for culture

The isolation, characterization, and growth of bacteria from cervical mucus were accomplished using various bacteriological culture media and reagents. The culture media and materials used in this experiment are listed below.

3.6.3.1 Liquid media (Broth)

The liquid media used for bacteriological analysis were (1) Nutrient broth (HIMEDIA, India), (2) Methyl Red-Voges Proskauer (MR-VP) broth (HIMEDIA, India), (3) Buffered Peptone Water (HIMEDIA, India), (4) Indole broth (HIMEDIA, India).

3.6.3.2 Solid media

The solid media used for bacteriological analysis were (1) Nutrient agar base (Scharlau, Spain), (2) MacConkey Agar medium (HIMEDIA, India), (3) Eosin Methylene Blue (EMB) Agar (HIMEDIA, India), (4) Mannitol salt agar (Scharlau, Spain), (5) Triple Sugar Iron (TSI) Agar slant (HIMEDIA, India), (6) Simmons citrate agar (HIMEDIA, India), (7) Blood agar (HIMEDIA, India), (8) Mueller-Hinton agar (MHA) (HIMEDIA, India).



Figure 5: Different kinds of Media

3.6.3 Chemicals, reagents, and solutions

The reagents used during the study were crystal violet, iodine solution, gram's iodine, acetone alcohol, normal saline, distilled water, ethidium bromide, safranin, methyl red solution, catalase test reagent hydrogen peroxide, (3% solution), kovac's reagent, ethyl alcohol (70% and 95%), gel-loading dye, tae buffer, wash buffer, elusion buffer, pcr master mix and other common laboratory chemicals and reagents.



Figure 6: Different types of chemical reagents.

3.6.4 Antibiotic discs

To identify the antibiotic sensitivity profile of several bacterial isolates with various classes of antibiotics. The antibiotic discs used were readily accessible commercially. By measuring the size of the zone of inhibition that forms when different amounts of the agent diffuse into the medium around the disc, this method made it possible to figure out how sensitive different antibiotics are. The antibiotics were examined in tests against the chosen bacterium are listed below, along with their disc concentrations in table 2.

Table 1. Antibiotics with their disc concentration

Sl. No.	Name of the Antibiotics	Concentrations (µg/disc)	Company Name
01	Ampicillin (AM)	10	Bioanalyse® Turkey
02	Vancomycin (VA)	30	
03	Erythromycin (E)	15	
04	Amoxicillin (AX)	30	
05	Levofloxacin (LEV)	5	
06	Ciprofloxacin (CIP)	5	
07	Ceftriaxone (CRO)	30	
08	Penicillin G (P)	10	
09	Tetracycline (TE)	10	
10	Gentamicin (CN)	10	



Figure 7: Antibiotics discs

3.7 Preparation of culture media, broth, and media plate

According to the manufacturer's instructions, all the media, broth, and reagents such as nutrient agar, EMB agar, MacConkey agar, mannitol salt agar, blood agar, nutrient broth etc. used in this experiment were weighed and dissolved in distilled water in a sterilized conical flask. After proper plugging of the flask, these were autoclaved at 121°C temperature and 15 lb. pressure for 15 min. After autoclaving, media were cooled up to 45-50°C. About 15-20 ml of particular media was poured in each sterilized petri dishes. After pouring media, plates were kept for solidification. After solidification these plates were kept in incubator at 37°C for 24 hours for sterility testing.

3.7.1 Nutrient agar

Nutrient agar is a general-purpose medium that supports the growth of a wide range of non-fastidious organisms. A variety of non-fastidious organisms can grow in nutrient agar, which is an all-purpose medium. On nutrient agar different bacteria show different cultural characters such as white, glistening, large mucoid, circular, rough, opaque, fuzzy white or slightly yellow with jagged edges etc. (Carter, 1967).

3.7.2 MacConkey agar (MAC)

MacConkey agar is both selective and differential. It has bile salt and the dye crystal violet, which stop gram-positive (G+) bacteria from growing and favor gram-negative (G-) bacteria. Additionally, it includes lactose, a carbohydrate that enables the classification of Gram-negative (G-) bacteria according to how well they can ferment lactose. On MacConkey agar gram-negative organisms show large mucoid, rose-pink colony (Carter, 1967).

3.7.3 Eosin Methylene Blue agar

Escherichia coli was grown on an Eosin Methylene Blue (EMB) agar medium to be observed (Cheesebrough, 1984). On EMB agar *E. coli* produces green metallic sheen with reflected light (Carter, 1967).

3.7.4 Mannitol Salt agar

Mannitol salt agar or MSA is a commonly used selective and differential growth medium in microbiology. It promotes the expansion of some bacteria while restraining the expansion of others. This medium is crucial in laboratories because it quickly distinguishes harmful organisms. *Staphylococcus aureus* creates colonies that are yellow with yellow zones, as opposed to other coagulase-negative Staphylococci cause little change in the medium's color while producing tiny pink or red colonies. Mannitol can be fermented by an organism, and when this happens, an acidic byproduct is produced, turning the phenol red in the agar yellow. Presumptive pathogenic (PP) *Staphylococcus* species are isolated using this method. Gram positive *Staphylococcus*: fermenting Mannitol: medium turns yellow (*S. aureus*) Gram-positive: *Staphylococcus*: not fermenting mannitol; medium does not change color (*S. epidermidis*); Gram-positive *Streptococcus*: inhibited growth (Wikipedia, 2023).

3.7.5 Blood agar

Blood agar (BA) is an enriched medium used to culture those bacteria or microbes that do not grow easily. Such bacteria are called "fastidious" as they demand a special, enriched nutritional environment as compared to the routine bacteria. It is necessary to identify and locate hemolytic bacteria, particularly those from the *Streptococcus* genus. It is also a differential medium because it permits the detection of hemolysis, or the

destruction of RBCs, brought on by cytolytic toxins generated by particular bacteria, such as particular strains of *Bacillus*, *Streptococcus*, *Enterococcus*, *Staphylococcus*, and *Aerococcus*. A versatile, enriched blood agar medium is frequently used to cultivate picky organisms. To distinguish between bacteria with distinct hemolytic capacities (β -hemolysis, α -hemolysis and γ -hemolysis (or non-hemolytic)) (Sagar Aryal, 2022).

3.7.6 Muller-Hinton agar (MHA)

Muller-Hinton Agar is a microbiological growth medium that is commonly used for antibiotic susceptibility testing.

3.8 Isolation and identification of bacteria

The samples were collected for the isolation and identification of bacteria by morphology, cultural characteristics, and staining characteristics. The characterization of bacteria was done by cultural, and biochemical reactions. The isolated bacteria were tested for the sensitivity patterns of different antibiotics and finally performed the molecular test by PCR.

3.8.1 Culture of bacteria

Samples (uterine pus) were collected in a sterile cotton swab stick containing PBS in a sterile tube from different farms. Samples were brought to the medicine, surgery, and obstetrics laboratory to maintain an aseptic condition. Samples were cultured on primary culture (nutrient agar) and incubated at 37°C for 24 hours. Then secondary culture was done on MacConkey blood agar from nutrient agar and incubated at 37°C for 24 hours. Again, subculture on selective media (MSA, EMB, Blood agar) and incubation at 37° for 24 hours was done. The isolation of the pure cultures was done. Gram's staining was performed to identify gram-positive and gram-negative bacteria, their morphology, and staining characteristics. Biochemical characterization of isolates by catalase, TSI, Indole, Simmons citrate, MR, and VP tests was done. After that, the determination of antibiotic sensitivity patterns was done by the disc diffusion method. Finally, molecular characterization by DNA extraction, reaction mixing, PCR amplification, agar-rose gel preparation, and electrophoresis was performed.



Inoculation of bacteria in culture media



Incubation of bacteria

Figure 8: Culture of Bacteria

3.8.2 Morphology study by gram's staining method under microscope

To identify whether bacteria were gram positive or gram negative, their size, shape, and organization, Gram's staining was carried out in accordance with the steps outlined by (Merchant and Packer, 1967). Gram-positive organisms had spherical, purple-colored shapes and were organized in clusters, raising concerns. The procedure was created by a Danish bacteriologist named Hans Christian Gram, hence the name.

3.8.2.1 The Procedure of gram's staining method

- A small colony was picked up with a bacteriological loop, smeared on a glass slide, air dried and then fixed by gentle heating.
- Crystal violet (primary stain) was applied over the smear for 2 min and then washed with running tap water.
- Gram's iodine (mordant) was then applied over the smear for 1 min and then washed with running tap water.
- Ethyl alcohol 95% (decolorizing agent) was applied over the smear for 10 seconds and then washed with running tap water.
- Counter stains were done with Safranin for 1 minute followed by gentle wash with running tap water.
- The slide was allowed to air dry and then examined under a microscope with the help of oil immersion.



Preparation of slide by gram's staining Observation of slide under microscope

Figure 9: Gram's staining

3.8.3 Biochemical test

3.8.3.1 Catalase test

Catalase is a common enzyme found in all living organisms exposed to oxygen. It is a crucial enzyme for defending the cell against reactive oxygen species' (ROS') oxidative damage. Catalase, which catalyzes the oxygen release from hydrogen peroxide (H_2O_2), is shown to be present in this test. It is used to distinguish between bacteria that produce the enzyme Catalase, such as staphylococci, and those that do not, such as streptococci. For ordinary culture, 3% H_2O_2 is often utilized, while 15% H_2O_2 is employed to identify catalase in anaerobes. A drop of 3% H_2O_2 was placed on the glass slide. A loopful of culture was added into the 3% solution. Observed for the evolution of oxygen bubbles. Positive results indicate copious bubbles produced, active bubbling, and Negative results indicate no or very few bubbles were produced.

3.8.3.2 Indole production test

The indole test was used to assess an organism's capacity to break down the amino acid tryptophan to produce the chemical indole. Indole is one of the three probable end products of tryptophan hydrolysis, which can result from tryptophan. With the help of Kovac's or Ehrlich's reagent, which contains 4 (p)-dimethyl amino Benzaldehyde, which combines with indole to create a red-colored molecule, indole synthesis can be identified. The identification of Enterobacterium required the use of the indole production test. The amino acid tryptophan is broken down by the majority of *E. coli* strains, *P. vulgaris*, *P. rettgeri*, and *M. morganii* species in order to liberate indole.

That was carried out by a network of several intracellular enzymes, collectively known as "tryptophan's." It was a component of the IMVIC assays, which were used to discriminate between Enterobacteriaceae family members. The bacteria to be tested was inoculated into tryptophan broth. Incubated overnight at 37°C. Then added a few drops of Kovac's reagent. Without shaking the tube finally, the result was observed. Positive results indicate the formation of a red or pink-colored ring at the top of the media, and negative results indicate no color change in the media after the ad dilution of the reagent.

3.8.3.3 Triple Sugar Iron (TSI) test

The Enterobacteriaceae, a family of gram-negative bacilli capable of fermenting glucose with the production of acid, were to be distinguished from other gram-negative intestinal bacilli using the Triple Sugar Iron Agar test, which was created to distinguish between the various groups or genera of the family. The main types of intestinal organisms were differentiated based on variations in their carbohydrate fermentation behaviors and hydrogen sulfide generation. The TSI agar slants contain lactose, sucrose, and glucose at concentrations of 1% and 0.1%, respectively, to simplify monitoring of the pattern of carbohydrate utilization and to enable detection of the use of this substrate exclusively. To recognize fermentation of carbohydrates, the phenol red acid-base indicator was also included. With a sterilized straight inoculation needle touched the top of a well-isolated colony. Inoculated TSI Agar by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant. Leave the cap on loosely and incubate the tube at 35°C in ambient air for 18 to 24 hours.

Table 2. Indication of TSI test.

Sl. No.	(slant/butt)	Interpretation
01	Yellow/Yellow	Glucose and lactose and/or sucrose fermentation.
02	Yellow/Yellow with bubbles	Glucose and lactose and/or sucrose fermentation, Gas produced.
03	Red/Yellow with bubbles and black precipitate	Glucose fermentation only, Gas produced, H ₂ S produced.

3.8.3.4 Methyl Red test

The methyl red test (MR) makes use of a glucose-containing liquid medium. The reagent methyl red is added to the medium after the medium has been infected and incubated to allow bacteria to proliferate and conduct fermentation. Methyl red is a pH indicator that turns medium red when it detects the synthesis of mixed acids (methyl red positive). Methyl red will not create a red color in the absence of mixed acid fermentation (methyl red negative). Firstly, the tube was Inoculated containing MR Broth with a pure culture of the microorganism under investigation. Then it was incubated at 37 °C for 24 hours. Finally, about 5 drops of the methyl red indicator solution was added to the tube. Positive results indicate a distinct red color at the top of the media, and negative results indicate a yellow color and/or no color change in the media.

3.8.3.5 Voges-Proskauer (VP) test

The Voges-Proskauer (VP) test is used to determine if an organism produces acetyl methyl carbinol from glucose fermentation. Acetyl methyl carbonyl, if present, is transformed to diacetyl in the presence of α -naphthol, strong alkali (40% KOH), and ambient oxygen. Although it wasn't used in the original method, the α -naphthol was discovered to have color-changing properties. Fermentation of glucose, lactose, and/or sugar generated H_2O_2 and gas. Fermentation of glucose, lactose, and/or sucrose results in the production of water. First, an intensifier by Barritt's chemicals is required. The diacetyl and guanidine-containing chemicals in the broth's peptones then condense to create a pinkish-red polymer. The tube was inoculated with a pure culture of bacteria containing V-P broth. Incubated at 37 °C for 24 hours. After incubation 10 drops of Barritt's reagent A were added to the V-P broth and shake the cultures. Then the tube was inoculated immediately with 10 drops of Barritt's reagent B. Positive results indicate a pink-red color on the surface of the media, and negative results indicate a lack of a pink-red color on the surface of the media.

3.8.3.6 Citrate utilization test

After incubating the Simmon's citrate agar medium for 96 hours at 37° C with a culture inoculation, the media turned blue and streaks of growth appeared. This indicated a favorable reaction. The original green color signified a negative test.

3.9 Antibiotic sensitivity tests

The standard Kirby-Bauer disk diffusion method was used to determine the antimicrobial susceptibility profile of the isolates (Bauer *et al.*, 1966) according to the recommendations of the National Committee for Clinical Laboratory Standard (CLSI-2022) Sensitivity to antibiotics was studied on Muller-Hinton Agar plate used the different types of commercial antibiotic discs. The antibacterial used are Ampicillin (AM) -10, Vancomycin (VA) -30, Erythromycin (E) -15, Amoxicillin (AX) -30, Levofloxacin (LEV) – 5, Ciprofloxacin (CIP) -5, Ceftriaxone (CRO) -30, Penicillin G (P) -10, Tetracycline (TE) -10, Gentamicin (GN) -10. Antibiotic disks were applied using some sterile forceps. Agar plates were incubated at 37°C for 24 hours. After overnight incubation at 37°C, the diameter in millimeters of the zones of inhibition around each of the antimicrobial discs was recorded and categorized as resistant or sensitive in accordance with company recommendations. All isolates were tested for sensitivities to 10 routine and practical antibiotics. Antibiotic discs are used shown in table 5.

Table 3. Antimicrobial agents with their disc concentration and diameter of zone of inhibition

Antimicrobial Agents	Sensitive or more	Intermediate	Resistant or less
Ampicillin (AM) -10	≥ 17	15-16	≤ 14
Vancomycin (VA) -30	≥ 17	15-16	≤ 14
Erythromycin (E) -15	≥ 23	14-22	≤ 13
Amoxicillin (AX) -30	≥ 18	14-17	≤ 13
Levofloxacin (LEV) - 5	≥ 17	14-16	≤ 13
Ciprofloxacin (CIP) -5	≥ 21	16-20	≤ 15
Ceftriaxone (CRO) -30	≥ 23	20-22	≤ 19
Penicillin G (P) -10	≥ 29	-	≤ 28
Tetracycline (TE) -10	≥ 15	12-14	≤ 11
Gentamicin (GN) -10	≥ 15	13-14	≤ 12

Source: The Clinical and Laboratory Standards Institute (CLSI-2022), revised paper

3.9.1 Antimicrobial susceptibility testing of isolated bacteria

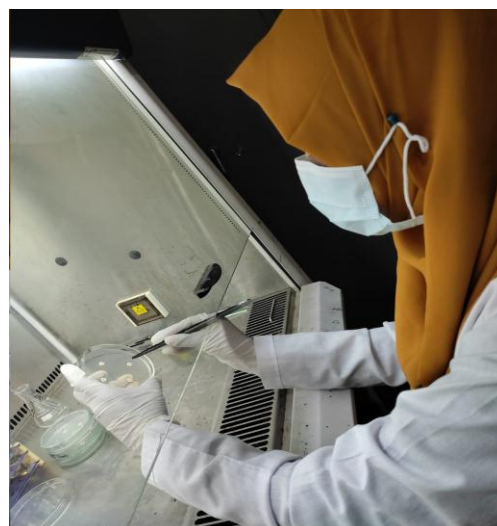
Antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection in vivo.

The procedure involved the following steps-

- The bases of each of the agar plates were labeled with the name of the test organism to be inoculated.
- A sterile cotton swab was dipped into a well-mixed saline test culture and by pressing the saturated swab against the inner wall of the culture tube excess inoculum was removed.
- Using the swab, the entire agar surface horizontally, vertically, and around the outer edge of the plate to ensure a heavy growth over the entire surface was streaked.
- All the culture plates are allowed to dry for about 5 minutes.
- The individual discs were placed at equal distances with forceps dipped in alcohol and flamed.
- Each disc was pressed down with sterile forceps to ensure that the discs adhere to the surface of the agar.
- All the culture plates were incubated in an inverted position for 24 hours at 37°C.
- All the culture plates are examined for the presence or absence of a zone of inhibition surrounding each disc after completion of incubation.



Media preparation for sensitivity test



Antibiotic disc placement on media

Figure 10: Antibiotic sensitivity test.

3.10 DNA extraction

3.10.1 Materials used for isolation of bacterial genomic DNA

The materials used for bacterial genomic DNA isolation were QIAamp® DNA mini kit, 1.5 ml Eppendorf tubes, Water bath, 56°C, Centrifuge machine, Micropipette with tip, Vortex machine etc.



Figure 11: QIAamp® DNA mini kit (QIAGEN) used in DNA extraction

3.10.2. Procedure of DNA Extraction

At first, 200 μ l of PBS was taken in an Eppendorf tube with the help of a micropipette. Then, one bacterial colony was taken from pure culture and added to the Eppendorf tube containing PBS with the help of inoculum. Then 130 μ l of Buffer ATL was added to each tube and mixed gently. Then 15 μ l of proteinase K was added to the tube, vortexed, and incubated at 56°C for three hours invert mixed occasionally. Then 200 μ l of Buffer AL was added to the sample, pulsed for 15 seconds, and incubated at 70°C for 10 minutes. Then 200 μ l of ethanol (100%) was added, pulse-mixed, and touch-spin. Then samples were transferred to a Qiagen mini spin column, spin maximum for 60 seconds, and the filtrate discarded. Then 500 μ l of Buffer AW1 was added to each tube, spin maximum for 60 seconds, and the filtrate was discarded. Then the transfer of the column to a fresh collection tube was done. Then 500 μ l of Buffer AW2 was added to each tube; spin max for 60 sec; discard the filtrate. The transfer of the spin column to a fresh 1.5-ml labeled Eppendorf tube was performed. Then 100 μ l of Buffer AE was added to the membrane. Then the tube was incubated at room temperature for a minimum of 10–15 minutes. Spin at 6000 rpm for 60 seconds was done. Store the collected DNA at -20°C until PCR amplification is done.



**Sample collection in
Eppendorf tube**



Boiling



Spinning

Figure 12: DNA extraction

3.11 Polymerase Chain Reaction (PCR)

3.11.1 Selection of primer for detecting *E. coli* and *Staphylococcus aureus*

There were two types of primer used in this study. One was for *E. coli*, and another was for *Staphylococcus aureus*

A) Eco 223 (F) and Eco 455 (R) primer: The band size was 232 bp to confirm the presence of *E. coli*.

B) Sau 234 (F) and Sau 1501 (R) primer: The band size was 1267 bp to confirm the presence of *Staphylococcus aureus*

Table 4. Properties of primer used in this study

Specificity	Primer name	Primer sequence (5′-3′)	Target gene	Annealing temp (°C)	Size of product amplified (bp)	Reference
<i>E. coli</i>	Eco 223 (F)	ATC AAC CGA GAT TCC CCC AGT	16S & 23S rRNA	55	232	Riffon <i>et al.</i> , (2001)
	Eco 455 (R)	TCA CTA TCG GTC AGT CAG GAG				
<i>S. aureus</i>	Sau 234 (F)	CGA TTC CCT TAG TAG CGG CG	16S & 23S rRNA	58	1267	
	Sau 1501 (R)	CCA ATC GCA CGC TTC GCC TA				

3.11.2 Amplification of extracted DNA for confirmation *E. coli* and *Staphylococcus aureus*

PCR product was formed by mixing all the essential reagents (master mix, forward primer “Eco 223, Sau 234”, reverse primer “Eco 455, Sau 1501”, nuclease free water and

extracted DNA) in a PCR tube (Table 5). Then the tubes were placed in the well of PCR machine and optimum condition for PCR was commanded (Table 6 and table 7). The overall procedure was followed according to Riffon *et al.*, (2001).



Mixing of master mix



PCR

Figure 13: PCR procedure

Table 5. Preparation of PCR product for Eco 223, Eco 455, Sau 234, Sau 1501 primer

Sl. No.	Name of the Products	Volume (µl)
01	Master mix	12.5
02	Extracted DNA	5
03	Primer F (Eco 223, Sau 234)	1
04	Primer R (Eco 455, Sau 1501)	1
05	Water	5.5
Total		25

Table 6. Condition of PCR for *Staphylococcus aureus*

Step name	Temperature	Duration time	Number of cycles
Preheat	94°C	2 minutes	1
Denaturation	94°C	45 seconds	35
Annealing	58°C	45 seconds	
Extension	72°C	2 minutes	
Final extension	72°C	6 minutes	1
Holding	4°C	overnight	1

Table 7. Condition of PCR for *E. coli*

Step name	Temperature	Duration time	Number of cycles
Preheat	94°C	3 minutes	1
Denaturation	94°C	30 seconds	35
Annealing	55°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	3 minutes	1
Holding	4°C	overnight	1

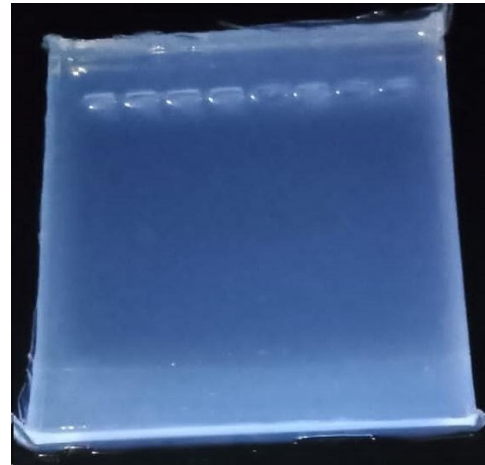
3.11.3 Preparation of gel and gel-electrophoresis

0.75 g of agarose powder was measured. Agarose powder was mixed with 50 mL 1xTAE in a microwavable flask. Microwave for 2 min was performed until the agarose was completely dissolved. The agarose solution was cooled down to about 50 °C for about 5 mins. Ethidium bromide (EtBr) (2.5 µl) was added to a final concentration of approximately 0.2-0.5 µg/ml. The agarose was poured into a gel tray with the well comb in place. Newly poured gel was placed at room temperature for 30-45 mins until it had completely solidified. Sample was applied in the gel. Adjustment of voltage or current (gel-electrophoresis about 70-100 volts) was performed. Set up of run time about 30-45 minutes was done. When DNA migrated sufficiently, as judged from the migration of bromophenol blue of loading buffer, the power supply was switched off. The gel was placed on the imaging system in the dark chamber of the image documentation. The UV

light of the system was switched on; the image was viewed on the monitor, focused, acquired and saved in a USB flash drive.



Gel running of PCR product



PCR gel

Figure 14: Gel-electrophoresis

3.12. Statistical analysis

The generated data was compiled in MS Excel sheet and analyzed using the statistical analytical software SPSS (version 29.0.1.0). Prevalence was analyzed by one way ANOVA. All significant differences were considered at $p < 0.05$.

CHAPTER IV

RESULTS

The research was aimed at the diagnosis, isolation and identification of causal agents of repeat breeding in dairy cows.

4.1 Prevalence of major reproductive disorders in dairy cattle in Rangpur Division

The prevalence of major reproductive disorders of dairy cattle is presented in table 8. The major reproductive problems were anestrus, repeat breeding syndrome, dystocia, endometritis, retained fetal membrane, abortion, uterine and vaginal prolapse. Among all the reproductive disorders were found in this study, repeat breeding syndrome was found to be the significantly ($p<0.05$) highest (41.33%) and anestrus was the second (31.33%) common reproductive disorders. The prevalence of retained placenta, abortion, dystocia, uterine prolapse, endometritis, vaginal prolapse was 9.66%, 5.33%, 7.00%, 1.33%, 2.66%, 1.33% respectively.

Table 8: Prevalence of major reproductive disorders in dairy cattle in Rangpur Division

Name of reproductive disorders (n=300)	Frequency	Percent of total affected animals (%)
Anestrus	94	31.33 ^b
Abortion	16	5.33 ^c
Endometritis	8	2.66 ^d
Dystocia	21	7.00 ^c
Repeat breeding syndrome	124	41.33 ^a
Retained placenta	29	9.66 ^c
Uterine prolapse	4	1.33 ^d
Vaginal Prolapse	4	1.33 ^d

^{abcd} different superscript letters in same column differs significantly (at least $p<0.05$)

4.2 Prevalence of Different Types of Bacteria Found in Repeat Breeding

Prevalence of different types of bacteria isolated from Repeat Breeder cows is shown in table 9. The prevalence of *E. coli* (40%) , *Styphylococcus aureus* (40%) and *Klebsiella spp.* (10%) was most common in all repeat breeder cows.

Table 9: Prevalence of Different Types of Bacteria Found in Repeat Breeding

Types of Bacteria	Total sample (n)	Positive (n)	Prevalence (%) (n/n)
<i>E. coli</i>	10	4	40 ^a
<i>Staphylococcus aureus</i>	10	4	40 ^a
<i>Klebsiella spp.</i>	10	1	10 ^b

^{ab} different superscript letters in same column differs significantly (at least $p < 0.05$)

4.3 Cultural characteristics of Bacteria

The cultural characteristics of *E. coli*, *Staphylococcus aureus*, and *Klebsiella spp.* on various culture media were presented in table 10 and figure 15-23.

Table 10. The cultural characteristics of *E. coli*, *Staphylococcus aureus*, and *Klebsiella spp.* on various culture media

Types of media	Cultural properties	Morphological properties	Remarks
Nutrient agar	White, glistening colonies	Purple/violet colored and small round and coccus shaped organism. Arranged in single, pairs and cluster	<i>Staphylococcus aureus</i>
Mannitol salt agar	Small whitish or yellowish colony		
Blood agar	Produce small white colony		
Nutrient agar	Large mucoid, white colony	Pink colored, small rod-shaped organism. Arranged in single, pair or short chain.	<i>E. coli</i>
MacConkey agar	Produce large mucoid, rose-pink colony.		
Eosin Methylene Blue agar	Green metallic sheen with reflected light.		
Nutrient agar	Creamy white, circular, raised, fluidal colony	Pink color, small, rod shaped, arranged in single.	<i>Klebsiella spp.</i>
MacConkey agar	Sticky, wet appearing, mucoid colony		
Eosin Methylene Blue agar	Large, mucoid, pink to purple colonies with no metallic sheen		

The bacteria were isolated from cervical mucous samples by using spread plate methods on NA, MAC, MSA, EMB, BA. All the isolates produced different kinds of colonies on the different agar plate.

E. coli produced large mucoid, white colony on nutrient agar (Figure-15), large mucoid, rose-pink colony on MacConkey agar (Figure-16), and green metallic sheen with reflected light on Eosin methylene blue agar (Figure-17).

Staphylococcus aureus produced white, glistening colony on nutrient agar (Figure-18), small white colony on blood agar (Figure-19) and yellowish colonies yellow zone with on mannitol salt agar (Figure-20).

Klebsiella spp. Produced creamy white, circular, raised, fluidal colony on nutrient agar (Figure-21), Sticky, wet appearing, mucoid colony on MacConkey agar (Figure-22), Large, Mucoid, Pink to purple colonies with no metallic sheen on EMB agar (Figure-23).

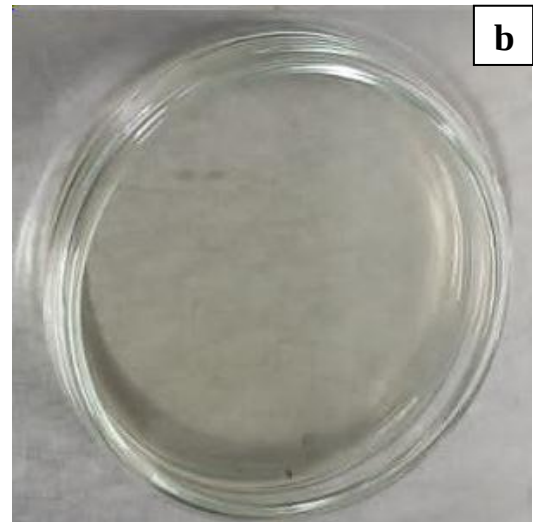
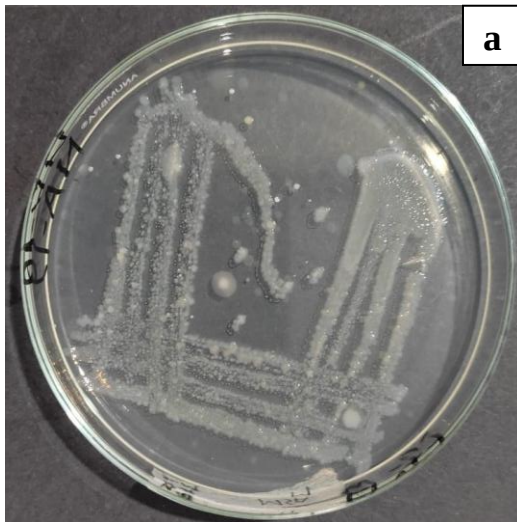


Figure 15: Growth of *E. coli* on (a) nutrient agar (b) negative control

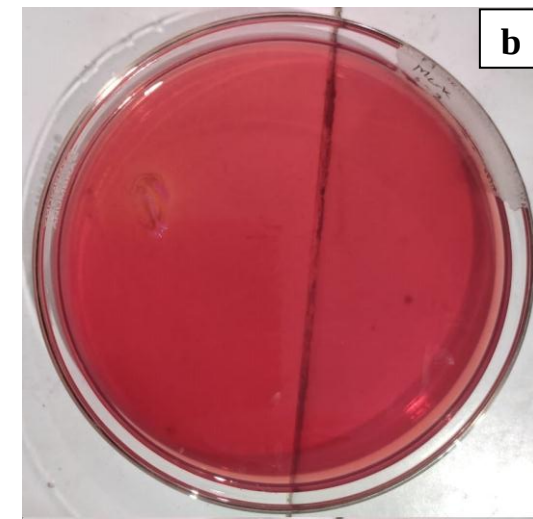
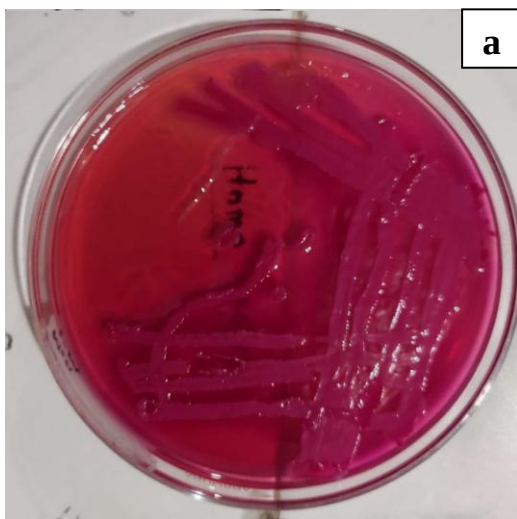


Figure 16. (a) Growth of *E. coli* on MacConkey agar (b) negative control

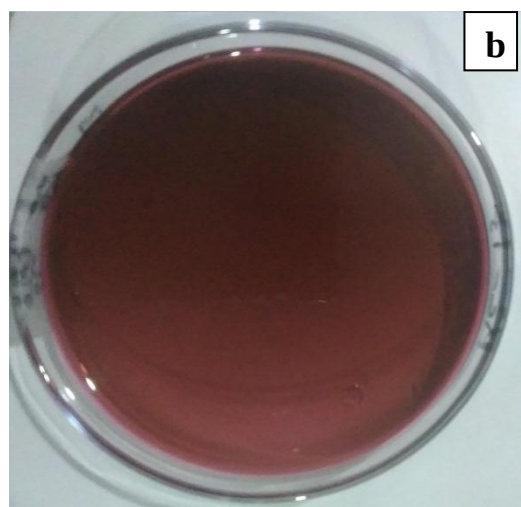
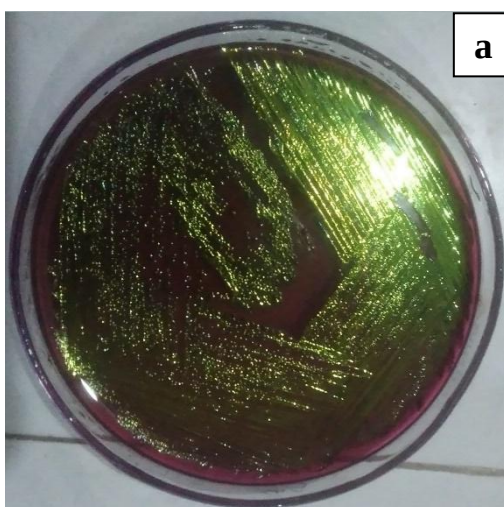


Figure 17: Growth of *E. coli* on (a) Eosin Methylene Blue agar (b) negative control

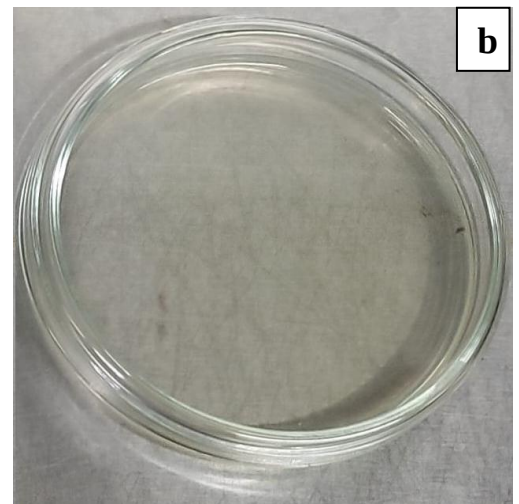


Figure 18: Growth of *Staphylococcus aureus* on (a) nutrient agar (b) negative control

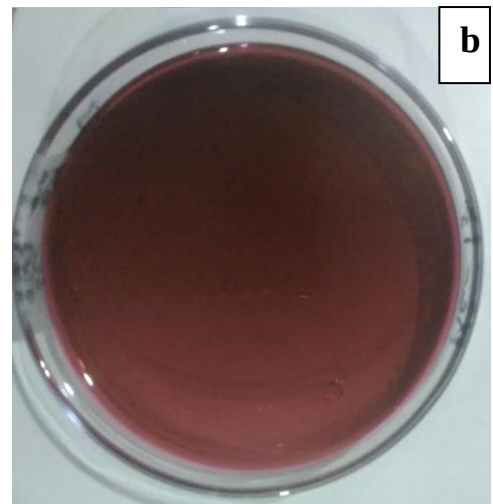
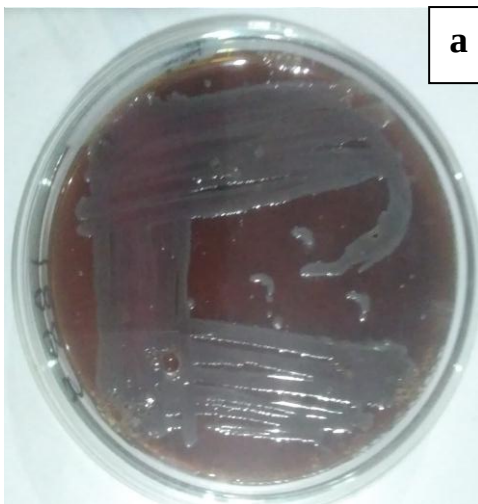


Figure 19: Growth of *Staphylococcus aureus* on (a) blood agar (b) negative control

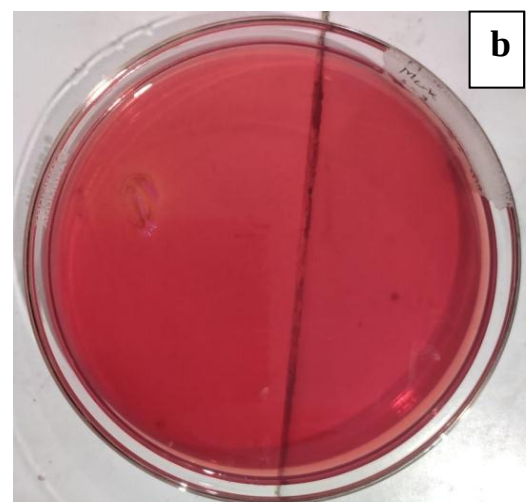
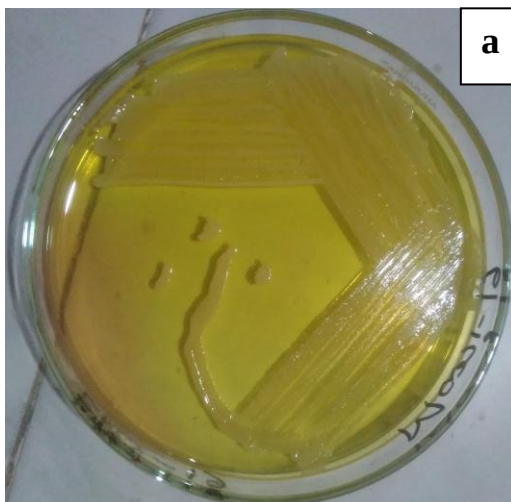


Figure 20: Growth of *Staphylococcus aureus* on (a) Mannitol salt agar (b) negative control

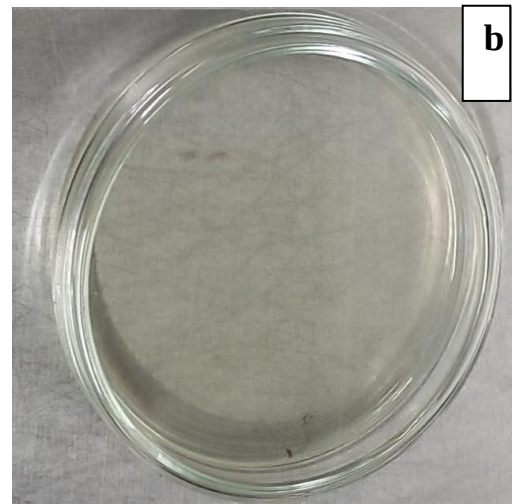
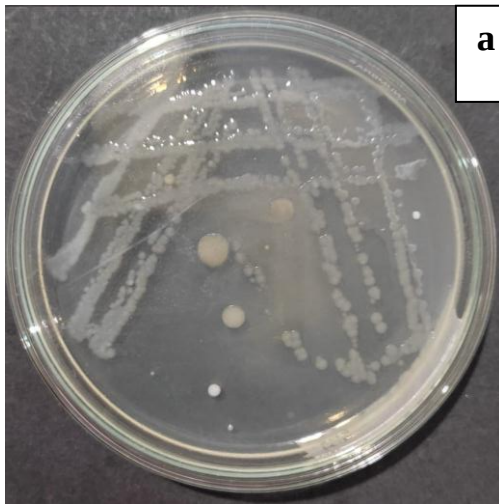


Figure 21: Growth of *Klebsiella* spp. on (a) nutrient agar (b) negative control

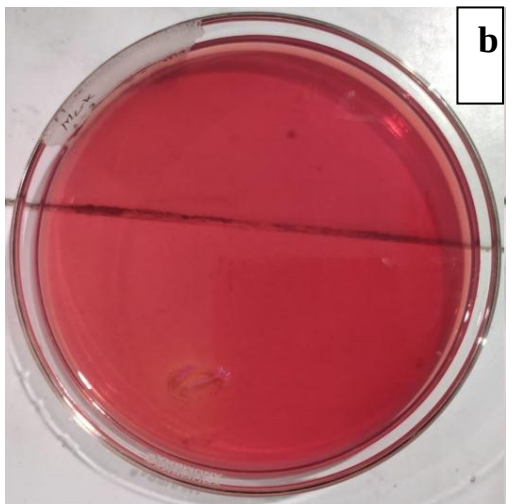


Figure 22: Growth of *Klebsiella* spp. on (a) MacConkey agar (b) negative control

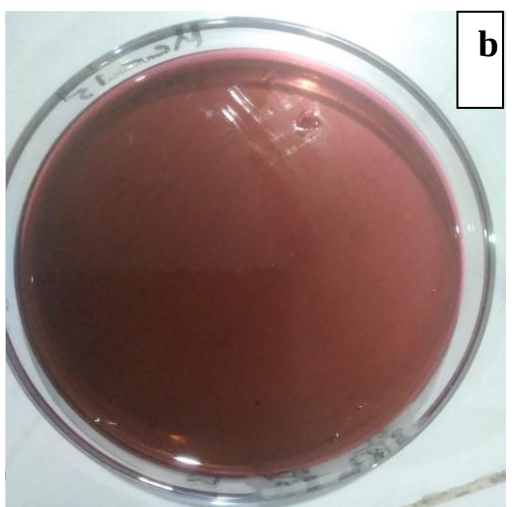
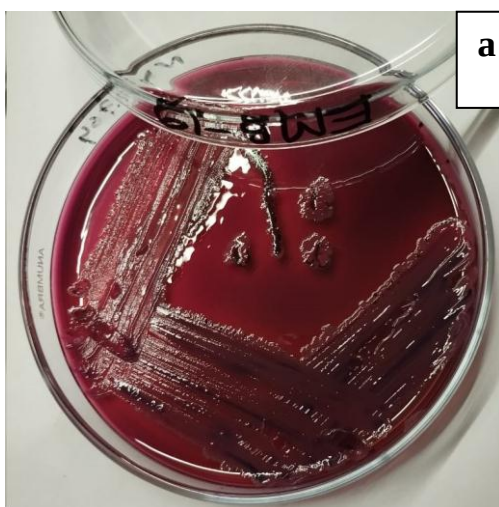


Figure 23: Growth of *Klebsiella* spp. on (a) Eosin Methylene Blue agar (b) negative control

4.3 Characterization of bacteria by using different biochemical tests.

- The identified isolates were characterized by using different biochemical tests (MR, VP, TSI, Indole, Catalase test Citrate test etc.) This observation also revealed that isolated organisms were *Staphylococcus aureus*, *Escherichia coli*, *klebsiella spp.*
- Catalase test was performed by placing a drop of hydrogen peroxide on a slide and mixing the colony of the bacteria to be tested thoroughly. Catalase test was positive (presence of bubble) for *Staphylococcus aureus*, *Klebsiella spp.*, and *E. coli* (Figure-24).
- MR reactions were positive (presence of a red colored colony on the surface of the media) for *Staphylococcus aureus*, *Escherichia coli* and MR reactions were negative (absence of a red colored colony on the surface of the media) for *Klebsiella spp.* (Figure-25).
- Indole tests were negative (absence of a cherry red colored ring on the surface of the media) for *Staphylococcus aureus* and *Klebsiella spp.*, on the other hand positive (presence of a cherry red colored ring on the surface of the media) for *Escherichia coli* (Figure-26).
- VP tests were positive (presence of a red colored colony on the surface of the media) for *Staphylococcus aureus*, *Klebsiella spp.* and negative (absence of a red colored colony on the surface of the media) for *Escherichia coli* (Figure-27).
- Simon's citrate agar was positive (formation of Prussian blue color on the slant) for *Staphylococcus aureus*, *Klebsiella spp.* and negative (no colored change of the medium) for *E. coli* (Figure-28).
- TSI was positive (yellow butt, yellow slant, presence of gas and absence of H₂S) for *E. coli* and *Klebsiella spp.* (Figure-29).

Table 11. Characterization of *Staphylococcus aureus*, *Escherichia coli* and *klebsiella* spp. using different biochemical tests.

Name of the Test		Observation			Remarks
Catalase test		Presence of bubbles.			<i>Staphylococcus aureus</i> , <i>Klebsiella</i> spp., <i>E. coli</i>
Indole test		Presence of cherry red colored ring on surface of the media.			<i>Escherichia coli</i> ,
		Absence of cherry red colored rings on the surface of the media.			<i>Staphylococcus aureus</i> , <i>Klebsiella</i> spp.
MR test		Presence of a red colored ring on the surface of the media.			<i>Escherichia coli</i> , <i>Klebsiella</i> spp.
		Absence of a red colored ring on the surface of the media.			<i>Staphylococcus aureus</i>
VP reaction		Presence of red colored rings on the surface of the media.			<i>Staphylococcus aureus</i> , <i>Klebsiella</i> spp.
		Absence of red colored ring on the surface of media			<i>Escherichia coli</i>
Simon's citrate test		Formation of Prussian blue color on the slant.			<i>Staphylococcus aureus</i> , <i>Klebsiella</i> spp.
		No color change of the medium			<i>Escherichia coli</i>
TSI Test	Slant	Butt	Gas	H ₂ S	<i>Escherichia coli</i> , <i>Klebsiella</i> spp.
	Yellow	Yellow	Present	Absent	

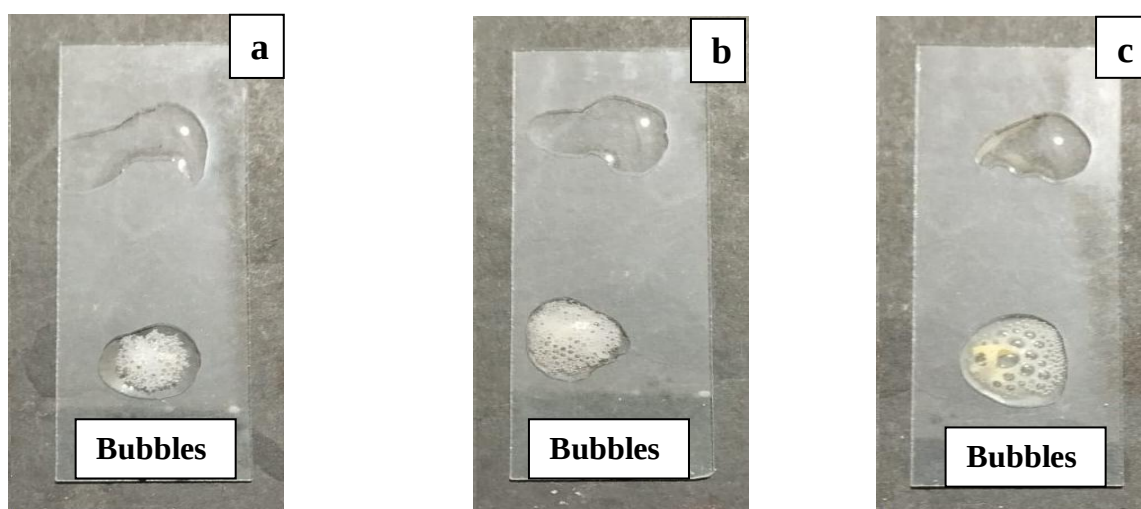


Figure 24. Catalase test positive for (a) *E. coli*, (b) *S. aureus* and (c) *Klebsiella spp.* showing presence of bubbles.

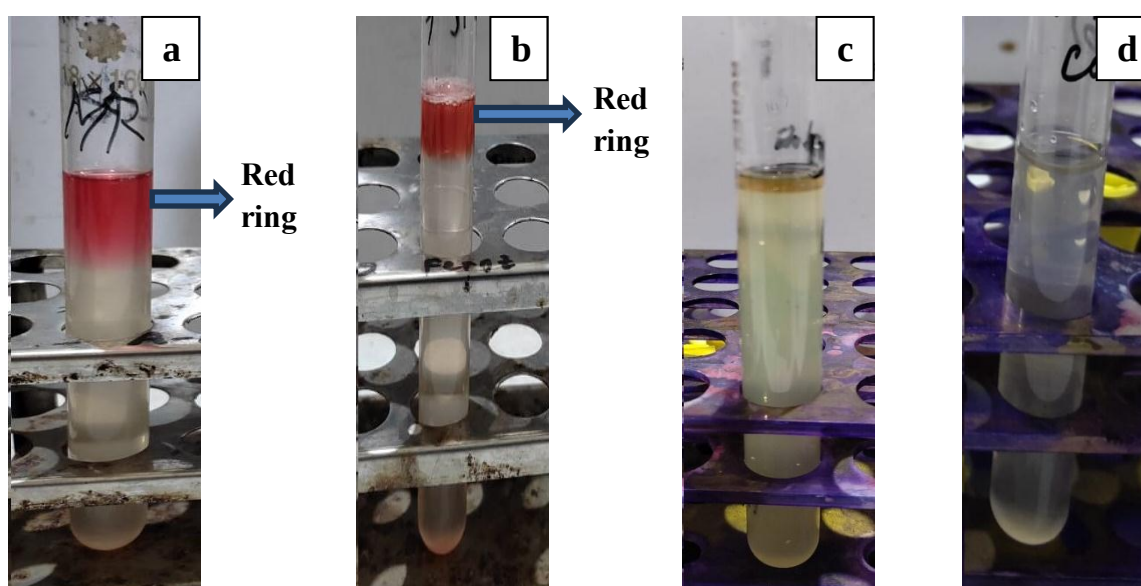


Figure 25. Methyl Red test positive for (a) *E. coli* and (b) *Staphylococcus aureus* showing presence of cherry red colored ring on surface of the media, negative for (c) *Klebsiella spp.* showing absence of cherry red colored ring on surface of the media (d) negative control.

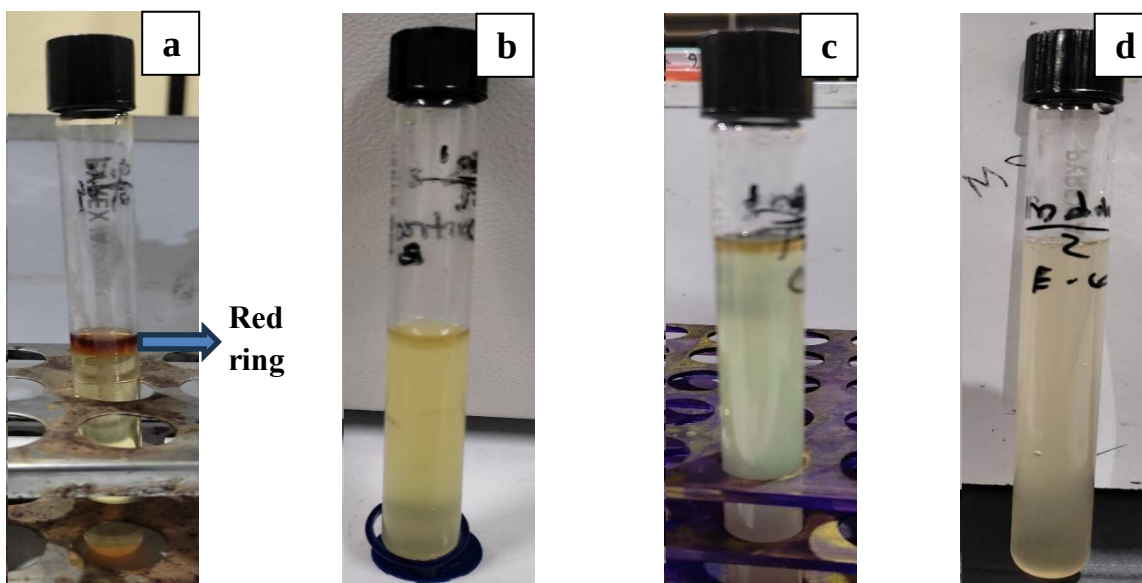


Figure 26: Indole test positive for (a) *E. coli* showing presence of cherry red colored ring on surface of the media and negative for (b) *Staphylococcus aureus* and (c) *Klebsiella spp.* showing absence of cherry red colored ring on surface of the media (d) negative control.

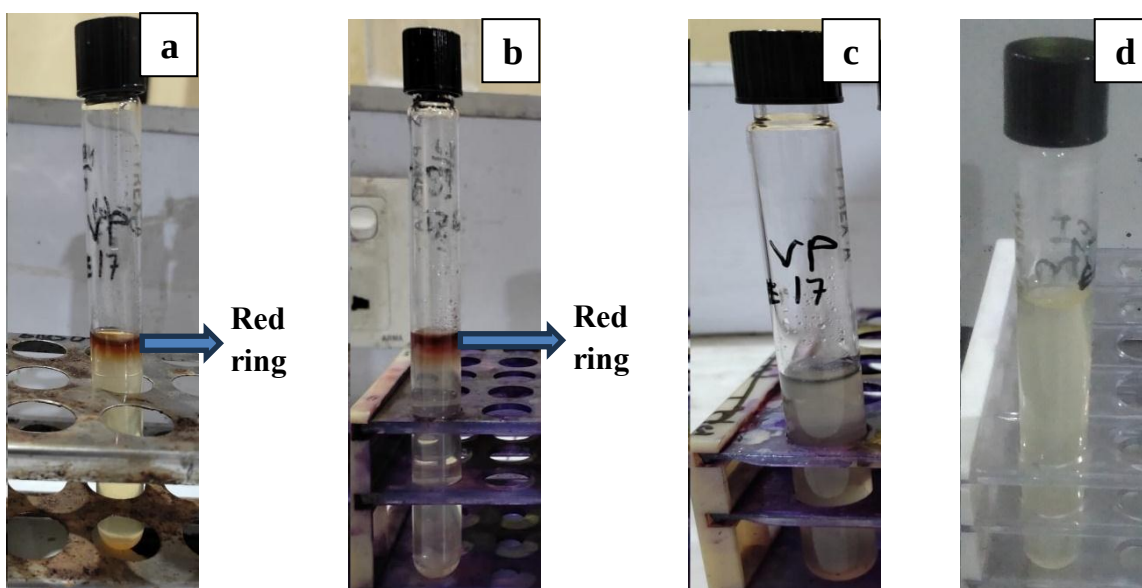


Figure 27: Voges-Proskauer test positive for (a) *Staphylococcus aureus* and (b) *Klebsiella spp.* showing presence of cherry red colored ring on surface of the media and negative for (c) *E. coli* showing absence of cherry red colored ring on surface of the media (d) negative control.

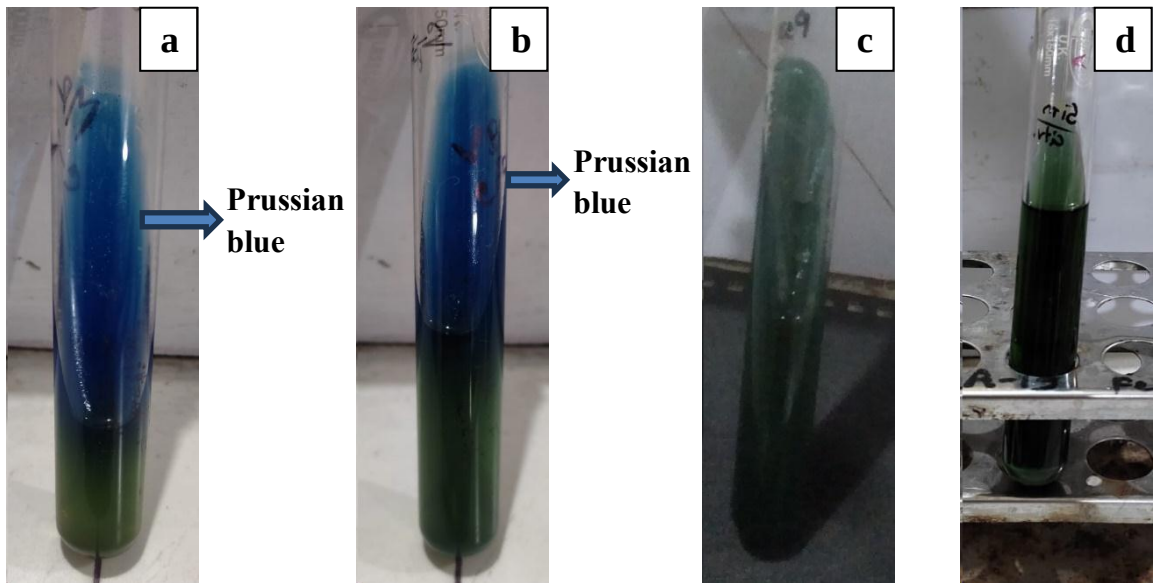


Figure 28: Citrate Utilization test positive for (a) *Staphylococcus aureus* and (b) *Klebsiella spp.* showing formation of Prussian blue on the slant and negative for (c) *E. coli* showing absence of Prussian blue on the slant (d) negative control.

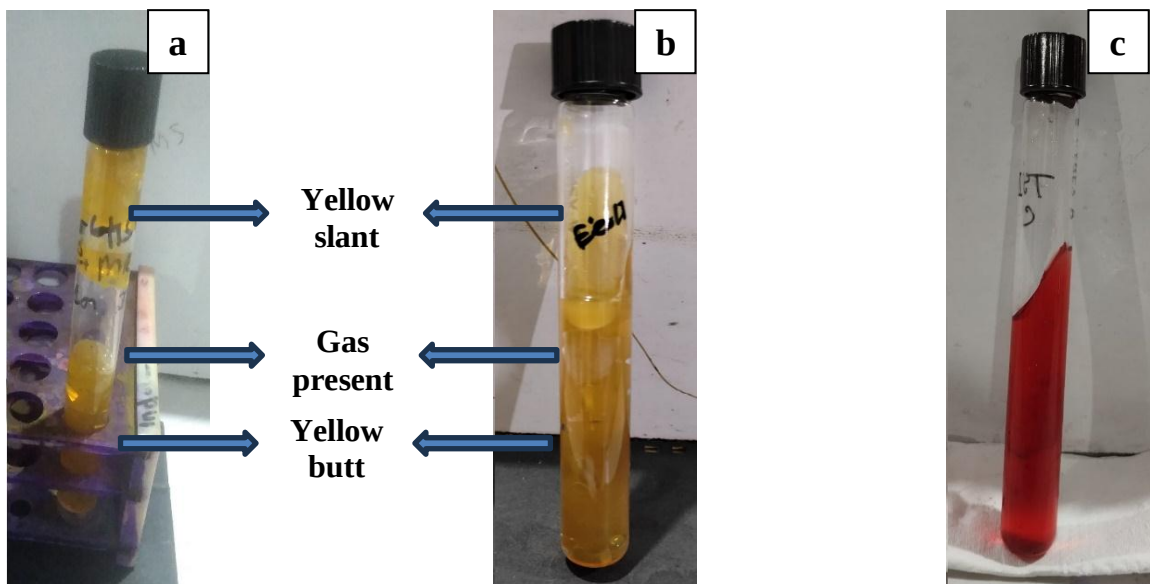


Figure 29: Triple Sugar Iron test positive for (a) *E. coli* and (b) *Klebsiella spp.* showing yellow slant, yellow butt and presence of gas (c) negative control.

4.4 Results of morphological properties by Gram's Staining Technique

The obtained results revealed that gram positive violet color, cocci shaped arranged in cluster indicated *Staphylococcus aureus* (Figure-31), Pink color, small, rod shaped, arranged in single, indicated gram negative *Klebsiella spp.* (Figure-32) It also observed that gram's negative pink color, small, rod-shaped organisms arranged in single, pair, short chain indicated *E. coli* (Figure-30).

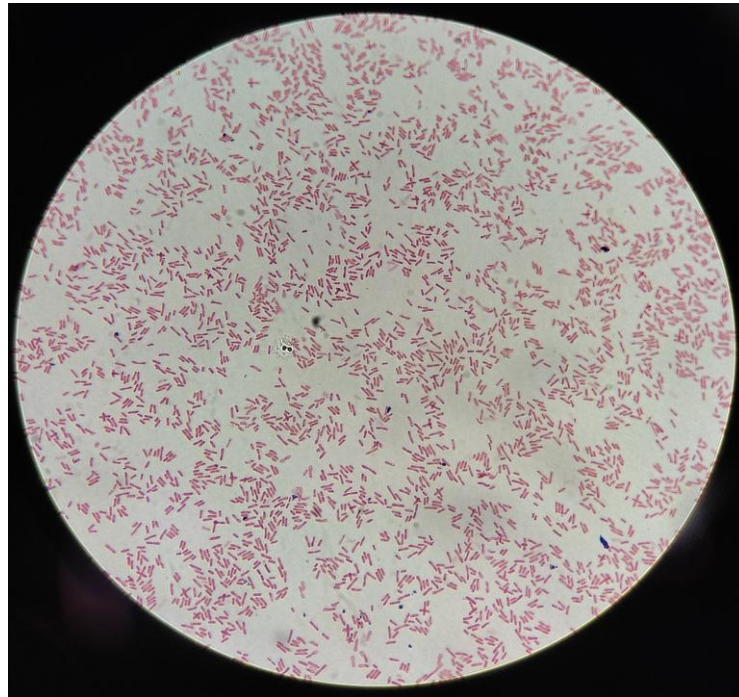


Figure 30: Pink color, rod shaped, arranged in single, indicated gram negative *Escherichia coli*

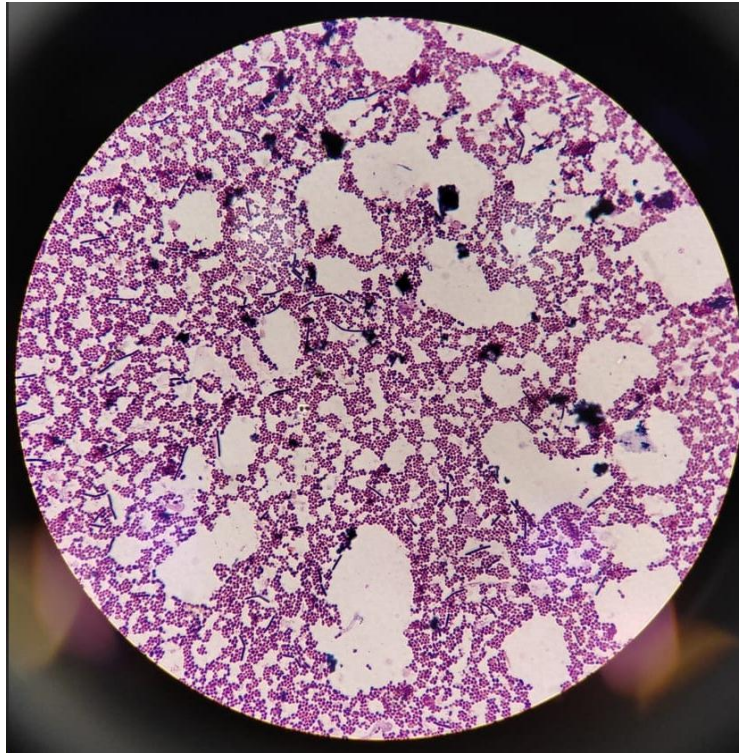


Figure 31: Violet color, cocci shaped, arranged in cluster, indicated gram positive *Staphylococcus aureus*

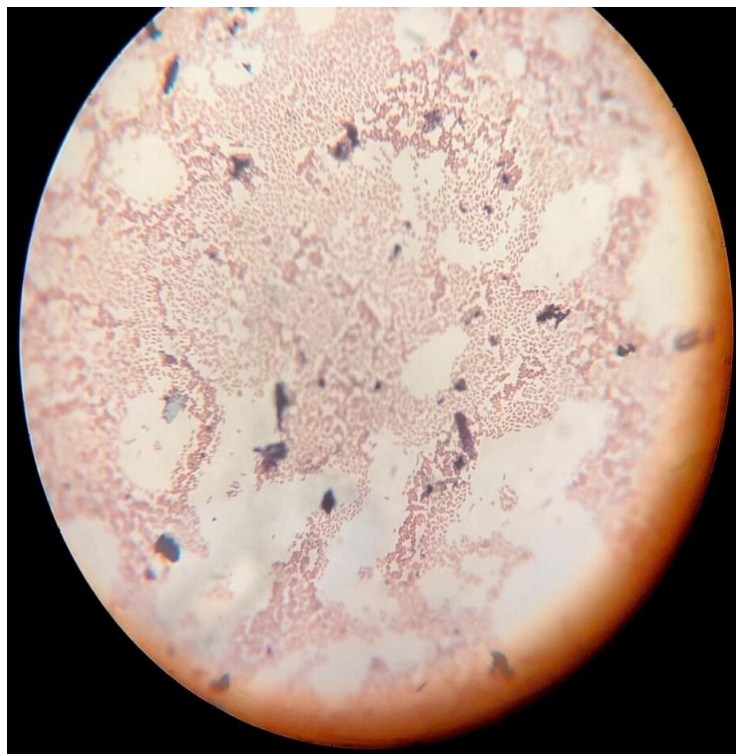


Figure 32: Pink color, small, rod shaped, arranged in single, indicated gram negative *Klebsiella spp.*

4.5 Result of antibiotic sensitivity test of isolated bacteria

To ascertain the sensitivity and resistance pattern against frequently used antibiotic discs, the isolated *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella spp.* were treated with antibiotics. The antibiogram profile revealed that *E. coli* was sensitive to Ceftriaxone, Gentamicin and resistant to Ciprofloxacin, Penicillin, Tetracycline, Amoxicillin, Erythromycin. *Staphylococcus aureus* was sensitive to Ciprofloxacin, Ceftriaxone, Levofloxacin intermediately resistant to Vancomycin and Amoxicillin and resistant to Ampicillin, Erythromycin. *Klebsiella spp.* was sensitive to Ciprofloxacin, Tetracycline intermediately resistant to Erythromycin, Gentamicin and resistant to Ceftriaxone, Penicillin, Amoxicillin. The results of antibiotic sensitivity tests are shown in table 12, 13 & 14. Antibiotic sensitivity patterns are shown in figure 31, 32 and 33.

Table 12. Zone diameter interpretative standards for *E. coli*

Antimicrobial agents	Diameter of zone of inhibition (mm)	Interpretation
Ciprofloxacin (CIP)-5	0	R
Ceftriaxone (CRO)-30	28	S
Penicillin G (P)-10	0	R
Tetracycline (TE)-10	0	R
Amoxicillin (AX)-30	0	R
Gentamicin (GN)-10	16	S
Erythromycin (E)-15	6	R

Source: The Clinical and Laboratory Standards Institute (CLSI-2022), revised paper

Table 13. Zone diameter interpretative standards for *Staphylococcus aureus*

Antimicrobial agents	Diameter of zone of inhibition (mm)	Interpretation
Ciprofloxacin (CIP)-5	26	S
Ceftriaxone (CRO)-30	20	S
Levofloxacin (LEV)-5	26	S
Vancomycin (VA)-30	15	I
Amoxicillin (AX)-30	17	I
Ampicillin (AM)-10	6	R
Erythromycin (E)-15	8	R

Source: The Clinical and Laboratory Standards Institute (CLSI-2022), revised paper

Table 14. Zone diameter interpretative standards for *Klebsiella spp.*

Antimicrobial agents	Diameter of zone of inhibition (mm)	Interpretation
Ciprofloxacin (CIP)-5	26	S
Ceftriaxone (CRO)-30	15	R
Penicillin G (P)-10	0	R
Tetracycline (TE)-10	19	S
Amoxicillin (AX)-30	0	R
Gentamicin (GN)-10	14	I
Erythromycin (E)-15	20	I

Source: The Clinical and Laboratory Standards Institute (CLSI-2022), revised paper

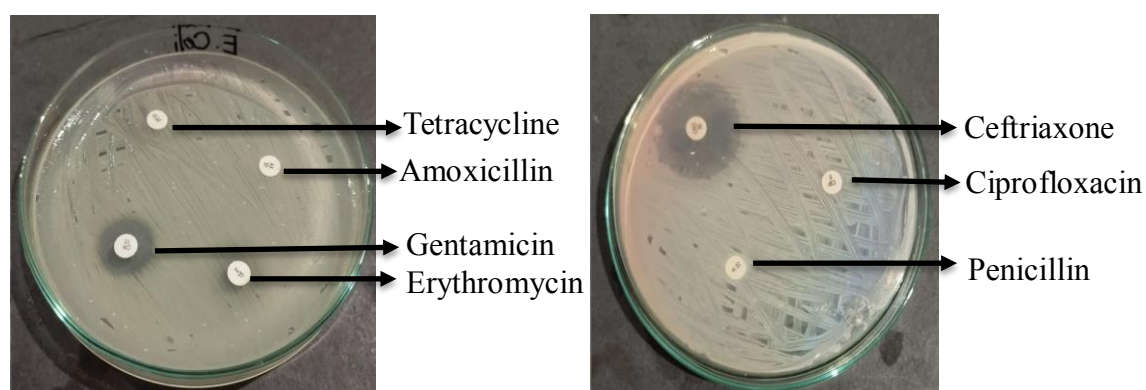


Figure 33: Representative picture of antibiotic sensitivity test of *E. coli*

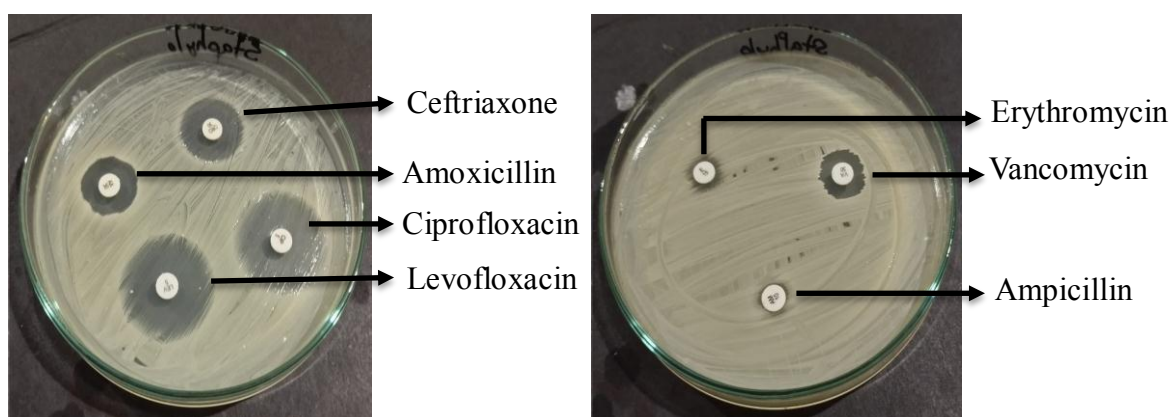


Figure 34: Representative picture of antibiotic sensitivity test of *S. aureus*

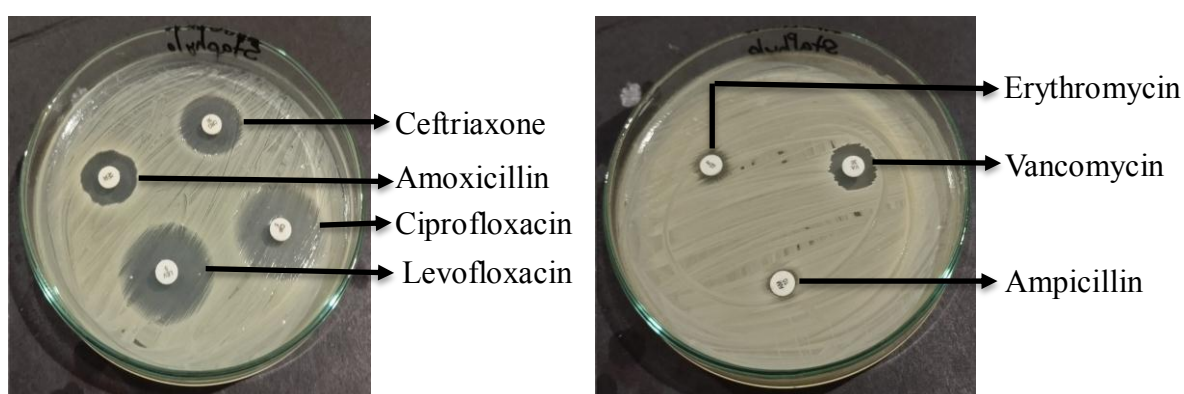


Figure 35: Representative picture of antibiotic sensitivity test of *Klebsiella spp.*

4.6 Molecular confirmation of *E. coli* and *Staphylococcus aureus* by PCR technique

Molecular confirmation of *E. coli* and *Staphylococcus aureus* was confirmed by using Eco 223, Eco 455, Sau 234, and Sau 1501 primers. The target genes were 16S and 23S rRNA, and the size of the product amplified at 232 bp and 1267 bp respectively. Result of PCR for *E. coli* and *Staphylococcus aureus* was shown in figure 34 and figure 35 respectively.

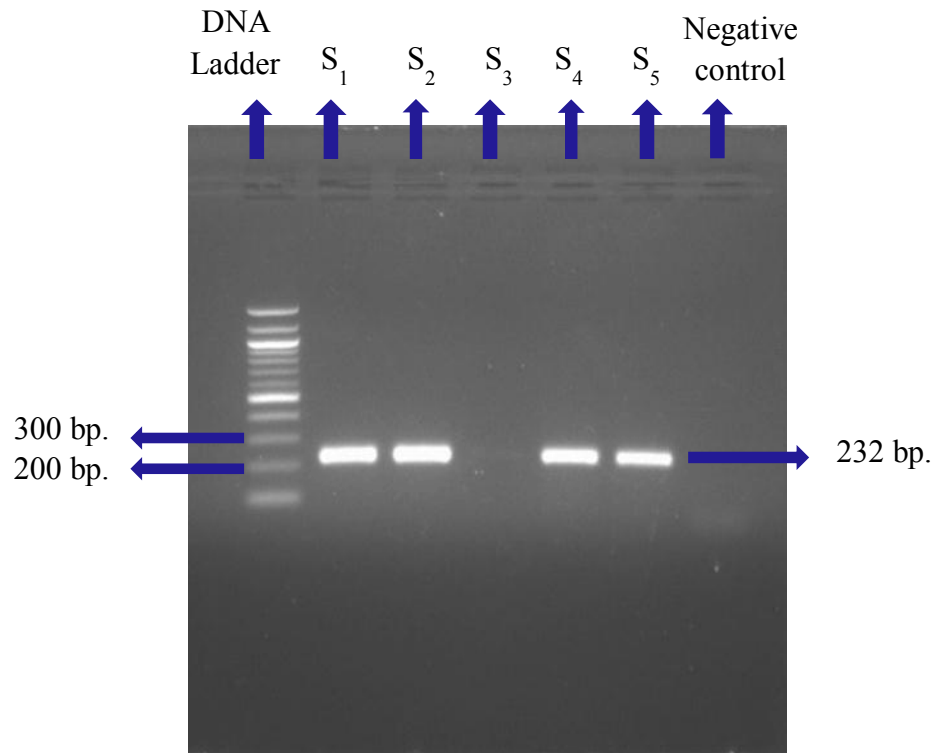


Figure 36: PCR amplification of *E. coli*. Well 1 contain DNA ladder, well 2-6 contain different sample, well 7 contain negative control. *E. coli* DNA shown at 232 bp. 100 bp. ladder was used.

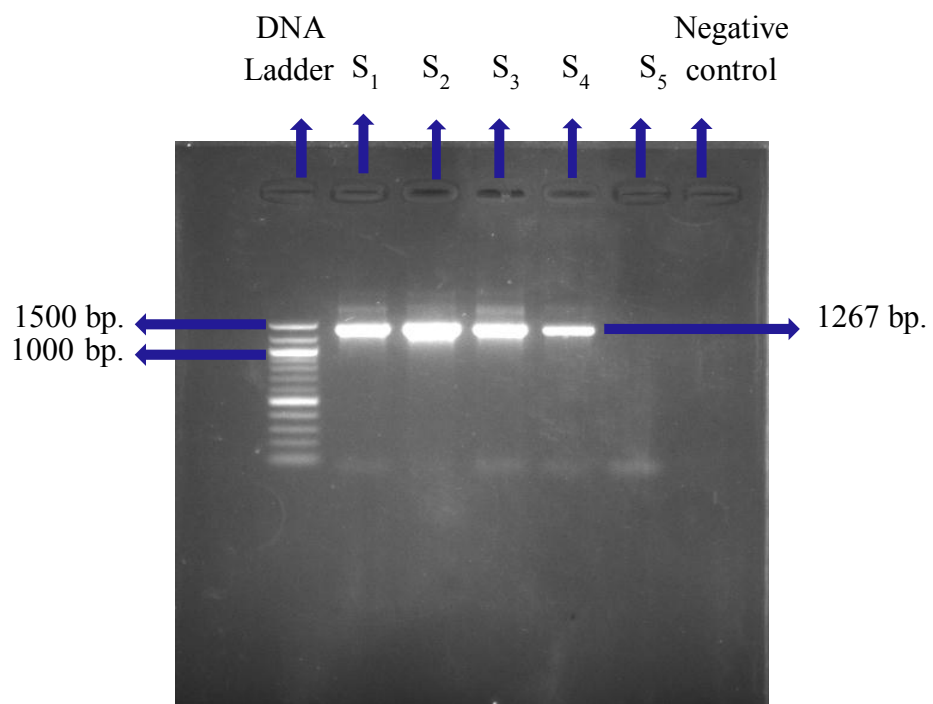


Figure 37: PCR amplification of *Staphylococcus aureus*. Well 1 contain DNA ladder, well 2-6 contain different sample, well 7 contain negative control. *Staphylococcus aureus* DNA shown at 232 bp. 100 bp. ladder was used.

CHAPTER V

DISCUSSION

The experiment was carried out to isolate and identify the bacteria with molecular confirmation causing repeat breeding syndrome in cows. Three different bacterial species (*Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella spp.*) were identified and isolated in this research. Based on the morphology of the colony, gram reaction, microscopic features, biochemical properties and molecular confirmation was done to identify the bacteria from the isolates. The selected isolates were further tested for antibiotic sensitivity using commercially available antibiotics and the conventional Kirby-Bauer disk diffusion method.

The results of present research revealed that the major reproductive problems were anestrus, repeat breeding syndrome, dystocia, endometritis, retrained fetal membrane, abortion, uterine and vaginal prolapse. Among all the reproductive disorders found in this study, repeat breeding syndrome was highest (41.33%) and anestrus was the second (31.33%) common reproductive disorders. The prevalence of retained placenta, abortion, dystocia, uterine prolapse, endometritis, and vaginal prolapse was 9.66%, 5.33%, 7.00%, 1.33%, 2.66%, 1.33%; respectively. The prevalence of repeat breeding syndrome was higher in this study than the findings of others (Islam *et al.*, 2023; Asaduzzaman *et al.*, 2017; Alemselem *et al.*, 2015; Mesafint & Guesh, 2014; Adane *et al.*, 2014; Maruf *et al.*, 2012; Bitew and Prasad, 2011). The causes of the highest prevalence found in this research might be due to improper management of farms, lack of hygienic condition of farm, improper insemination, failure to detection of ovulation and poor semen quality, age, nutrition, and lack of skilled artificial inseminator in Rangpur division.

The prevalence of *E. coli* was 40%, *Staphylococcus aureus* 40% and *Klebsiella spp.* 10% found in the present study. Compared to our findings, similar results were reported in the other studies (Gani *et al.*, 2008; Singh *et al.*, 1996).

In the present study, cultural characterization of isolated *Staphylococcus aureus* on nutrient agar produced white, glistening colonies; on mannitol salt agar exhibited yellow colonies with yellow zones; and on blood agar produced small white colonies (β -hemolysis). The cultural characterization of *Escherichia coli* on nutrient agar produced

large mucoid, white colonies; on EMB agar produced green metallic sheen with reflected light and on MacConkey agar produced large mucoid, rose-pink colonies. Cultural characterization of *Klebsiella spp.* on nutrient agar produced creamy white, circular, raised, fluidal colonies and on MacConkey agar produced sticky, wet appearing, mucoid colonies and on EMB agar produced large mucoid pink to purple colonies with no metallic sheen. These characteristics of different bacteria observed in this research were consistent with previous studies (Gani *et al.*, 2008; Cheesbrough, 2005).

The biochemical tests showed that *Staphylococcus aureus* made catalase (+ve), methyl red (MR) (+ve), Voges-Proskauer (VP) (+ve), Simon's Citrate Utilization (+ve) and indole (-ve). Catalase (+ve), methyl red (+ve), indole (+ve), Triple sugar iron (+ve), Voges-Proskauer (VP) (-ve) and Simon's Citrate Utilization (-ve) were all made by *Escherichia coli*. *Klebsiella spp.* produced catalase (+ve), Voges-Proskauer (VP) (+ve), Simon's Citrate Utilization (+ve), indole (-ve) and Methyl Red (MR) (-ve), Triple sugar iron (+ve). These results showed similarity with previous studies (Gani *et al.*, 2008; Cheesbrough, 2005).

In gram's staining, the obtained results revealed that gram-positive violet color, cocci-shaped arranged in clusters, indicated *Staphylococcus aureus*. Gram negative, pink-colored, small rod-shaped, arranged in single rods indicated *Klebsiella spp.* It also revealed that the gram's negative pink color and small, rod-shaped organisms arranged in single, pair and short chains indicated *E. coli* which is agreed by others (Gani *et al.*, 2008; Cheesbrough, 2005).

In recent years, there has been a surge in antibiotic resistance (ABR) throughout the world (Luepke and Mohr, 2017; Spellberg *et al.*, 2004). ABR poses a significant risk in terms of mortality and economic burden worldwide. However, the developing countries are more affected because of the widespread misuse of antibiotics, non-human antibiotic use, poor quality of drugs, inadequate surveillance, and factors associated with individual and national poverty (poor healthcare standards, malnutrition, chronic and repeated infections, unaffordability of more effective and costly drugs) (Ayukekbong *et al.*, 2017; Sosa *et al.*, 2010). Bangladesh, a developing country of Southeast Asia with a high degree of ABR, poses a regional and global threat. To ascertain the sensitivity and resistance pattern against frequently used antibiotic discs, the isolated *Staphylococcus*

aureus, *Escherichia coli*, and *Klebsiella spp.* were treated with antibiotics. The antibiogram profile revealed that *E. coli* was sensitive to Ceftriaxone, Gentamicin and resistant to Ciprofloxacin, Penicillin, Tetracycline, Amoxicillin, Erythromycin. *Staphylococcus aureus* was sensitive to Ciprofloxacin, Ceftriaxone, Levofloxacin intermediately resistant to Vancomycin and Amoxicillin and resistant to Ampicillin, Erythromycin. *Klebsiella spp.* was sensitive to Ciprofloxacin, Tetracycline intermediately resistant to Erythromycin, Gentamicin and resistant to Ceftriaxone, Penicillin, Amoxicillin. These results are similar with previous studies (Gani *et al.*, 2008; Rahman *et al.*, 2002b). In a human study performed in Chittagong in 2003, typhoid patients were found to be unresponsive to second-line therapy (ciprofloxacin). First-line therapy was not even attempted because of existing resistance (Asna *et al.*, 2003). Therapeutic failures like this are not rare at all. Furthermore, in relation to this, multiple studies have demonstrated irrational antibiotic prescribing by physicians, a habit of self-medication among patients, and the indiscriminate use of antibiotics in agriculture and farming in different parts of the country (Biswas *et al.*, 2014a,b; Sutradhar *et al.*, 2014). The susceptibility pattern of *E. coli* showed resistance to sulphamethaxazole (40%), polymixin (100%), tetracycline (100%), oxacillin (40%), gentamycin (40%) and cefoxitin (100%) (Moges *et al.*, 2013). The isolates of *A. pyogenes* showed resistance to polymixin (66.66%), tetracycline (66.66%), oxacillin (16.66%), gentamycin (50%) and cefoxitin (16.66%). *Klebsiella spp.* showed resistance to only cefoxitin (100%) and susceptible for all the tested antimicrobials (Moges *et al.*, 2013). The isolates of *Streptococcus spp.* and *C. fetus* were susceptible to all the tested antimicrobial (Moges *et al.*, 2013).

Molecular detection of *E. coli* was confirmed by using Eco 223 (F) primer and primer sequence (5'-3') ATC AAC CGA GAT TCC CCC AGT, Eco 455 (R) primer and primer sequence (5'-3') TCA CTA TCG GTC AGT CAG GAG. The target genes were 16S and 23S rRNA and the size of the product amplified at 232 bp. *Staphylococcus aureus* was confirmed by using Sau 234 (F) primer and primer sequence (5'-3') CGA TTC CCT TAG TAG CGG CG and Sau 1501 (R) primer and primer sequence (5'-3') CCA ATC GCA CGC TTC GCC TA. The target genes were 16S and 23S rRNA and the size of the product amplified at 1267 bp. Other studies had reported similar results (Liu *et al.*, 2002; Riffon, 2001).

Molecular approaches have been shown to be highly effective tools in the advancement of novel diagnostic tests. PCR-based approaches utilizing the 16S or 23S rDNA region sequences have been effectively employed in the identification of numerous bacterial species, particularly in cases where labor-intensive techniques like ribotyping prove to be time-consuming (Bes *et al.*, 2000; Bentley and Leigh, 1995). One of the primary benefits of polymerase chain reaction (PCR) is in its capacity to utilize minute quantities of nucleic acid materials, hence obviating the need for culture, while also offering advantages in terms of speed and ease of analysis. Hence, we opted for polymerase chain reaction (PCR) amplification of DNA regions that encode ribosomal RNA (rRNA) due to the existence of hypervariable areas, which aid in the creation of oligonucleotide probes with high specificity (Giovannoni *et al.*, 1988), as well as common regions that enable the design of universal probes (Gray *et al.*, 1984). Furthermore, the presence of several copies of rDNA allows for signal augmentation (Whitehead and Cotta, 2000).

CHAPTER VI

CONCLUSION

The prevalence of repeat breeding syndrome was (41.33%). *E. coli*, *Styphylococcus aureus* and *Klebsiella spp.* are the most common bacteria isolated from repeat breeder cows. PCR bands of *Escherichia coli* and *Staphylococcus aureus* was shown at 232 bp and 1267 bp, respectively. *E. coli* was sensitive to Ceftriaxone, Gentamicin and resistant to Ciprofloxacin, Penicillin, Tetracycline, Amoxicillin, Erythromycin. *Staphylococcus aureus* was sensitive to Ciprofloxacin, Ceftriaxone, Levofloxacin intermediately resistant to Vancomycin and Amoxicillin, however, resistant to Ampicillin, Erythromycin. *Klebsiella spp.* was sensitive to Ciprofloxacin, Tetracycline; intermediately resistant to Erythromycin, Gentamicin and resistant to Ceftriaxone, Penicillin, Amoxicillin. Treatment for repeat breeding syndrome should be done after performing antibiotic sensitivity test. The results should be useful for farmers and veterinary practitioners to assess bacterial load in genital tract causing repeat breeding syndrome and uterine infections.

REFERENCES

- AA Maruf, MR Islam, MM Rahman, MMU* Bhuiyan and M Shamsuddin. (2012). Prevalence of Reproductive Disorders of Dairy Cows in Chittagong District of Bangladesh. *Bangladesh Vet J* (2012) 46(1-4):11-18.
- Acevedo, N. (2001). *Effects of scrotal insulation on spermatozoal morphology and chromatin stability to acid denaturation in the bovine* (Doctoral dissertation, Virginia Tech).
- Adane H, Yisehak T, Niguse T (2014). Assessment of major reproductive disorders of dairy cattle in Urban and Per-Urban area of Hosanna, Southern Ethiopia. *J. Anim. Vet. Sci.* 2 (5): 135-14.
- Ahmed FO, Elsheikh AS. 2014. Treatment of repeat breeding in dairy cows with Lugol's Iodine. *IOSR Journal of Agriculture and Veterinary Science.* 7 (4): 22-26.
- Ahmed WM, El-khadrawy HH, Hanafi EM, Ali AH, Shalaby SA. 2010. Clinical perspective of repeat breeding syndrome in buffaloes. *Journal of Animal Sciences.* 6(11): 661- 666.
- Ahuja AK, Cheema RS, Narang D, Dhindsa SS. 2017. Bacterial pathogens and antibiotic susceptibility patterns of cervico-vaginal discharges in cross bred repeat breeding heifer cows. *International Journal of Current Microbiology and Applied Sciences.* 6: 1769-1775.
- Albihn, A. (1991). Standing oestrus, ovarian function and early pregnancy in virgin and repeat breeder heifers. *Journal of Veterinary Medicine Series A*, 38(1-10), 212-221.
- Alemselam, B.M., Christopher, R. H., Goitom, G., Desalew, T., Gidena, D., Tadesse, G. and Simon, C. R. (2015): Assessment of Reproductive Performance and Problems in Crossbred (Holstein Friesian X Zebu) Dairy Cattle in and Around Mekelle, Tigray, Ethiopia. *Animal and Veterinary Sciences*, 3(3): 94-101.
- Ali A, Hassanein KM, Al-Sobayil FA, Tharwat M, Al-Hawas A, Ahmed AF. 2010. Relationship between characters of vaginal discharges and uterine bacterial isolates recovered from repeat breeding female camels (*Camelus dromedarius*). *Journal of Agriculture and Veterinary Science.* 2: 87-97.

- Ali, M. Y., Ershaduzzaman, M., Khatun, R., Alam, U. S., Akter, S., Roy, R. R., ... & Sarker, N. R. (2020). Ovarian physiology of repeat breeder cows at Bathan area of Bangladesh. *Bangladesh Journal of Livestock Research*, 95-104.
- Amiridis, G. S., Tsiligianni, T. H., Dovolou, E., Rekkas, C., Vouzaras, D., & Menegatos, I. (2009). Combined administration of gonadotropin-releasing hormone, progesterone, and meloxicam is an effective treatment for the repeat-breeder cow. *Theriogenology*, 72(4), 542-548.
- Anjum IA, Usmani RH, Tunio MT, Abro SH. 2010. Improvement of conception rate in crossbred cattle by using GnRH analogue therapy. *Pakistan Veterinary Journal*. 29(2):6-10.
- Arora, A. K., Jagir, S., Pangaonkar, G. R., & Nanda, A. S. (2000). Bacteriological studies on the genital infection in repeat breeder bovines. *International Journal of Animal Sciences*, 15(2), 205-207.
- Asaduzzaman K. M., Mohammad Musharraf Uddin Bhuiyan, Rahman M. M., Jayonta Bhattacharjee (2017). Prevalence of Repeat Breeding and its Effective Treatment in Cows at Selected Areas of Bangladesh. *Bangladesh Journal of Veterinary Medicine* 14(2):183. DOI: 10.3329/bjvm.v14i2.31391
- Asaduzzaman KM, Bhuiyan MM, Rahman MM, Bhattacharjee J. 2016. Prevalence of repeat breeding and its effective treatment in cows at selected areas of Bangladesh. *Bangladesh Journal of Veterinary Medicine*. 14 (2):183-190.
- Asna, S. M. Z. H., Haq, J. A., & Rahman, M. M. (2003). Nalidixic acid-resistant *Salmonella enterica* serovar Typhi with decreased susceptibility to ciprofloxacin caused treatment failure: a report from Bangladesh. *Japanese journal of infectious diseases*, 56(1), 32-33.
- Ayalon, N. (1978). A review of embryonic mortality in cattle. *Reproduction*, 54(2), 483-493.
- Ayukekbong, J. A., Ntemgwa, M., & Atabe, A. N. (2017). The threat of antimicrobial resistance in developing countries: causes and control strategies. *Antimicrobial Resistance & Infection Control*, 6(1), 1-8.

- Azizunnesa, Joy SD, Faruk MO. 2011. Isolation and Identification of Uterine Bacteria in Postpartum Dairy Cows. *Bangladesh Journal of Microbiology*. 28(1): 19-23.
- Båge, R., Gustafsson, H., Larsson, B., Forsberg, M., & Rodriguez-Martinez, H. (2002). Repeat breeding in dairy heifers: follicular dynamics and estrous cycle characteristics in relation to sexual hormone patterns. *Theriogenology*, 57(9), 2257-2269.
- Bailie JH. 1982. Management and economic effects of different levels of oestrus detection in the dairy herd. *The Veterinary Record*. 110(10): 218-21.
- Barman P, Yadav MC, Bangthai A, Kumar H. 2013. Antibigram of bacteria isolated from bovine endometritis. *Veterinary Research International*. 1(1): 20-24.
- Bartlett, P. C., Kirk, J. H., & Mather, E. C. (1986). Repeated insemination in Michigan Holstein-Friesian cattle: Incidence, descriptive epidemiology and estimated economic impact. *Theriogenology*, 26(3), 309-322.
- Bauer, A. W., Kirby, W. M. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American journal of clinical pathology*, 45(4-ts), 493-496.
- Bentley, R. W., & Leigh, J. A. (1995). Determination of 16S ribosomal RNA gene copy number in *Streptococcus uberis*, *S. agalactiae*, *S. dysgalactiae* and *S. parauberis*. *FEMS Immunology & Medical Microbiology*, 12(1), 1-7.
- Bes, M., Guerin-Faublee, V., Meugnier, H., Etienne, J., & Freney, J. (2000). Improvement of the identification of staphylococci isolated from bovine mammary infections using molecular methods. *Veterinary Microbiology*, 71(3-4), 287-294.
- Bhat FA, Bhattacharyya HK, Khan MZ. 2012. Studies on prevalence of repeat breeding in crossbred cattle of Kashmir valley. *Indian Journal of Animal Research*. 46(3): 306- 309.
- Bicalho ML, Machado VS, Oikonomou G, Gilbert RO, Bicalho RC. 2012. Association between virulence factors of *Escherichia coli*, *Fusobacterium necrophorum*, and *Arcanobacterium pyogenes* and uterine diseases of dairy cows. *Veterinary microbiology*. 157(1-2): 125-131.

- Bisinotto, R.S.; Greco, L.F.; Ribeiro, E.S.; Martinez, N.; Lima, F.S.; Staples, C.R.; Thatcher, W.W.; Santos, J.E.P. Influences of nutrition and metabolism on fertility of dairy cows. *Anim. Reprod.* 2012, 9, 260–272.
- Biswas, M., Roy, D. N., Tajmim, A., Rajib, S. S., Hossain, M., Farzana, F., & Yasmen, N. (2014). Prescription antibiotics for outpatients in Bangladesh: a cross-sectional health survey conducted in three cities. *Annals of clinical microbiology and antimicrobials*, 13, 1-7.
- Biswas, M., Roy, M. N., Manik, M. I. N., Hossain, M. S., Tapu, S. T. A., Moniruzzaman, M., & Sultana, S. (2014). Self-medicated antibiotics in Bangladesh: a cross-sectional health survey conducted in the Rajshahi City. *BMC public health*, 14, 1-7.
- Bitew M, Prasad S (2011). Study on major reproductive health problems in indigenous and cross breed cows in and around Bedelle, South West Ethiopia. *J. Anim. Vet. Adv.* 10: 723-727.
- Boettcher, P. J., & Perera, B. M. A. O. (2007). Improving the reproductive management of smallholder dairy cattle and the effectiveness of artificial insemination: a summary. *Reproductive Management of Dairy Cattle Subjected to Artificial Insemination*, 1.
- Butler, W.R. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. *Livest. Prod. Sci.* 2003, 83, 211–218.
- Butt BM, Besser TE, Senger PL, Widders PR. 1993. Specific antibody to *Haemophilus somnus* in the bovine uterus following intramuscular immunization. *Infection and immunity*. 61(6): 2558-2562.
- Carter, G. R. (1967). Diagnostic procedures in veterinary bacteriology and mycology. *Diagnostic procedures in veterinary bacteriology and mycology*.
- Ceylan A, Serin I, Aksit H, Seyrek KA. 2008. Concentrations of some elements in dairy cows with reproductive disorders. *Bulletin of the Veterinary Institute in Pulawy*. 52: 109- 112.

- Chandrakar D, Tiwari RP, Awasthi MK, Hirpurkar SD. 2002. Microbial profile and their antibiogram pattern in cervico-uterine content and conception rate following treatment in repeat breeder crossbred cows. *Indian Journal of Animal Reproduction*. 23(2): 148-150.
- Cheesbrough, M. (2005). *District laboratory practice in tropical countries, part 2*. Cambridge university press.
- Cummins, S.B.; Lonergan, P.; Evans, A.C.; Butler, S.T. Genetic merit for fertility traits in Holstein cows: II. Ovarian follicular and corpus luteum dynamics, reproductive hormones, and estrus behavior. *J. Dairy Sci.* 2012, 95, 3698–3710.
- Das JM, Dutta P, Deka KC, Biswas RK, Sarmah BC, Dhali A. 2009. Comparative study on serum macro and micro mineral profiles during oestrus in repeat breeding crossbred cattle with impaired and normal ovulation. *Livestock Research for Rural Development*. 21(5): 1-8.
- Day, M. L., & Anderson, L. H. (1998). Current concepts on the control of puberty in cattle. *Journal of Animal Science*, 76(suppl_3), 1-15.
- De Kruif A. 1978. Factors influencing the fertility of a cattle population. *Reproduction*. 54(2): 507-518.
- Deka KC, Chakrabarty AK, Boro BR, Nath KC. 1979. Studies on the microflora in the cervico-vaginal mucus of the repeat breeder cows. *Vetco India*. 19: 40-46.
- Deka, R.P.; Magnusson, U.; Grace, D.; Randolph, T.F.; Shome, R.; Lindahl, J.F. Estimates of the economic cost caused by five major reproductive problems in dairy animals in Assam and Bihar, India. *Animals* 2021, 11, 3116.
- Dhaliwal GS, Murray RD, Woldehiwet Z. 2001. Some aspects of immunology of the bovine uterus related to treatments for endometritis. *Animal Reproduction Science*. 67(3-4): 135-152.
- Dhara, S.; Mahajan, M.; Arya, D.; Kumar, S. Surgical management of pneumovagina in a cross bred cow: A case report. *Indian J. Anim. Health* 2022, 61, 368–370.

- Dholakia PM, Shah NM, Purohit JH, Kher HN. 1987. Bacteriological study of non-specific genital infection and its antibiotic spectra in repeat breeders. *Indian Veterinary Journal*. 64(8): 637-640.
- Dovensky T, Popovsky K, Mickousky G, Kocosky LJ, Trojancanec P, Petkov V, Popovska F. 1996. Serum concentration of several biochemical parameters in cows with reproductive disorders. In 13th International Congress on Animal Reproduction. Australia.
- Eduvie, L. O. (1985). Factors affecting postpartum ovarian activity and uterine involution in Zebu cattle indigenous to Nigeria. *Animal Reproduction Science*, 8(1-2), 123-128.
- Eid, L. N., & Parrish, J. J. (1995). Duration of G 2-phase and onset of M-phase during the first cell cycle of the bovine embryo is dependent on bull in vivo fertility. *Theriogenology*, 1(43), 205.
- Eid, L. N., Lorton, S. P., & Parrish, J. J. (1994). Paternal influence on S-phase in the first cell cycle of the bovine embryo. *Biology of reproduction*, 51(6), 1232-1237.
- El-Azab MA, Whitmore HL, Kakoma I, Brodie BO, McKenna DJ, Gustafsson BK. 1988. Evaluation of the uterine environment in experimental and spontaneous bovine metritis. *Theriogenology*. 29(6): 1327-34.
- El-Khadrawy HH, Ahmed WM, Hanafi M. 2011. Observations on repeat breeding in farm animals with emphasis on its control. *Journal of reproduction and infertility*. 2(1): 1-7.
- Erb HN, Smith RD, Oltenacu PA, Guard CL, Hillman RB, Powers PA, Smith MC, White ME. 1985. Path Model of Reproductive Disorders and Performance, Milk Fever, Mastitis, Milk Yield, and Culling in Holstein Cows. *Journal of dairy science*. 68(12): 3337-3349.
- Eshete, T.; Demisse, T.; Yilma, T.; Tamir, B. Repeat breeding and its' associated risk factors in crossbred dairy cattle in Northern Central Highlands of Ethiopia. *Vet. Med. Int.* 2023, 2023, 1176924.
- Ferreira, R. M., Chiaratti, M. R., Macabelli, C. H., Rodrigues, C. A., Ferraz, M. L., Watanabe, Y. F., ... & Baruselli, P. S. (2016). The infertility of repeat-breeder

cows during summer is associated with decreased mitochondrial DNA and increased expression of mitochondrial and apoptotic genes in oocytes. *Biology of Reproduction*, 94(3), 66-1.

Ferreira, T. C., Krahmer, E., & Wubben, S. (2016, August). Towards more variation in text generation: Developing and evaluating variation models for choice of referential form. In *Proceedings of the 54th Annual Meeting of the Association for Computational Linguistics (Volume 1: Long Papers)* (pp. 568-577).

Foldi J, Kulcsar M, Pesci A, Huyghe B, DeSa C, Lohuis J, Cox P, Huszenicza G. (2006). Bacterial complication of postpartum uterus in cattle. *Animal Reproduction Science*. 96: 265-281.

Foote RH. 1979. Time of artificial insemination and fertility in dairy cattle. *Journal of Dairy Science*. 62: 355-358.

Foote, R. H., & Parks, J. E. (1993). Factors affecting preservation and fertility of bull sperm: a brief review. *Reproduction, fertility and development*, 5(6), 665-673.

Francos C. 1974. Observation on the frequency of reproductive disorders in dairy herds in Israel. *Deutsche Tierarztlliche Wochenschr.* 81: 135-138.

Francos G. 1979. The relationship between the incidence of endometritis and repeat breeders in dairy herds. *Refuah Veterinarith*. 36(4): 131-134.

Fredriksson G, Kindahl H, Sandstedt K, Edqvist LE. 1985. Intrauterine bacterial findings and release of PGF₂ α in the postpartum dairy cow. *Journal Veterinary Medical Association*. 32(1-10): 368-380.

Gani, M. O., Amin, M. M., Alam, M. G. S., Kayesh, M. E. H., Karim, M. R., Samad, M. A., & Islam, M. R. (2008). Bacterial flora associated with repeat breeding and uterine infections in dairy cows. *Bangladesh Journal of Veterinary Medicine*, 6(1), 79-86.

Garrido, M.R.; Peña, A.I.; Herradon, P.G.; Becerra, J.J.; Sande, J.; Quintela, L.A. Evaluation of tubal patency in repeat breeder Holstein cows. *Span. J. Agric. Res.* 2020, 17, e04SC02.

Gerasimova, A. A. (1938). Duration of Heat and Time of Ovulation in the Cow. *Probl. Zivotn.* 12: 126-129. *An. Breed. Abs*, 8: 32.

- Giovannoni, S. J., DeLong, E. F., Olsen, G. J., & Pace, N. R. (1988). Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. *Journal of bacteriology*, 170(2), 720-726.
- Gizaw Y., Bekana M., Abayneh T. Major reproductive health problems in small holder dairy production in and around Nazareth town, Central Ethiopia. *Journal of Veterinary Medicine and Research* . 2007;5(4)
- González Moreno, C.; Torres Luque, A.; Galvao, K.N.; Otero, M.C. Bacterial communities from vagina of dairy healthy heifers and cows with impaired reproductive performance. *Res. Vet. Sci.* 2022, 142, 15–23.
- Gray, M. W., D. Sankoff and R. J. Cedergren. 1984. On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in a small subunit ribosomal RNA. *Nucleic Acids Res.* 12:5837–5852.
- Guo M, Wang G, Shao G, Tian W, Xu Y, Xie G. 2012. Identification of intrauterine mycoplasma in endometritis of dairy cow isolates by 16S rDNA nested PCR-DGGE. *Journal of Animal and Veterinary Advances.* 11(10): 1745-1752.
- Gupta, A. G., & Deopurkar, R. L. (2005). Microbial study of gynaecological infection in cattle. *IJAR*, 14, 118-119.
- Gustafsson H, Emanuelson U. 2002. Characterisation of the repeat breeding syndrome in Swedish dairy cattle. *Acta Veterinaria Scandinavica.* 43(2): 115.
- Gustafsson, H. (1985). Characteristics of embryos from repeat breeder and virgin heifers. *Theriogenology*, 23(3), 487-498.
- Gustafsson, H., & Larsson, K. (1985). Embryonic mortality in heifers after artificial insemination and embryo transfer: differences between virgin and repeat breeder heifers. *Research in veterinary science*, 39(3), 271-274.
- Gustafsson, H., Larsson, K., Kindahl, H., & Madej, A. (1986). Sequential endocrine changes and behaviour during oestrus and metoestrus in repeat breeder and virgin heifers. *Animal Reproduction Science*, 10(4), 261-273.

- Haile A., Tsegaye Y., Tesfaye N. Assessment of major reproductive disorders of dairy cattle in urban and per urban area of hosanna, southern Ethiopia. *Animal and Veterinary Sciences* . 2014;2(5):135–141.
- Hall, J. G., Branton, C. and Stone, E. J. (1959). Estrus, estrous cycles, ovulation time, time of service, and fertility of dairy cattle in Louisiana. *Journal of Dairy Science*, 42(6): 1086-1094.
- Hartmann, D.; Rohkohl, J.; Merbach, S.; Heilkenbrinker, T.; Klindworth, H.P.; Schoon, H.A.; Hoedemaker, M. Prevalence of cervicitis in dairy cows and its effect on reproduction. *Theriogenology* 2016, 85, 247–253.
- Hasib, F.M.Y.; Reza, M.M.B.; Alam, M.M.; Hasan, T.; Azizunnesa. Occurrence and risk factors of repeat breeding on household dairy cows of Hathazari in Chattogram. *Bangladesh J. Vet. Anim. Sci.* 2020, 8, 102–111.
- Hewett, C. D. (1968). A survey of the incidence of the repeat breeder cow in Sweden with reference to herd size, season, age, and milk yield. *British Veterinary Journal*, 124(8), 342-352.
- Hodel, F., Moll, J., & Kuenzi, N. (1995). Analysis of fertility in Swiss Simmental cattle—Genetic and environmental effects on female fertility. *Livestock Production Science*, 41(2), 95-103.
- Hossain, M. M. (2002). *Improvement of conception in repeat breeding cows using synthetic gonadotrophin relasing hormone (GnRH)* (Doctoral dissertation, MS Thesis. Department of surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh).
- Hussain AM. 1990. Defense mechanisms of the bovine uterus. *Selezione-Veterinaria*, 31: 1046-1048.
- Imakawa, K., Day, M. L., Zalesky, D. D., Garcia-Winder, M., Kitfok, R. J., & Kinder, J. E. (1986). Influence of dietary-induced weight changes on serum luteinizing hormone, estrogen and progesterone in the bovine female. *Biology of reproduction*, 35(2), 377-384.

- Ingawale MV, Dhoble RL, Sawle AG, Gacche MG, Rangnekar MN, Jadhav JM. (2001). Effect of fertagyl (GnRH) administration at the time of insemination on conception rate in prostaglandin induced estrus in cows. *Blue cross book*. 20: 8-11
- Ingawale, M. V., Dhoble, R. L., & Sawale, A. G. (2003). Isolation, identification and antibiotic sensitivity pattern of the uterine secretions in cows with metritis. *The Indian Journal of Animal Reproduction*, 24(1), 52-53.
- Islam MN, Habib MR, Khandakar MMH, Rashid MH, Sarker MAH, Bari MS, Islam MZ, Alam MK, Sarkar MM, Jahan R, Mahzabin R, Islam MA. Repeat breeding: prevalence and potential causes in dairy cows at different milk pocket areas of Bangladesh. *Trop Anim Health Prod*. 2023 Mar 17;55(2):120.
- Islam, R. (2011). Synchronization of estrus in cattle: a review. *Veterinary World*, 4(3). Itoh, K.; Endo, N.; Kataoka, S.I.; Tanaka, T. Assessment of tubal patency by hysterosalpingo–contrast sonography in cow. *J. Anim. Sci*. 2016, 94, 56–57.
- Jainudeen, M. R., & Hafez, E. S. E. (2000). Reproductive failure in females. *Reproduction in farm animals*, 259-278.
- Jayakumar C, Vahida AM. 2000. Effects of administration of a gonadotrophin releasing hormone analogue at mid-cycle post insemination. *Indian Veterinary Journal*. 77(5): 454-455.
- Jeffrey S, Stevenson and Edward P (1990). Double insemination and gonadotropin-releasing hormone treatment of repeat-breeding dairy cattle. *Journal of Dairy Science* 73: 1766-1772.
- Jeong, J.-K.; Kim, I.-H. Risk factors for repeat breeder dairy cows and their impacts on reproductive performance. *Korean J. Vet. Res*. 2022, 62, e15.
- Jit Yadav, C. (2020). Identification and characterization of uterine bacteria and selection of treatment strategy in repeat breeding cows to increase conception rate in dairy farms in Chattogram (Doctoral dissertation, Chattogram Veterinary and Animal Sciences University Chattogram-4225, Bangladesh).

- Jordan ER. 2003. Effects of heat stress on reproduction. *Journal of Dairy Science*. 86(1): 104-114
- Kafi, M.; Azari, M.; Chashnigir, O.; Gharibzadeh, S.; Aghabozorgi, Z.; Asaadi, A.; Divar, M.R. Inherent inferior quality of follicular fluid in repeat breeder heifers as evidenced by low rates of in vitro production of bovine embryos. *Theriogenology* 2017, 102, 29–34.
- Kaikini AS, Chikailikar GK, Dinodorkar CB. 1981. Reproductive disorders in HF and Gir F1 crossbred cows. *Indian Journal of Animal Reproduction*. 1: 43-46.
- Kamal MM, Parveen N, Saha S, Amin MM. 2001. Bacteriological study on uterine discharge in repeat breeder cows. *Bangladesh Journal of Veterinary Medicine*. 35: 49-52.
- Karwani A, Sharma JK. 2003. Incidence and epidemiology of abortions and repeat breeding in bovines at dairy farms in Punjab, India. In *The 11th international symposium of the world association of veterinary laboratory diagnosticians and oie seminar on biotechnology* pp. 9-13.
- Kasimanickam R, Duffield TF, Foster RA, Gartley CJ, Leslie KE, Walton JS, Johnson WH. 2004. Endometrial cytology and ultrasonography for the detection of subclinical endometritis in postpartum dairy cows. *Theriogenology*. 62(1-2): 9-23.
- Kauffold, J.; Groeger, S.; Bergmann, K.; Wehrend, A. Use of contrast sonography to test for tubal patency in dairy cows. *J. Reprod. Dev.* 2009, 55, 335–338.
- Kessy, B.M.; Noakes, D.E. The use of the starch grain and phenolsulphonphthalein tests to investigate infertile cows. *Vet. Rec.* 1979, 24, 489–491.
- Khair A (2005). Hormonal intervention to increase the fertility in anoestrus and repeat breeding cows at Bangladesh Agricultural University veterinary clinic. MS in *Theriogenology* Thesis. Department of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh agricultural University, Mymensingh, Bangladesh.

- Khair, A., Asaduzzaman, M., Sultana, Z., Talukder, A. K., Das, Z. C., Alam, M. G. S., & Shamsuddin, M. (2018). Economic benefit in repeat breeder cows using intrauterine infusion of penicillin and estrus synchronization followed by timed artificial insemination. *Journal of advanced veterinary and animal research*, 5(4), 454.
- Khan, I., Qureshi, M. S., Akhtar, S. and Ali, I. (2016). Fertility Improvement in Cross-Bred Dairy Cows Through Supplementation of Vitamin E as Antioxidant. *Pakistan Journal of Zoology*, 48(4): 923-930.
- King WA. 1990. Chromosome abnormalities and pregnancy failure in domestic animals. In *Advances in veterinary science and comparative medicine*. Academic Press. 34, pp. 229-250.
- Kumar R, Dharmi AJ, Butani MG, Kavani FS, Sarvaiya NP, Killedar A. 2010. Management of repeat breeding under field conditions using hormonal and non-hormonal drugs in cows. *Indian Journal of Animal Reproduction*. 31(1): 30-32.
- Lafi SQ, Kaneene JB. 1988. Risk factors and associated economic effects of the repeat breeder syndrome in dairy cattle. *Veterinary Bulletin (United Kingdom)*.
- Lafi, S. Q., & Kaneene, J. B. (1992). Epidemiological and economic study of the repeat breeder syndrome in Michigan dairy cattle. I. Epidemiological modeling. *Preventive Veterinary Medicine*, 14(1-2), 87-98.
- Lafi, S.Q.; Kaneene, J.B.; Black, J.R.; Lloyd, J.W. Epidemiological and economic study of the repeat breeder syndrome in Michigan dairy cattle. II. Economic modeling. *Prev. Vet. Med.* 1992, 14, 99–114.
- Levine, H.D. The repeat breeder cow. *Bov. pract.* 1999, 33, 97–105.
- Lewis, M., Gough, C., Martínez, R., Powell, M., Marks, J., Woolard, G. C., & Ribisch, K. H. (1997). *Implementing the lexical approach: Putting theory into practice* (Vol. 3, No. 1, pp. 223-232). Hove: Language Teaching Publications.
- Liang, D.; Arnold, L.M.; Stowe, C.J.; Harmon, R.J.; Bewley, J.M. Estimating US dairy clinical disease costs with a stochastic simulation model. *J. Dairy Sci.* 2017, 100, 1472–1486.

- Linares, T., & King, W. A. (1980). Morphological study of the bovine blastocyst with phase contrast microscopy. *Theriogenology*, 14(2), 123-133.
- Linares, T., King, W. A., Larsson, K., Gustavsson, I., & Bane, A. (1980). Successful, repeated non-surgical collection of blastocysts from virgin and repeat breeder heifers. *Veterinary Research Communications*, 4, 113-118.
- Liu MC, Wu CM, Liu YC, Zhao JC, Yang YL, Shen JZ. 2009. Identification, susceptibility, and detection of integron-gene cassettes of *Arcanobacterium pyogenes* in bovine endometritis. *Journal of dairy science*. 92(8): 3659-3666.
- Liu, J., Enzelberger, M., & Quake, S. (2002). A nanoliter rotary device for polymerase chain reaction. *Electrophoresis*, 23(10), 1531-1536.
- Livestock Economy at a glance 2022-2023. http://dls.portal.gov.bd/sites/default/files/files/dls.portal.gov.bd/page/ee5f4621_fa3a_40ac_8bd9_898fb8ee4700/2023-07-23-12-04-afbccb96f8b27d4bab6501aa8c2c2ff.pdf
- Lobago F., Bekana M., Gustafsson H., Kindahl H. Reproductive performances of dairy cows in smallholder production system in Selalle, Central Ethiopia. *Tropical Animal Health and Production* . 2006;4:333–342.
- Luepke, K. H., & Mohr III, J. F. (2017). The antibiotic pipeline: reviving research and development and speeding drugs to market. *Expert review of anti-infective therapy*, 15(5), 425-433.
- Mane PM, Dhoble RL, Chaudhari RJ, Dawane SC, Kolpe AB. 2009. Bacterial spectrum, antibiotic sensitivity pattern of bacterial isolates and conception rate in repeat breeders. *Intas Polivet*. 10 (1): 32-35.
- Mane PM. 2010. Clinical efficacy of different intrauterine preparations in repeat breeder bovines. *Research Journal of Animal Husbandry and Dairy Science*. 1 (2): 77-79.
- Mann, G.E.; Lamming, G.E.; Fray, M.D. Plasma oestradiol and progesterone during early pregnancy in the cow and the effects of treatment with buserelin. *Anim. Reprod. Sci.* 1995, 37, 121–131.

- Maruf, A. A., Islam, M. R., Rahman, M. M., Bhuiyan, M. M. U., & Shamsuddin, M. (2012). Prevalence of Reproductive Disorders of Dairy Cows in Chittagong District of Bangladesh. *Bangladesh Vet J*, 46(1-4), 11-18.
- Maurer RR, Echternkamp SE. 1985. Repeat-breeder females in beef cattle: Influences and causes. *Journal of Animal Science*. 61(3): 624-636.
- Merchant, I. A., & Packer, R. A. (1967). *Veterinary bacteriology and virology*.
Veterinary bacteriology and virology.
- Mesafint, M., & Guesh, N. (2014). Repeat breeder syndrome in dairy cows: influence of breed and age on its prevalence. *World Journal of Agricultural Sciences*, 10(4), 200-203.
- Mesafint, M., & Guesh, N. (2014). Repeat breeder syndrome in dairy cows: influence of breed and age on its prevalence. *World Journal of Agricultural Sciences*, 10(4), 200-203.
- Moges, N., Regassa, F., Yilma, T., & Unakal, C. G. (2013). Isolation and antimicrobial susceptibility of bacteria from dairy cows with clinical endometritis. *Journal of Reproduction and Infertility*, 4(1), 04-08.
- More RM, Patil AD, Kumbhar UB, Mugale RR. 2012. Fertility improvement by hormonal therapies in repeat breeding cows. *Indian Journal of Animal Reproduction*. 33(2): 61- 63.
- Moss, N.; Lean, I.J.; Reid, S.W.J.; Hodgson, D.R. Risk factors for repeat-breeder syndrome in New South Wales dairy cows. *Prev. Vet. Med.* 2002, 54, 91–103.
- Murthy GVK, Nanjaiah RD, Keshava Murthy BS. 1974. Bacterial flora of cervical mucus in repeat breeding bovines. *Indian Veterinary Journal*. 51: 264-268
- O'Farrell KJ. 1975. Role of management in dairy herd fertility. *Irish veterinary journal*. 29: 118-124.
- Panangala VS, Fish NA, Barnum DA. 1978. Microflora of the cervico-vaginal mucus of repeat breeder cows. *The Canadian Veterinary Journal*. 19(4): 83.

- Patel JA, Dharni AJ, Kavani FS, Panchal MT, Ghodasara DJ. 2010. Therapeutic management and changes in the endometrium of repeat breeding cows. *Indian Journal of Animal Reproduction*. 31(1): 1-6.
- Perez-Marin CC, Espana F. 2007. Oestrus expression and ovarian function in repeat breeder cows, monitored by ultrasonography and progesterone assay. *Reproduction in Domestic Animals*. 42: 449-456.
- Pérez-Marín, C. C., & Quintela, L. A. (2023). Current Insights in the Repeat Breeder Cow Syndrome. *Animals*, 13(13), 2187.
- Perez-Marin, C. C., Moreno, L. M., & Calero, G. V. (2012). Clinical approach to the repeat breeder cow syndrome. *A Bird's-Eye View of Veterinary Medicine*, 337-362.
- Raghavan R, Nilakantan PR, Uppal PK. 1971. Studies on the bacteriology of bovine genital tract. *Indian veterinary journal*. 48 (8): 779-783
- Rahman A, Rahman A, Rahman H, Ahmed MU 1996. Anestrus and repeat breeding problems in indigenous cattle in Bangladesh. *Tropical Animal Health Production*. 7: 605-609.
- Rahman MA, Alam MGS, Shamsuddin M and Rahman MM (2002b). Antibiotic sensitivity of the bacteria isolated from endometrial biopsies of cows. *The Bangladesh Veterinarian* 19 (1): 1-11.
- Rahman, M. F., Islam, M. S., Hossain, M. A., Prodhan, M. A. M. and Rahman, A. (1993). Reproductive patterns of different breeds of cows in Bangladesh. *Bangladesh Journal of Livestock Research*.
- Ramaswamy V, Latha N, Gnanasubramanian T, Jesudass JA, Manickam R.1998. Multiple drug resistant bacteria in repeat breeder bovines. *Indian Journal of Animal Reproduction*. 19(2): 123-125.
- Rangnekar MN, Dhobli R, Gacche MG, Ingawale MV, Sawale AG, Jadhav JM. 2002. Physical properties of estrual cervical mucus in repeat breeding crossbred (Holstein- Friesian) cows with reference to fertility. *The Indian Journal of Animal Sciences*. 72(12).

- Rao AR, Naidu KV. 1987. Effect of Receptal on conception rate of crossbred cattle. Indian Veterinary Journal. 64: 1077
- Rao YS, Naidu KV, Kumar RP, Reddy VT. 2001. Microbial flora and antibiogram pattern of the uterine isolates from crossbred cows with endometritis. Indian Journal of Animal Reproduction. 22: 54-56.
- Riffon, R., Sayasith, K., Khalil, H., Dubreuil, P., Drolet, M., & Lagacé, J. (2001). Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. *Journal of clinical microbiology*, 39(7), 2584-2589.
- Roberts SJ. 1986. Veterinary Obstetrics and Genital diseases, 2nd Ed. S.J. Roberts-Woodstock, New York. pp 381-459.
- Roche, J. F., Bolandl, M. P., & McGeady, T. A. (1981). Reproductive wastage following artificial insemination of heifers. The Veterinary Record, 109(18), 401-404.
- Rodrigues CA, Teixeira AA, Ferreira RM, Ayres H, Mancilha RF, Souza AH, Baruselli PS. 2010. Effect of fixed-time embryo transfer on reproductive efficiency in high-producing repeat-breeder Holstein cows. Animal reproduction science. 118(2-4): 110-117.
- Roine K, Saloniemi H. 1978. Incidence of infertility in dairy cows: disease incidence, infertility, herd size, housing type. Acta Veterinaria Scandinavica.
- Romaniukowa K. 1984. Phagocytosis in the uterus. Proceedings of the 10th International Congress on Animal Reproduction and AI. Urbanachampaign, Illinois, USA. 4: 33- 38.
- Roy GP, Akhtar MH, Singh AP, Prasad KM, Singh RB. 1995. Effect of Receptal to improve the fertility in bovines. Indian Journal of Animal Reproduction. 16: 131.
- Ryan DP, Kopel E, Boland MP, Godke RA. 1991. Pregnancy rates in dairy cows following the administration of a GnRH analogue at the time of artificial insemination or at mid-cycle post insemination. Theriogenology. 36(3): 367-377.
- Sagar Aryal, 2022. Blood Agar- Composition, Preparation, Uses and Pictures (microbiologyinfo.com).

- Sah SK, Nakao T. 2006. Characteristics of repeat breeding buffaloes in Nepal. *Journal of Reproduction and Development*. 52(3): 335-341.
- Salasel, B.; Mokhtari, A.; Taktaz, T. Prevalence, risk factors for and impact of subclinical endometritis in repeat breeder dairy cows. *Theriogenology* 2010, 74, 1271–1278.
- Samad MA. 1996. *Poshu Palon O Chikitsavidya (Animal Husbandry and Medicine)*.
- Samatha V, Srilatha C, Anjaneyalu YSR, Kumar SRV. 2013. Ultrastructural and immune histochemical studies of endometrial biopsies in repeat breeder buffaloes. *International Journal of Food, Agriculture and Veterinary Sciences*. 3(1): 203-206.
- Sangsrivong, S.; Combs, D.K.; Sartori, R.; Armentano, L.E.; Wiltbank, M.C. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17 β in dairy cattle. *J. Dairy Sci.* 2002, 85, 2831–2842.
- Sankar UM, Dhoble RL, Agarwal SK, Srivastav SK, Nautiyal L. 1987. Influence of synthetic gonadotropin releasing hormone (GnRH) on pregnancy rate in post-partum cows. *Indian Journal of Dairy Science*. 42 (2): 210-211.
- Santana AO, Oba E., Lopes J, Uribe-Velasquez LF. 1998. Avalia ao histopatologica do endometrio em femeas bovinas repetidoras de cio. *Archieves of Reproduction in Animal*, 6: 58-61.
- Santos, J.E.P.; Rutigliano, H.M.; Sá Filho, M.F. Risk factors for resumption of postpartum cyclicity and embryonic survival in lactating dairy cows. *Anim. Reprod. Sci.* 2009, 110, 207–221.
- Sarder MJU, Moni, MIZ, Aktar S. 2010. Prevalence of reproductive disorders of crossbreed cows in the Rajshahi district of Bangladesh. *SAARC Journal of Agriculture*. 8(2): 65- 75.
- Saxena MS. 2005. Current strategies towards treatment and control of infertility and other genital disorder. In: *Seminar on recent trends in diagnosis of emerging and re- emerging animal disease*, Pantnagar, Uttarakhand, pp. 71-73.

- Shah NM, Dholakia PM. 1983. Microflora of the cervicovaginal mucus of Surti buffaloes and their drug resistance pattern. *Indian Journal of Animal Sciences*. 53(2): 147-150.
- Sharda R, Moghe MN, Tanwani SK. 1991. Antibiotic sensitivity pattern of bacteria isolated from repeat breeding animals. *Indian Veterinary Journal*. 68(3): 197-200.
- Sharma VK, Yngard RA, Lin Y (2009) Silver nanoparticles: green synthesis and their antimicrobial activities. *Adv Colloid Interface Sci* 145:83–96
- Shelton K, Gayerie D, Abreu MF, Hunter MG, Parkinson TJ, Lamming GE. 1990. Luteal inadequacy during the early luteal phase of subfertile cows. *Journal of Reproduction and Fertility*. 90: 1-10.
- Shiferaw Y., Tenhagen B.-A., Bekana M., Kassa T. Reproductive disorders of crossbred dairy cows in the central highlands of Ethiopia and their effect on reproductive performance. *Tropical Animal Health and Production* . 2005;37(5):427–441.
- Short, R. E, Bellows, R. A., Staigmiller, R. B., Berardinelli, J. G. and Custer, E. E. (1990). Physiological mechanisms controlling anestrus and infertility in postpartum beef cattle. *Journal of Animal Science*, 68(3): 799-816.
- Shrestha HK, Nakao T, Higaki T, Suzuki T, Akita M. 2004. Resumption of postpartum ovarian cyclicity in high-producing Holstein cows. *Theriogenology*. 61(4): 637-49.
- Singh CS, Singh SK, Singh B. 1981. Studies on the incidence of infertility in cows. *Indian Veterinary Journal*. 58: 909-912.
- Singh J, Dadarwal D, Honparkhe M, Kumar A. 2009. Incidences of various etiological factors responsible for repeat breeding syndrome in cattle and buffaloes. *The International Journal of Veterinary Medicine*, 6:1.
- Singh M and Pant HC (1998) Factors responsible for AI failure in the field. *Indian Vet J* 75: 1128-1129.
- Singh NJ, Singh A, Patel AK. 2015. Employing the effect of gentamycin and enrofloxacin treatment on pregnancy rate of repeat breeder dairy cross bred cows. *World Journal of Pharmaceutical Research*. 4(8): 1144-1148.

- Singh NP, Chaturvedi VK and Singh DP (1996). Bacteriological studies on repeat breeder bovines. *Indian Veterinary Journal* 73 (4): 462-463.
- Singh NP, Chaturvedi VK, Singh DP. 1996. Bacteriological studies on repeat breeder bovines. *Indian veterinary journal*.73 (4): 462-463.
- Singh RB, Sharma RD, Singh GB. 1983. Incidence of repeat breeding in cows and buffaloes. *Indian journal of dairy science*.
- Singh, M., Kapoor, S., Sharma, S. and Vasishta, N.K. (2004). Studies on the clinical efficacy of ciprofloxacin administered through intrauterine route in repeat breeder cows suffering from endometritis in Himachal Pradesh. *IntasPoli vet*, 5: 209–210.
- Singh, J, Dadarwal, D., Honparkhe, M. and Kumar, A. (2008). Incidences of various etiological factors responsible for repeat breeding syndrome in cattle and The Inter. J. Vet. Med., 6(1): 1-6.
- Singh, M. and Pant, H.C. (1998a). Blood biochemical profile of normal and repeat breeder cows in Himachal Pradesh. *Indian J. Anim. Reprod*. 19: 156-157.
- Singh, M. and Pant, H.C. (1998b), Factors responsible for AI failure in the field. *Indian Vet J.*, 75: 1128- 1129.
- Singh, M. and Pant, H.C. (1999). Factors associated with repeat breeding in Himachal
- Sonwane NS. 2000. Effect of gonadotropin releasing hormone (GnRH) analogue on improvement of conception rate in crossbred cows. M.V.Sc. Thesis submitted to MAFSU, Nagpur.
- Sood, P.; Zachut, M.; Dube, H.; Moallem, U. Behavioral and hormonal pattern of repeat breeder cows around estrus. *Reproduction* 2015, 149, 545–554.
- Sosa, A. D. J., Byarugaba, D. K., Amábile-Cuevas, C. F., Hsueh, P. R., Kariuki, S., & Okeke, I. N. (Eds.). (2010). Antimicrobial resistance in developing countries (pp. 3-7). New York: Springer.

- Spellberg, B., Powers, J. H., Brass, E. P., Miller, L. G., & Edwards Jr, J. E. (2004). Trends in antimicrobial drug development: implications for the future. *Clinical infectious diseases*, 38(9), 1279-1286.
- Stevenson, J. S. and Britt, J. H. (2017). A 100-year review: Practical female reproductive management. *Journal of dairy science*, 100(12): 10292-10313.
- Stevenson, J. S., Johnson, S. K. and Milliken, G. A. (2003). Incidence of postpartum anestrus in suckled beef cattle: treatments to induce estrus, ovulation, and conception. *The Professional Animal Scientist*, 19(2): 124-134.
- Stone GG, Oberst RD, Hays MP, Mcvey S, Chengappa MM. 1994. Detection of *Salmonella* serovars from clinical samples by enrichment broth cultivation-PCR procedure. *Journal of Clinical Microbiology*. 32(7): 1742-1749.
- Sullivan, J. J., & Elliott, F. I. (1968). Bull fertility as affected by an interaction between motile spermatozoa concentration and fertility level in artificial insemination. *Int Congr Anim Reprod Artif Insemination [Proc]*.
- Sutradhar, K. B., Saha, A., Huda, N. H., & Uddin, R. (2014). Irrational use of antibiotics and antibiotic resistance in southern rural Bangladesh: perspectives from both the physicians and patients. *Annual Research & Review in Biology*, 1421-1430.
- Talukdar, D.J.; Papori, T.; Ahmed, K. Minerals and its impact on fertility of livestock: A review. *Agr. Rev.* 2016, 4, 333–337.
- Tanimura, K.; Uematsu, M.; Kitahara, G.; Osawa, T.; Sasaki, Y. Longitudinal effect of repeat breeding in Japanese Black beef cattle at a low parity on subsequent fertility in commercial cow-calf operations. *Theriogenology* 2022, 189, 177–182.
- Thakur, S., Singh, M. and Vasishta, N.K. (2006a). Study on etiology of repeat breeding.
- Thrusfield MV. 2005. Criteria for success of questionnaire. *Veterinary Epidemiology*. 3: 189- 213.
- Tibary A, Anouassi A. 1997. Breeding soundness examination of the female camelidae In: Tibary, A. ed. *Theriogenology in Camelidae. Anatomy, physiology. BSE, pathology and artificial breeding. Actes Editions. Institute Agronomique et Veterinaire Hassan II.* pp: 243-310.

- Vandeplassche M. 1984. Stimulation and inhibition of phagocytosis in domestic animals. Proceedings of 10th International Congress on Animal Reproduction and AI. Urbana- campaign, Illinois, USA. 3: 475-477.
- Vasconcelos JL, Sartori R, Oliveira HN, Guenther JG, Wiltbank MC. 2001. Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. Theriogenology. 56(2): 307-314.
- Vlasova, A.N.; Saif, L.J. Bovine immunology: Implications for dairy cattle. Front. Immunol. 2021, 12, 643206.
- Walsh, S.W.; Williams, E.J.; Evans, A.C. A review of the causes of poor fertility in high milk producing dairy cows. Anim. Reprod. Sci. 2011, 123, 127–138.
- Warriach HM, Ahmad N, Ahmad G, Khan MS, Rabbani M, Ahmad I. 2009. Effect of antibiotic treatment on pregnancy rate of repeat breeder dairy cross bred cows with sub-clinical uterine infection. Pakistan Veterinary Journal. 29(1): 40-42.
- Watson ED, Diehl NK, Evans JF. 1990. Antibody response in the bovine genital tract to intrauterine infusion of *Actinomyces pyogenes*. Research in veterinary science. 48(1):70-75.
- Westwood, C.T.; Lean, I.J.; Garvin, J.K. Factors influencing fertility of Holstein dairy cows: A multivariate description. J. Dairy Sci. 2002, 85, 3225–3237.
- Whitehead, T. R., & Cotta, M. A. (2000). Development of molecular methods for identification of *Streptococcus bovis* from human and ruminal origins. *FEMS microbiology letters*, 182(2), 237-240.
- Wikipedia , 2023. Mannitol salt agar. https://en.wikipedia.org/wiki/Mannitol_salt_agar
- Williams GL, Amstalden M, Garcia MR, Stanko RL, Nizielski SE, Morrison CD, Keisler DH. 2002. Leptin and its role in the central regulation of reproduction in cattle. Domestic Animal Endocrinology. 23(1-2): 339-349.
- Wolfenson D, Roth Z, Meidan R. 2000. Impaired reproduction in heat-stressed cattle: basic and applied aspects. Animal reproduction science. 60: 535-547.

Yusuf, M., Nakao, T., Ranasinghe, R. B. K., Gautam, G., Long, S. T., Yoshida, C., ... & Hayashi, A. (2010). Reproductive performance of repeat breeders in dairy herds. *Theriogenology*, 73(9), 1220-1229.

Zemjanis R. 1980. Repeat breeding or conception failure in cattle. In: Morrow DA edited. Current Therapy in Theriogenology: diagnosis, treatment and prevention of reproductive diseases in animals. 2nd ed. WB Saunders, Philadelphia. pp. 205-213.

APPENDIX-1
QUESTIONNAIRE

1. Particulars of the owner:

Serial No.:

i. Name:

ii. Village:

iii. Upazila:

2. Particulars of dairy cattle:

Name of the reproductive disorder:

General Information:

i. Breed:

ii. Age:

iii. Parity:

iv. Body condition score:

Diagnosis:

i. History:

ii. Clinical signs:

Treatment:

Date:

Signature of Investigator

Note: If necessary Bengali version of the questionnaire will be used while interviewing beneficiaries.