

IMPACT OF LUTEAL CYST ON LOCAL IMMUNE REGULATION IN UTERUS IN CATTLE

A THESIS

BY

MD. MUSFIQUR RAHMAN

Student ID. 1604115

Session: January–June, 2022-2023

Semester: July- December

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IN

PHARMACOLOGY



DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY

**MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY,
DINAJPUR-5200**

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**DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY
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DECEMBER, 2023

DEDICATED
TO MY
BELOVED PARENTS, TEACHERS AND
WELLWISHERS

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ABSTRACT

The present study was undertaken to investigate the local immune response of the uterus bearing a persistent luteal cyst in the ovary in cow. The under diagnosed immune effect of persistent luteal cyst could be one that directly or indirectly regulates reproductive performance. This study aimed to see the effect of luteal cyst on local immune response of the uterus. First, uteri were collected (n=36) from different slaughter houses and observed grossly ovary to detect the cystic and control ovary. Uterine swab was collected from the endometrial of the uterus by using sterilized swab sticks and mixed with 10 ml PBS solution for bacterial cell culture. Microbiological identification of uterine sample showed *E. coli* and *Staphylococcus aureus* bacteria isolation in uterus having luteal cystic in the ovary, whereas in normal uterus showed no growing bacteria. Further, bacterial antibiotic sensitivity test was perform to detect sensitiveness of that bacteria. Result showed there was no antimicrobial resistance, all were found susceptible. Uterine fluid sample were collected from uterus, mixed with 20 ml sterilized normal saline solution, then staining the fluid with giemsa's stain. The result showed presence of immune cells especially neutrophil and lymphocytes in uterus having luteal cyst in the ovary and control sample had no appearance of immune cells. also histopathological examination of ovary and uterus were performed. Histopathological observation showed inflammatory condition exists in the uterine wall in cystic uterus compare to normal uterus. All together it can be concluded that persistent luteal cyst suppressed the local immunity and favor inflammatory condition in the uterine wall.

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CHAPTER 1

INTRODUCTION

CHAPTER I

INTRODUCTION

Ovarian cyst (OC) has been defined as an anovulatory follicular structure with a diameter that exceeds 20 mm (Silva *et al.*, 2012) or 25 mm, lasting at least 10 days on the ovary in the absence of a functional corpus luteum (Murayama *et al.*, 2015). OC is to be developed as a consequence of either absent or insufficient LH surge due to alteration of the hypothalamic- pituitary axis (Baravalle *et al.*, 2015). Long time presence of anovulatory follicular structure in the cyst results persistent release of ovarian steroid hormone specially estrogens (Robinson *et al.*, 2006) and progesterone. Ovarian hormone regulates the growth and development of uterine wall depending on the status of estrus cycle. In cattle, 17 β -estradiol (E2) induces a 4- fold increase in proliferation of luminal and glandular epithelial cells (Martin *et al.*, 1973) and progesterone (P4) suppresses epithelial cell proliferation and enhance stromal cell proliferation for embryo attachment (Martin *et al.*, 1969 and Tong *et al.*, 1999). Ovarian hormones regulate accompanying changes in the uterine vasculature, with an E2- induced increase in vascular permeability followed by a P4- induced induction of angiogenesis to expand the vascular supply required for implantation success (Ma *et al.*, 2001). Previous study reported that ovarian steroid regulates the transcription factor of uterine physiology (Shimizu *et al.*, 2010). However, ovarian steroid is the key contributors to innate and acquired immune responses to bacterial and viral infections (reviewed in Bouman *et al.*, 2005). It regulates the immune status of the uterus by regulating the chemokine and cytokine regulation. Estrogen is a immune stimulator and promotes production of proinflammatory cytokines through enhancing TLR4 signaling in macrophages in female (Calippe *et al.*, 2010). Progesterone is a potent immunosuppressive molecule to inhibit the release of pro-inflammatory cytokine from the leukocytes in mouse uteri (Hunt *et al.*, 1997). Recently, we have shown in vivo & in vitro evidence that day-7 embryo secretes IFNT that induces an anti-inflammatory immune response in local immune cells of the uterus in cows (Rashid *et al.*, 2018, Talukder *et al.*, 2017). It can be concluded that though many attempt has been taken to treat cyst by hormonal therapy but no data is available on immune status of uterus in presence of ovarian cyst. Therefore, our study will provide new information for effective treatment of cystic cow along with hormonal treatment.

An ovarian cyst is a fluid-filled sac within the ovary. Often they cause no symptoms. Occasionally they may produce bloating, lower abdominal pain, or lower back pain. The majority of cysts are harmless. If the cyst either breaks open or causes twisting of the ovary, it may cause severe pain. This may result in vomiting or feeling faint, and even cause headaches.

Most ovarian cysts are related to ovulation, being either follicular cysts or corpus luteum cysts. Other types include cysts due to endometriosis, dermoid cysts, and cystadenomas. Many small cysts occur in both ovaries in polycystic ovary syndrome (PCOS). Pelvic inflammatory disease may also result in cysts. Rarely, cysts may be a form of ovarian cancer. Diagnosis is undertaken by pelvic examination with an ultrasound or other testing used to gather further details.

Often, cysts are simply observed over time. If they cause pain, medications such as paracetamol (acetaminophen) or ibuprofen may be used. Hormonal birth control may be used to prevent further cysts in those who are frequently affected. However, evidence does not support birth control as a treatment of current cysts. If they do not go away after several months, get larger, look unusual, or cause pain, they may be removed by surgery.

There are several types of functional cysts:

Follicular cyst: the most common type of ovarian cyst. In cattle, a follicle containing the ovum, an unfertilized egg, will rupture during ovulation. If this does not occur, a follicular cyst of more than 2.5 cm diameter may result.

Corpus luteum: cysts appear after ovulation. The corpus luteum is the remnant of the follicle after the ovum has moved to the fallopian tubes. This normally degrades within 5 to 9 days. A corpus luteum that is more than 3 cm is defined as cystic.

Theca lutein cysts: occur within the thecal layer of cells surrounding developing oocytes. Under the influence of excessive hCG, thecal cells may proliferate and become cystic. This is usually on both ovaries.

The majority of the literature found to be reviewed was primarily concerning follicular cysts, rather than the other two varieties. Due to this uneven concentration of information, the largest section of the review with the most information is the section on follicular cysts. Of the three conditions, the least information was found on cystic corpora luteum, with luteal cysts falling in the middle. Because luteinized cysts are often considered to be just the later form of follicular cysts, it is likely that researchers find it more important to examine the initial cause of the cystic follicle than the pathogenesis of the luteal cyst. A likely explanation for the lack of literature on the cystic CL is the harmlessness of the condition when compared to follicular and luteal cysts. The first is considered to be a variation of a normal corpus luteum that does not affect cyclicity, while the latter conditions both cause a state of anestrus in affected animals. In order to minimize losses due to ovarian cysts, it is imperative that one is able to differentiate between the different types of cysts and other conditions that lead to anestrus. It is also necessary to be aware of other abnormalities of the ovary so that a lesion found during palpation is not immediately diagnosed as cystic, but rather examined completely so that the proper diagnosis can be made.

All of this should be taken into consideration before decisions on treatments are made in order to effectively cure animals of the condition they have. Cystic ovaries significantly decrease reproductive efficiency of affected animals, causing losses in milk production and, overall, monetary losses to the dairy. Therefore, ovarian cysts should be continually researched in order to assure that they do not pose a serious problem to the dairy industry.

Among many factors that attribute for reproductive failure, ovarian cyst (OC) is one of the most important reproductive disorders affecting the fertility of dairy cows. Long time presence of anovulatory follicular structure in the cyst results persistent release of ovarian steroid hormone. Ovarian hormone regulates the growth and development of uterine wall depending on the status of estrus cycle. This study hypothesize that ovarian follicular cyst modulated hormonal condition which affect the mucosal immunological environment in oviduct and uterus in cow. Therefore, our present study aimed to

- i. Investigate the effect of luteal cystic condition on presence of bacteria in bovine uterus
- ii. Explore the infections as well as immune response in bovine uterus at luteal cystic condition.
- iii. Draw the histopathological pattern /changes of cystic ovary infections at luteal cystic condition



CHAPTER 2

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Purpose of this chapter is to provide a selective review of the research works performed in relation to the present study. Literature on consequences that impact on ovarian cyst which is related to this study has been reviewed under the following heading.

2.1 Ovarian cyst

There are several types of cysts that can be found on the ovaries of the cow, which can have a significant impact on the reproductive efficiency of the animal. The cystic structures that were studied included follicular cysts, luteal cysts, and cystic corpora luteal. The objective was to determine the potential causes of and factors affecting incidence of ovarian cysts in dairy cattle and to examine functionality of various preventative methods and treatments of cysts. This was performed through the review and presentation of pertinent information on the cystic ovarian conditions. Each type of cyst is defined and its characteristics described in addition to its causes, preventative methods and treatments. There is also information presented on the methods of differentiation between the cystic conditions as well as other anovulatory conditions that provide similar symptoms. Throughout the report, the different types of cysts are compared and contrasted in their characteristics, causes, and treatments so that connections are made between the different, yet remarkably similar, conditions. The importance of studying cystic conditions lies in the consequences they have for the dairy producer. As most cysts are anovulatory, they cause severe delays in the reproductive efficiency of affected animals, leading to decreased milk production over time. This inevitably can affect the bottom line and cause the dairy producer to lose money. Because of the significant impact that cysts can have, they should be understood completely so that the most effective diagnoses can be made and treatments administered in order to prevent unnecessary losses from the farm.

2.2 Follicular cysts

Definitions and Characteristics There are several definitions used to describe ovarian follicular cysts, and the traditionally accepted definition is that they are –follicular structures of 2.5 cm or larger that persist for a variable period in the absence of a corpus luteum (Youngquist and Threlfall, 2007). There are several sources however who, due to new data,

define cysts differently. A 2003 study by Hatler et al. pointed out that ‘follicles typically ovulate at 17 mm in diameter, so follicles that persist at that diameter or greater may be considered to be ‘cystic.’ Vanholder et al. (2006) suggested that cystic ovarian follicles (COF) should be defined as ‘follicles with a diameter of at least 2 cm that are present on one or both ovaries in the absence of any luteal tissue and that clearly interfere with normal ovarian cyclicity’ So, though experts may argue on the specific wording used and the exact diameter that a follicle must reach to be considered cystic, we can consider ovarian follicular cysts to be any follicular structure on the ovary in the absence of luteal tissue, larger than normal follicular size, that persists for a significant amount of time and affects the estrus cycle of the animal. Though we cannot determine the exact cause of ovarian follicular cysts, we can recognize that they ‘develop when one or more follicles fail to ovulate and subsequently do not regress but maintain growth and steroidogenesis’ (Vanholder et al., 2006). It has also been determined that follicular cysts are anovulatory structures so, as long as they persist, cows will remain infertile (Youngquist and Threlfall, 2007). Cystic ovarian disease (COD is the ‘most common endocrine pathology to be found in dairy cows, and incidence is ‘believed to vary from 1 to 30% depending on herd and breed conditions (Gordon, 1996). Holsteins are the most susceptible to develop a cystic condition compared to other breeds, and the most likely time of diagnosis is 30-60 d. after parturition in high-yielding dairy cows (Gordon, 1996). Follicular cysts, when compared to other ovarian cystic conditions, are characterized by thin walls and produce very small amounts of progesterone. Occasionally, a persistent condition can lead to increased testosterone levels, causing some cows to exhibit masculine aggressive and sexual behavior. However, most cystic cows will remain in anestrus as long as the condition persists. (Ball and Peters, 2004) The fluid in follicular cysts contains many components, including hormones like estradiol, progesterone, and insulin. Anti-Müllerian hormone, a hormone strongly linked with polycystic ovarian syndrome in humans, is also present in follicular cysts, but at such a low concentration suggesting that there is no correlation between the hormone and incidence of COD in cattle (Monniaux, 2008). There are also a variety of proteins present in the fluid, 8 of which were identified in a study by Maniwa et al. (2005) as ‘BMFA, EAF, MeS, VEGF-receptor, GAPDH, HSP70, BLG, and SD. All that is currently known concerning the general functions of each protein is described in the study, but it is imperative that these proteins be researched extensively as well as any other components identified in follicular cysts in the future so that the pathology of the condition is fully understood. One proposed explanation for the development of follicular cysts is that ‘the positive feedback of estradiol on release of gonadotropin-releasing hormone (GnRH) is compromised’ (Bartolome et al., 2005). This would not interfere with the ability of the pituitary gland to release luteinizing hormone (LH) in cystic animals, but the overall function of the hypothalamic pituitary-ovarian axis would be altered, and the preovulatory LH surge that normally induces ovulation does not occur. In cystic animals, despite the failure of ovulation by the pre-ovulatory

4 follicle, a surge of follicle-stimulating hormone (FSH) would occur, and under conditions of low progesterone concentrations and high LH concentrations, dominant follicles would be caused to grow to a larger size. These oversized follicles are termed follicular cysts, that produce high concentrations of estradiol and inhibin, cause a delay in follicular turnover and are responsible for the persistent cystic condition. (Bartolome et al., 2005; K Causes While there are many factors that have been linked to the predisposition of cattle to develop follicular cysts, also referred to as Cystic Ovarian Disease, the exact cause has not been defined. The factors that do predispose cows to develop follicular cysts have been suggested as genetics, hormonal imbalances, or exogenous factors, among others. And –the most widely accepted hypothesis is that COD results from a neuroendocrine imbalance involving the hypothalamic-hypophyseal-gonadil-axis (Hooijer et al., 2001). In other words, the most likely cause of COD is a disruption along the pathway from the hypothalamus, a part of the brain that regulates hormones through the pituitary gland, to the gonads, which are the ovaries in this situation. While this general hypothesis may be accepted, it is vital to determine the specific disruption and where along the pathway it occurs in order to fully understand how follicular cysts develop and how they can be treated and prevented. Hooijer et al. (2001) performed a study examining the correlation between genetics and incidence of COD. They initially mention that, through previous studies, the heritability of the trait has been estimated as 0.00-0.13. This means that, if there is indeed a correlation, the probability of genetically selecting against the COD trait and successfully producing desired offspring is very low. However, it has also been recognized that there is an association between high milk yield and increased incidence of COD, and that cows genetically selected for producing more milk will also be more likely to develop multiple follicular cysts over their lifetimes (Hooijer et al., 2001; Brito and Palmer, 2004; Fleischer et al., 2001). In fact, the study performed by Hooijer et al. (2001) led to the conclusion that selection for milk yield leads to an increase in COD incidence by 1.5% per 500 kg increase in milk yield. The researchers hypothesized that a possible explanation for the direct correlation between high milk yield and 6 increased COD incidence –is that cows in early lactation, which are trying to meet the increased requirements for milk production, are more susceptible to environmental changes with hormonal implications as a result|| (Hooijer et al., 2001). In other words, it is possible that the correlation between milk yield and COD incidence is based on the state of stress on the animal during early lactation rather than a genetic correlation. While incidence of ovarian follicular cysts is likely due to both environmental and genetic factors, more research should be done in this area to identify the specific relationships between these factors. I would suggest that a good direction to pursue would be to examine the bovine genome in order to determine if there are any qualitative trait loci (QTLs) or single nucleotide polymorphisms (SNPs) that code for ovarian cysts. Doing this would be the most effective way to truly determine if there is a genetic factor affecting the incidence of COD, and specifically whether or not the possible factor is genetically tied to the genes coding for milk production. Putting all that aside, based on the study performed by Hooijer et al. (2001), it can be concluded

that there is definitely a heritability factor for cystic ovaries determined by a genetic predisposition for COD in Dutch Black and White dairy cattle, and that without a reexamination of genetic selection methods, incidence of cysts will increase as long as selection for milk production traits continues. In a 2002 study by Gümen et al, researchers hypothesized that -follicular cysts would develop if cows experienced an estradiol-induced GnRH/LH surge in the absence of an ovulatory follicle. They further suggested that -estradiol would fail to induce a subsequent GnRH/LH surge in these cows until they were treated with progesterone (Gümen et al., 2002). Their theories were based on the dynamic nature of cysts, evident by follicular waves due to increases in FSH, even in the presence of large anovulatory cysts. Three experiments were performed during the study to test the. Results from the experiments indicated that cows treated with 7 estradiol would develop an anovulatory condition after only one treatment. Researchers suggested that if estradiol induced a GnRH/LH surge at a time when an ovulatory follicle was not present then the cow would subsequently become anovulatory due to the lack of an increase in circulating progesterone following the LH surge (Gümen et al., 2002). The more simple interpretation is that a progesterone deficiency during the estradiol- induced LH surge is a probable cause of the development of follicular cysts on the ovaries. In addition to the study performed by Gümen et al. (2002), there has been much investigation on the relationship between progesterone and ovarian follicular cysts. A study by Hatler et al. (2003) looked more closely at this relationship. Like many other sources, the study points out that the basic reason cysts form is because of an endocrine imbalance, and that the vast majority of data suggest that cysts form because of a failure of the preovulatory LH surge to occur at the appropriate time in follicular maturation (Hatler et al., 2003). The researchers wanted to further understand the role of progesterone in the formation of follicular cysts, stating that: The objectives of the present study were to determine how often cows diagnosed with ovarian follicular cysts have intermediate concentrations of progesterone and to relate the fate of follicles that form in the presence of a follicular cyst to circulating concentrations of progesterone during their development. (Hatler et al., 2003) Results of the aforementioned study suggested that the majority of cows with anovulatory cystic follicles did have intermediate concentrations of progesterone at the time of diagnosis. They also observed that developing dominant follicles, when in the presence of follicular cysts and especially those with intermediate progesterone concentrations, developed into new follicular cysts themselves. The ultimate conclusion was that intermediate levels of progesterone commonly associated with cysts may predispose subsequent follicles to form cysts and contribute to the phenomenon of cyst turnover (Hatler et al., 2003). My interpretation of this data is that there is a definite relationship between endogenous concentrations of progesterone and the incidence of follicular cyst formation.

Further research should be performed in order to distinguish the exact role of progesterone in the development of ovarian follicular cysts. A 2002 study performed by López-Gaitus et al. examined risk factors that lead to the development of COD. While the researchers did not determine any true cause of ovarian cysts, they examined several factors that would predispose cows to develop a cystic condition. One of the factors examined was body condition, and results suggested that a gain in nutritional status prepartum can impair postpartum ovarian function, thus being a major risk factor for early cyst development that continues to the late cystic condition (López-Gaitus et al., 2002). Another risk factor that was identified was calving season, and results indicated that cysts were 2.6 times more likely to develop in warm periods than during cooler seasons. The researchers also examined abnormal puerperium as a risk factor for ovarian cysts, defining the term broadly as any of the disorders that include twinning, retained placenta, primary metritis, and ketoneuria, or a combination of the conditions. The cows that did have an abnormal puerperium were about twice as likely to develop new follicular cysts, but the condition was not determined to be a risk factor for late ovarian cysts. López-Gaitus et al. (2002) also recognized the effect of high milk production on cyst incidence, and determined that older cows were more likely to develop a chronic cyst condition than those in earlier lactations. All of these risk factors should be thoroughly considered in the management of dairy facilities, and based on the knowledge of their existence, measures should be taken to prevent increased incidence of ovarian cysts. A thorough explanation for the biological cause of follicular ovarian cysts can be provided by Bartolome et al. (2005). The initial root of the problem is a dysfunction of the positive feedback of estradiol on release of GnRH. Because of this, function of the hypothalamic-pituitary-ovarian axis is altered, even though the pituitary gland is still able to release LH. Because of the hormonal imbalance, the dominant follicle does not ovulate before the transient increase in FSH occurs, stimulating a new wave of follicular development, which occurs under conditions of low progesterone concentrations and high LH concentration (Bartolome et al., 2005). This causes excessive growth of dominant follicles to become follicular cysts, which produce large amounts of estradiol and inhibin, therefore causing a delay in follicular turnover. Brito and Palmer's review on COD (2004) provided a succinct flow chart that hypothesizes the different mechanisms that potentially lead to the absence of the preovulatory surge of LH that ultimately causes follicular cyst Formation (Aneko et al., 2002)

2.3 Luteinized cysts

The Merck Veterinary Manual (Kahn, 2010) describes luteal cystic ovary disease as being characterized by enlarged ovaries with one or more cysts, the walls of which are thicker than those of follicular cysts because of a lining of luteal tissue. Ball and Peters (2004) call these cysts luteinized cystic follicles, describing them as cysts with thicker walls that produce high levels of progesterone. In appearance, they are smooth and rounded, with a spherical cavity that is lined by a layer of fibrous tissue surrounded by the luteinized cells (Schlafer, 2007).

Luteal cysts are considered anovulatory cysts and are associated with infertility and mucometra in cattle (Foley, 1996). When compared to follicular cysts, luteinized cysts are more likely to persist over long periods of time and can lead to nymphomania in some animals (Ball and Peters, 2004). Causes luteal cysts –develop when ovulation fails to occur and the theca undergoes luteinization (Schlafer, 2007). They are also often considered to be the later form of ovarian follicular cysts (Vanholder et al., 2005), and therefore the causes pertaining to follicular cysts can also be considered the original causes of luteal cysts. The luteal cyst occurs when the cells of the follicular cyst (granulosa and theca) become luteinized and start producing progesterone (Peter et al., 2009). Luteal cyst incidence increases with age and most often affects cows with high milk production (Foley, 1996).

2.4 Cystic corpora lutea

A cystic corpus luteum (CL) in cow is defined as luteal tissue initiating from a corpus hemorrhagicum and containing fluid in a central cavity greater than 7 mm in diameter (Chuang et al., 2010). The terms for cystic CLs can often be confused with those for luteal cysts, though the first is a normally functional structure and the latter a pathological condition. Because of this, the contemporary term _corpus luteum with a cavity has been suggested to replace the classical term cystic corpus luteum (Chuang et al., 2010). Cystic CLs occur spontaneously, when follicles become luteinized without ovulation (Pineda, 2003). Incidence of cystic CLs ranges from 25.2% to 78.8% during diestrus and decreases with progression of the estrous cycle (Kahn, 2010). Because cystic corpora lutea are found in cows that are normally cycling or pregnant, they are considered to be a normal stage or variation of CL development (Kahn, 2010). The 10th edition of the Merck Veterinary Manual thoroughly describes the physical properties of cystic CLs and their similarities and differences to normal corpora lutea: Cystic CL have a soft, mushy core area, due to presence of fluid from a degenerating blood clot, compared with the homogeneous, liver-like consistency of the base of a typical CL. The cystic CL as well as the typical CL may or may not have an ovulation crown or papilla at its apex. Absence of this ovulation crown or papilla should not be considered diagnostic of the cystic condition because 10-20% of functional, normal CL fail to develop this feature. (Kahn, 2010). Although diagnosis is not essential as cystic CLs are not pathological and therefore do not require treatment, the ideal time for detection of the structure is 5-7 d after estrus. At this point 22 in time, the ovarian structure is near the end of the corpus hemorrhagic stage of development. (Kahn, 2010)

Diagnosis of ovarian cysts differentiation of cystic conditions ss follicular cysts and luteal cysts are similar in size and function, it may be difficult to differentiate the two. The key to proper diagnosis is a combination of physical examination, ultrasonography, and plasma progesterone testing: The accuracy of diagnosing ovarian cysts and differentiating follicular and luteal cysts can be increased by combining per rectum palpation of the genital tract to determine that a corpus luteum is absent and the uterus lacks tone; ultrasonography to confirm that a corpus luteum is absent, to determine the size of follicles that are present, and to check for luteinization; and measurement of plasma progesterone concentration to determine the degree of luteinization. (Bartolome et al., 2005) Though it is important to understand the differences between these two types of cysts, treatment is similar between the two conditions so differentiation is not always necessary. (Bartolome et al., 2005) There is a significant difference between luteal cysts and cystic corpora lutea (cystic CLs). While luteal cysts are originally pathological follicular cysts that have luteinized with age, cystic CLs are physiologic and originate from follicles that have formed a cavity during corpus luteum development (Bartolome et al., 2005). These cystic structures can be differentiated through rectal palpation to determine if the structure is structurally characteristic of a corpus luteum. A cystic CL can be diagnosed if there is detection of a line of demarcation and distortion in the shape of the ovary. However, differentiation can only be successfully confirmed when rectal palpation and ultrasonography are combined. (Bartolome et al., 2005) 24 When differentiating between ovarian follicular cysts and normal pre-ovulatory follicles, one should look at the number and size of the structures but should especially base their judgement on the basis of uterine tonicity. Upon rectal palpation, cows with ovarian cysts will be found to have -multiple follicles that are typically larger-than-normal Graafian follicles with a flaccid uterus in the absence of a corpus luteum (Bartolome et al., 2005).

Contrarily, cows in proestrus with normal follicles will have an erect, turgid uterus. This uterine tonicity is in response to luteal oxytocin secreted by the ovaries of non-cystic cows. This method is the most effective at differentiating between follicular cysts and normal follicles, however an ultrasound should also be used to determine the number and size of ovarian follicles in order to confirm the diagnosis. (Bartolome et al., 2005) Some may have a difficulty determining whether a cow has ovarian cysts or it is in postpartum nutritional anestrus. Though a cystic condition may often be the first guess for the cause of prolonged anestrus, several physiologic (eg, pregnancy), pathophysiologic (eg, postpartum and nutritional anestrus and ovarian cysts), and pathologic (eg, pyometra, hydrometra, ovarian hypoplasia, and granulosa cell tumor) conditions result in a lack of cyclic activity, anovulation, or anestrus (Bartolome et al., 2005).

Shallow postpartum nutritional anestrus is an anovulatory condition that can occur during the early postpartum period, and it's characterized by regular follicular waves and lower than normal secretions of luteinizing hormone (LH) and insulin like growth factor 1 (IGF-1). These conditions cause the animal to be unable to support complete follicular development and lead to insufficient estradiol concentrations for ovulation. While this condition is associated with animals in negative energy balance, cows with cystic ovarian degeneration are in positive energy balance. Cows may also have deep postpartum nutritional anestrus, during which the ovaries are small with limited follicular development and follicle stimulating hormone (FSH) secretions are low. Because of this, a simple rectal palpation and/or ultrasound exam can identify whether or not the anovulatory condition is caused by deep postpartum nutritional anestrus. Ovarian cysts are differentiated from shallow anestrus on the basis of the number and size of the follicles, occurrence of follicular waves, body condition score (BCS), and stage of lactation. (Bartolome et al., 2005)

2.5 Antimicrobial sensitivity test

There are three test methods (disk diffusion, broth dilution and agar dilution). Antimicrobial susceptibility testing methods that consistently provide reproducible and repeatable results is when followed correctly.

Disk diffusion testing is one of several phenotypic assays which can be utilized to determine the antimicrobial resistance profile (anti-biogramme) of an organism. Disk diffusion tests estimate in vitro susceptibility. The principle of agar diffusion is simple: Agar plates are inoculated with standardized inoculums of the bacteria and an antimicrobial disk is placed on the inoculated agar plate. The disks used for a disk diffusion assay contain a standardized known amount of an antimicrobial agent, which diffuses into the agar when in contact with the agar surface. The plate is incubated under standardized conditions following Clinical and Laboratory Standards Institute (CLSI) guidelines. During incubation, the antimicrobial agent diffuses into the agar and inhibits growth of the bacteria, producing a zone of inhibition around the disk. Following incubation, the diameter of this zone is measured and the results are interpreted as resistant, intermediate, or susceptible using standard guidelines (e.g. CLSI M100). The size of the inhibition zone indicates the degree of resistance, and might also give important information about the resistance mechanism and the resistance genes involved. In addition, the disk diffusion method can be used for determination of MIC values provided the necessary reference curves for conversion of inhibition zones into MIC values are available. Highly standardized methods are essential for all types of susceptibility testing. These assays are highly sensitive to variations in: inoculums density, media formulation, media pH, and incubation conditions. In addition, agar diffusion methods are strongly influenced by agar depth, diffusion rate of the antimicrobial agent and growth rate of the specific bacteria. To insure the accuracy and reproducibility of antimicrobial susceptibility test results, internal quality control testing must be regularly performed as recommended by CLSI (CLSI M2, 2017).

2.6 Immune cell

Granulocytes include basophils, eosinophils, and neutrophils. Basophils and eosinophils are important for host defense against parasites. They also are involved in allergic reactions. Neutrophils, the most numerous innate immune cell, patrol for problems by circulating in the bloodstream. They can phagocytose, or ingest, bacteria, degrading them inside special compartments called vesicles.

Mast cells also are important for defense against parasites. Mast cells are found in tissues and can mediate allergic reactions by releasing inflammatory chemicals like histamine.

Monocytes, which develop into macrophages, also patrol and respond to problems. They are found in the bloodstream and in tissues. Macrophages, "big eater" in Greek, are named for their ability to ingest and degrade bacteria. Upon activation, monocytes and macrophages coordinate an immune response by notifying other immune cells of the problem. Macrophages also have important non-immune functions, such as recycling dead cells, like red blood cells, and clearing away cellular debris. These "housekeeping" functions occur without activation of an immune response.

Neutrophils accumulate within minutes sites of local tissue injury. They then communicate with each other using lipid and other secreted mediators to form cellular "swarms." Their coordinated movement and exchange of signals then instructs other innate immune cells called macrophages and monocytes to surround the neutrophil cluster and form a tight wound seal.

Dendritic cells (DC) are an important antigen-presenting cell (APC), and they also can develop from monocytes. Antigens are molecules from pathogens, host cells, and allergens that may be recognized by adaptive immune cells. APCs like DCs are responsible for processing large molecules into "readable" fragments (antigens) recognized by adaptive B or T cells. However, antigens alone cannot activate T cells. They must be presented with the appropriate major histocompatibility complex (MHC) expressed on the APC. MHC provides a checkpoint and helps immune cells distinguish between host and foreign cells.

Natural killer (NK) cells have features of both innate and adaptive immunity. They are important for recognizing and killing virus-infected cells or tumor cells. They contain intracellular compartments called granules, which are filled with proteins that can form holes in the target cell and also cause apoptosis, the process for programmed cell death. It is important to distinguish between apoptosis and other forms of cell death like necrosis. Apoptosis, unlike necrosis, does not release danger signals that can lead to greater immune activation and inflammation. Through apoptosis, immune cells can discreetly remove infected cells and limit bystander damage. Recently, researchers have shown in mouse models that NK cells, like adaptive cells, can be retained as memory cells and respond to subsequent infections by the same pathogen.



CHAPTER 3

MATERIALS & METHODS

CHAPTER III

MATERIALS AND METHODS

3.1 Study area

This study was conducted in collaboration with the department of physiology and pharmacology; and department of pathology and parasitology at Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur from January 2023 to June 2023 to evaluate the impact of ovarian cyst in dairy cattle. And the samples were collected from different abattoirs in Dinajpur district.

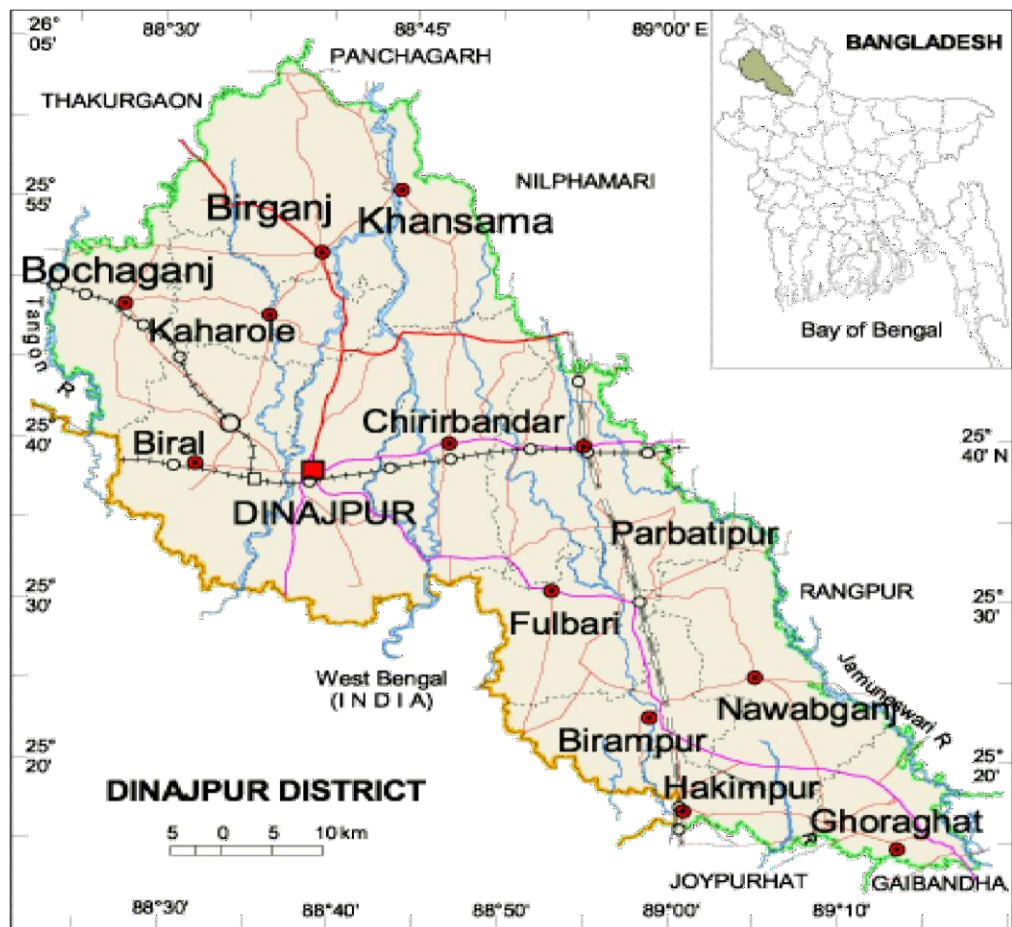


Fig. 1: Map of Dinajpur district.

3.2 Experimental layout

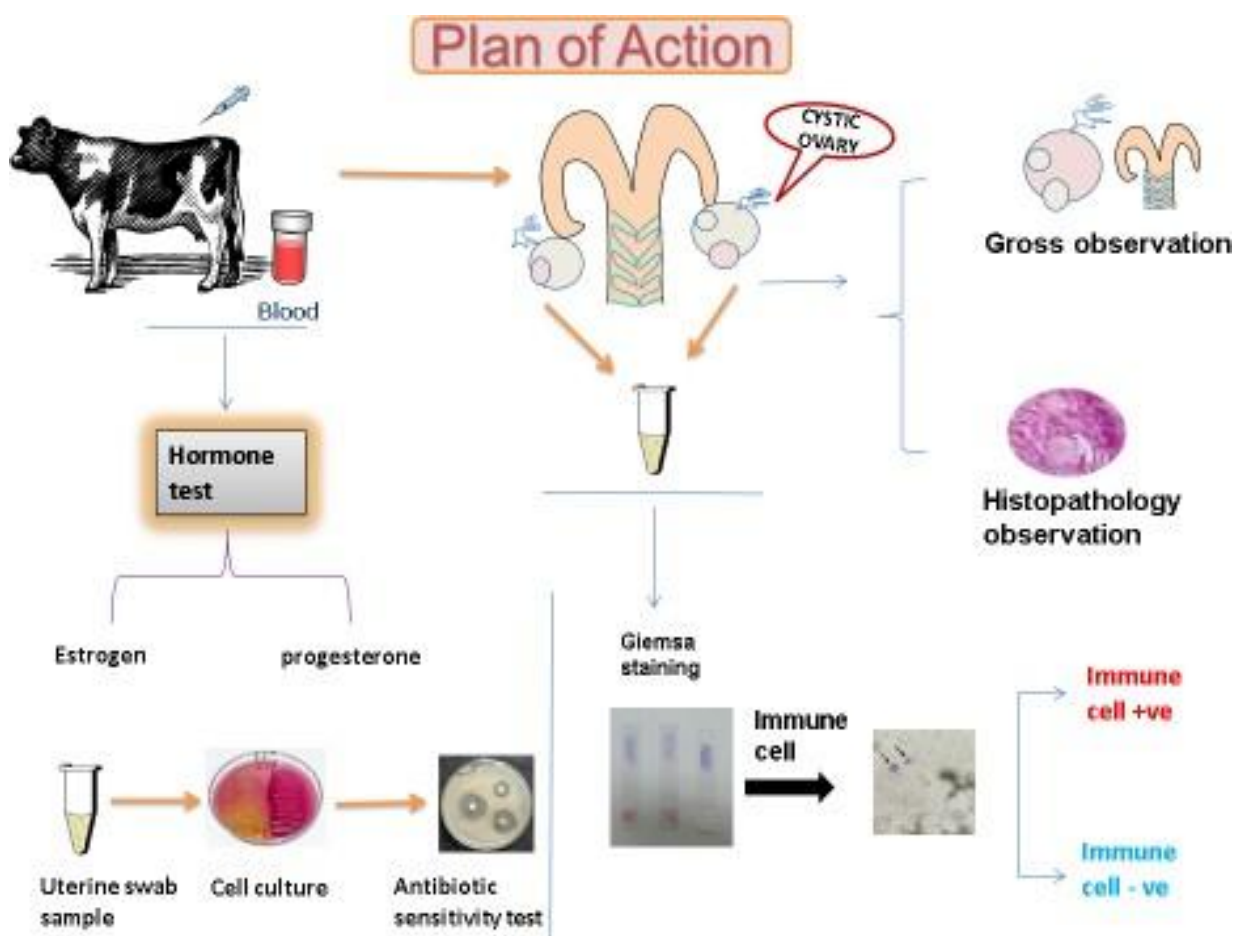


Fig. 2: Experimental layout

3.3 Sample collection

About 2.5 ml of peripheral blood samples and uterus samples were collected from different dairy farm and abattoir of Dinajpur Sadar upazila, Dinajpur within Ethylene Diamine Tetra Acetate (EDTA) tube and the uterus within saline solution and shifted to the department of physiology and pharmacology.



Figure 3: Collection of blood sample and uterus sample

3.4 Transportation and preservation of samples

The samples were carried out to the laboratory in an ice box contained ice and processed for the isolation, identification and characterization of bacteria subsequently. And the remaining samples were stored at 4 for future use. The histopathological samples are preserved in formalin solution.

3.5 Laboratory identification of immune cell through Giemsa's stain method

Giemsa stained uterine fluid smear microscopy was used to evaluate blood samples. In this study, the effects of geography and season were kept in consideration. Giemsa staining was used to create thick and thin uterine fluid smears. Air drying and absolute methanol fixing were carried out. After methanol fixation, the smear was stained with 10% Giemsa's stain. Magnification under 100x objectives after washing, air drying, and emulsification. The immune cell were identified microscopically using the methods given by Benjamin (1978) and Soulsby (1982).

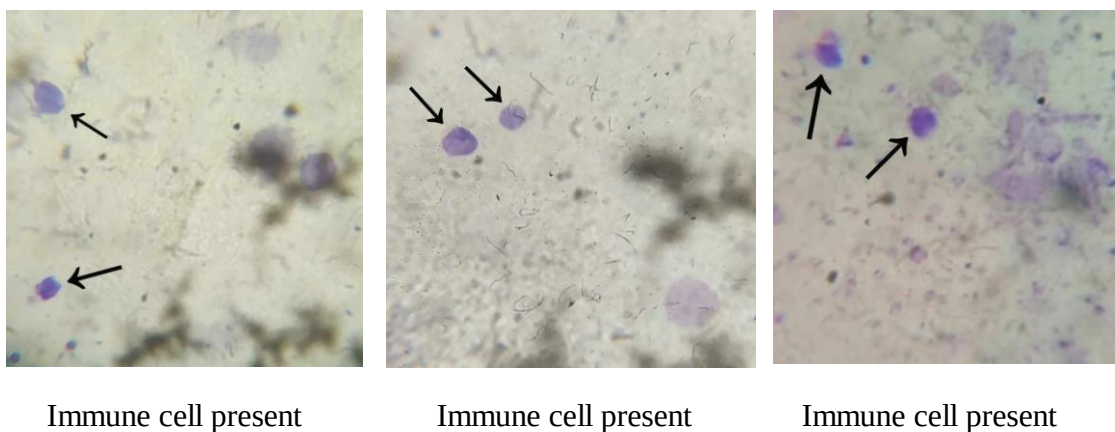


Figure 4: Identification of Immune cell

3.6 Media for culture

Different bacteriological culture media and reagents were used for isolation and identification and also propagation of bacteria from collected samples. The culture media and reagents used in this experiment are mentioned below:

3.6.1 Liquid media

1. Nutrient broth (Difco)

3.6.2 Solid media for culture

1. Nutrient Agar Medium, (HI-MEDIA, India)
2. Eosin Methylene Blue, (EMB) (HI-MEDIA, India)
3. Mannitol salt agar

3.6.3 Reagents

1. Crystal violet dye
2. Gram's iodine
3. Alcohol
4. Safranin
5. Saline
6. Iodine solution
7. Kovac's reagent
8. Methyl- red solution
9. 3% H₂O₂
10. P – Amino dimethylaniline oxalate
11. Phenol red
12. Phosphate buffered saline (PBS) solution
13. Potassium tellurite (1%)

3.6.4 Glassware and appliances

The different types of important equipment used for this work are listed as follow down

1. Distilled water
2. Sterile bent glass or plastic spreader rods.
3. Micropipette (1-5µl; 5-50µl; 10-100µl; 50-500µl; 100-1000µl)
4. Freeze (-20°C)
5. Refrigerator (40C)
6. Spirit lamp

7. Water bath
8. Vortex Mixture
9. Labeling tape
10. Experimental test tube
11. Stopper
12. Petri dish
13. Conical flask.
14. Durham's tube
15. Slide
16. Microscope
17. Cotton
18. Immersion Oil
19. Toothpick
20. Autoclave
21. Thermometer
22. Incubator
23. Jar
24. Beaker
25. Cylinder
26. Electric Balance
27. Filter paper
28. Spirit lamp and
29. Bacteriological loop etc.

3.7 Materials used for antibiogram test

- Test tube rack
- Bunsen burner
- Inoculating loop or needle
- Forceps
- Sterile swabs
- Mueller-Hinton or Nutrient agar plates
- Antibiotic disks
- Stock culture of experimental bacteria

3.7.1 Antibigram discs

To determine the drug resistance pattern of different bacterial isolate the followings are the antibiotics that were tested against the selected organism with their disc concentration

Table 3: Antimicrobial agents with their disc's concentration

Antimicrobial class	Name of antimicrobial agents	Disc code	Disc concentration (µg/disc)	Company Name
Aminoglycosides	Amikacin	AK	30	HIMEDIA
Folate pathway Inhibitor	Clotrimazole	COT	1.25/23.75	HIMEDIA
Aminopenicillin	Ampicillin	AMP	25	HIMEDIA
Cephalosporin	Ceftriaxone	CTR	30	HIMEDIA
Phenicol	Chloramphenicol	C	30	HIMEDIA
Fluroquinolone	Ciprofloxacin	CIP	5	HIMEDIA
Aminoglycoside	Streptomycin	ST	10	HIMEDIA
Aminoglycosides	Neomycin	NE	30	HIMEDIA
Tetracycline	Tetracycline	TE	30	HIMEDIA

Note: µg = Microgram

3.8 Methods

The following methods were used for the isolation and identification of bacteria.

3.8.1 Laboratory preparations

All items of required glassware including test tubes, pipettes plate, slides, cylinder, flasks, conical flasks, glass and vials soaked in a household dishwashing detergent solution (Trix recket and colman Bangladesh ltd.) overnight. Contaminated glassware was disinfected with 2% sodium hypochlorite solution prior to cleaning. The glassware was then cleaned by brushing, washed thoroughly in running tape water, rinsed within distilled water and finally sterilized either by dry heat at 160°C for 2 hours or by autoclaving for 15 minutes at 121°C under 15 lbs. pressure per sq inch. Autoclaved items were dried in a hot air oven over at

50°C. Disposable plastic (items e.g., micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50°C for future use.

3.8.2 Sampling and processing of samples

Proper care was taken during the sampling procedure to prevent contamination of sample. The samples tubes were completely tied at the time of sampling that prevent contamination. After came to the laboratory 1g sample was taken from each sample and mixed with 9ml PBS (Phosphate Buffer Solution) in each test tube for serial dilution (10^{-1} to 10^{-7}) and 50 µl samples were seeded on nutrient agar, using spread plate method. The plating was done in the laminar flow to maintain aseptic conditions and the medium were then incubated at 37°C for 24 h.

3.8.3 Plan of the experiment work at a glance

All of those samples were collected from different surface environments of Dinajpur district with a ice containing ice box. Then all of the samples were transferred to the microbiological laboratory of department of microbiology, HSTU, Dinajpur, Bangladesh. 50 µl (0.05 ml) of samples were primarily inoculated into nutrient agar. Subsequently Nutrient agar, EMB agar, Mannitol salt agar, were employed and identification of bacteria. Molecular characterization also performed for genome sequencing. At last performed antibiotic sensitivity test with the pure isolated bacteria.

3.8.4 Preparation of culture media

All the media, broth and reagents used in this experiment were prepared according to instruction of the manufacturer.

3.8.5 Nutrient broth media

Thirteen grams of dehydrated nutrient broth (HI-MEDIA, India) was suspended into 1000 ml of distilled water and boil to dissolve it completely. The solution was then distributed in tubes, stopper with cotton plugs and sterilized in autoclaving at 121°C and 1.2 kg/cm² pressure for 15 minutes. The sterility of the medium was checked by incubating at 37°C for overnight and stored at 4°C in aerator for further use. (Cater 1979)

3.8.6 Nutrient agar media

Twenty-eight grams of nutrient agar powder (HI-MEDIA) was dissolved in 1000 ml of cold distilled water in a flask. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile Petri dish and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use. (Cater 1979).

3.8.7 Eosin methylene blue agar

Thirty-six grams of EMB agar base (HI-MEDIA, India) was added to 1000 ml of water in a flask and boil to dissolve the medium completely. The medium was sterilized by autoclaving at 1.2 kg/cm² pressure and 121°C for 15 minutes and I to 50°C and shake the medium in order to oxidize the methylene blue (i.e., to restore its blue color). Then 10 ml of medium was poured into each sterile Petri dish sized and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37° C for overnight to check their sterility and petri dishes without contamination were used for cultural characterization or stored at 4°C in refrigerator for future use. (Cater 1979).

3.8.8 Mannitol salt agar

Suspend 111 grams of mannitol salt agar in 1000mls of distilled water boil to dissolve the medium completely sterilize by autoclaving at 15lbs pressure (121 degree) for 15 minutes. 10 ml of medium was poured into each sterile Petri dish sized and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37° C for overnight to check their sterility and petri dishes without contamination were used for cultural characterization Store the dehydrated medium at 24 Degree Celsius and the prepared medium at 2-4 degree Celsius for further uses.

3.8.9 Preparation of reagents

3.8.10 Methyl- red solution

The indicator MR solution was prepared by dissolving 0.1 gm of Bacto methyl- red in 300 ml of 95% alcohol and diluted to 500 ml with the addition of distilled water.

3.8.11 Methyl- red

A quantity of 17 gm of MR-VP medium was dissolved in 1000 ml of distilled water, dispensed in 2 ml amount in each tube and the tubes were autoclaved. After autoclaving, the

tubes containing medium were incubated at 37 °C for overnight to check their sterility and then in refrigerator for future use.

3.8.12 Alpha- naphthol solution

Alpha- naphthol solution was prepared by dissolving 5 gm of Alpha- naphthol in 100 ml of 95% ethyl alcohol

3.8.13 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of Potassium hydroxide crystals in 100 ml of cooled water.

3.8.14 Phosphate buffered saline solution

Eight grams of sodium chloride (NaCl), 2.89 grams of di-sodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), 0.2 gram of potassium chloride (KCl) and 0.2 gram of potassium hydrogen phosphate (KHPO_4) were suspended in 1000 ml of distilled for the preparation of phosphate buffered saline solution. The solution was heated to dissolve completely.

Then the solution was sterilized by autoclaving at 1.2 kg / cm^2 pressure and 121° C for 15 minutes and stored for future use.

3.8.15 Indole reagent (Kovac's reagent)

This solution was prepared by dissolving 25 ml of concentrated Hydrochloride acid in 75 ml of amyl alcohol and to the mixture 5 grams of para dimethylamino benzaldehyde crystals were added. This was then kept in a flask equipped with rubber cork for future use.

3.8.16 Crystal violet solution (0.5%)

Dissolve 500 mg crystal violet mixed 75 ml distilled water then added 25 ml methanol and store at room temperature which acts as a primary coloring agent (Isenberg, 1998).

3.8.17 Gram's iodine

Dissolve 6.7 gram of potassium iodide in 100mL of demonized water, add 3.3 g of iodine; stir to dissolve, then dilute to 1 L. Store in a bottle which acts as a mordant. (Isenberg HD.; 1998)

3.8.18 Safranin

Dissolve 0.1 g safranin in 75 mL of distilled water, then dilute to 100 ml. Filter before use which acts as a counter stain (Isenberg, 1998).

3.9 Isolation of bacteria

3.9.1 Isolation of bacteria in pure culture

For isolation of bacteria in pure culture, the mixed culture was inoculated into nutrient agar media by streak plate technique to obtain isolated colonies as per suggested by poindexter (1986).

3.9.2 Procedure for isolation and identification of bacterial pathogens

Primary culture on nutrient agar

With the help of sterile inoculating loop the collected samples were directly inoculated into nutrient agar and incubated at 37 °C for 24 hours. The incubated media were then examined for growth of bacteria.

Inspection: Growth of microorganisms and their colony characteristics were recorded according to procedures described by (Carter, GR. 1979)

Secondary culture on differential media

The organisms were inoculated into MacConkey agar, and incubated at 37°C for 24 hours. The incubated media were then examined for growth of bacteria.

Step-1: A inoculum was picked up with a sterile inoculating loop and was spread on an area of the medium in the petri dish.

Step -2: The loop was sterilized by being heated as red hot in a flame.

Step -3: The inoculums was spread over the remainder of the plate by drawing the cooled, sterilized loop across the part of the inoculated area, then streaking in a single direction in each parallel line. This method was repeated as many times as necessary to obtain a culture containing only one type of colony and usually at least two more times to ensure purity.

Culture on selective media

1. EMB agar for *E. coli*
2. Mannitol salt agar for *Staphylococcus aureus*

3.10 Isolation of bacteria by colony morphological characteristics

Each of the colonies was seen for size, shape, pigment, diameter, form, margin and elevation.

3.11. Gram's staining

Gram's staining method was followed to study the morphological and staining characteristics bacteria and to provide information about the presumptive bacterial identification as per recommendation of Merchant and Packer (1967). A pure colony of each isolate was picked and gram staining was performed. Then the shape, arrangement and gram reaction of the isolates were observed under microscopic field.

Gram's staining method

Clean glass slides were obtained using sterile technique, smear of each of the organisms were prepared by placing a drop of normal saline on the slide, and then transferring each organism separately on the slide with a sterile, cooled loop and mixing organism by means of circular of the inoculating loop smears were allowed to air-dry and then heat fixed in the usual manner. Smears were flooded with crystal violet and let stood for one minute then washed with tap water. Smear were gently flooded with gram's iodine mordant and let stood one minute. Gently washed with tap water 95% alcohol-acetone was added for 10 seconds, gently washed with tap water. Safranin was added as counter stain for 1 minute. Gently washed with tap water and dry by air. Then examined under microscope with high power objects (100 X) using immersion oil

Gram's staining observation –

- Gram Negative: pale to dark red
- *E. coli*- Rod shaped, arranged singly or in pairs.
- *Staphylococcus aureus* – blue stained diplococcic
- *Klebsiella pneumonia*- rod shaped

3.12 Antibigram test

Antimicrobial sensitivity test was performed according to the procedure Kirby-bauer disk diffusion susceptibility test protocol suggested by Jan Hudzicki (2009). First developed in the 1950s, it was refined and by W. Kirby and A. Bauer, then standardized by the World Health Organization in 1961. The Kirby-Bauer (K-B) disk diffusion test is the most common method

for antibiotic resistance/susceptibility testing. Sensitivity to antibiotics was studied on Mueller-Hinton agar plate used the different types of commercial antibiotic discs. After the discs were placed on the plates, the plates were inverted and incubated at 37°C for 16 to 18 hours. After incubation, the diameter of the zones of complete inhibition (including the diameter of the disc) was measured and recorded in millimeters. The measurements were made with a ruler on the undersurface of the plate without opening the lid. The value was compared with the zone-size table. The zones of growth inhibition were provided by Clinical and Laboratory Standards Institute (CLSI, 2012). Isolates were classified as susceptible, intermediate and resistant categories based on the standard interpretation tables updated according to the Clinical and Laboratory Standards Institution (CLSI, 2012)

3.13 Histopathological examination

During necropsy, various organs having gross lesions were collected, preserved at 10% formalin. Formalin fixed samples of the ovary from the blood protozoa infected dairy cows were processed for paraffin embedding, sectioned and stained with hematoxylin and eosin according to standard method (Luna, 1998) for histopathological examination.

Details of tissue processing, sectioning and staining are given below.

3.13.1 Equipment and appliances

- Samples (Ovaries)
- 10% formalin
- Chloroform
- Paraffin
- Alcohol
- Tape water
- Xylene
- Hematoxylin and Eosin stain
- Distilled water
- Clean slides
- Cover slips
- Mounting media (DPX).
- Microscope
- Microtome

3.13.2 Processing of tissue for histopathology

3.13.3 Collection of tissue and processing

During tissue collection the following point were taken into consideration: The tissues were collected in conditions as fresh as possible.

Normal and diseased tissues were collected side by side.

The thickness of the tissues were as less as possible (5mm approximately).

3.13.4 Fixation

10% formalin was added in the plastic container (10 folds of the tissue size and weight) for fixation of samples.

3.13.5 Washing

The tissues were trimmed into a thin section and washed over night in running tape water to remove formalin.

3.13.6 Dehydration

The tissues were dehydrated by ascending ethanol series to prevent shrinkage of cells as per following schedule.

- 50% alcohol — one hour
- 70% alcohol — one hour
- 80% alcohol — one hour
- 95% alcohol — one hour
- Absolute alcohol — three changes (one hour for each change.)

3.13.7 Cleaning

The tissues were cleaned in chloroform for 3 hours to remove ethanol (1 and half hr in each, two changes).

3.13.8 Impregnation

Impregnation was done in melted paraffin (56- 60°C) for 3 hours.

3.13.9 Embedding

Paraffin blocks containing tissue pieces were made using templates and molten paraffin.

3.13.10 Sectioning

Then the tissues were sectioned with a microtome at 5-6 micrometer thickness. The sections were allowed to spread on lukewarm water bath (40-42°C) and taken on a glass slide. A small amount of gelatin was added to the water bath for better adhesion of the section to the slide. The slides containing sections were air dried and stored in cool place until staining.

3.14 Routine hematoxylin and eosin staining

procedure Preparation of ehrlich's hematoxylin

solution Hematoxylin crystals 4.0 g

Alcohol, 95% 200.0 ml

Ammonium or potassium alum 6.0 g

Distilled water 200.0 ml

Glycerin 200.0 ml

Glacial acetic acid 20.0 ml

Hematoxylin was dissolved in the alcohol and the alum was dissolve in distilled water and mixed thoroughly. After these were in complete solution the glycerin and acetic acid are added.

Preparation of eosin solution

1% stock alcoholic eosin

Eosin Y, water soluble 1 g

Distilled water 20 ml

95% alcohol 80 ml

Eosin was dissolved in water and then 80 ml of 95% alcohol was added.

Working eosin solution

Eosin stock solution 1part

Alcohol, 80% 3 parts

0.5ml of glacial acetic acid was added to 100 ml of working eosin solution just before use.

Staining protocol

The sectioned tissues were stained as described below:

The sectioned tissues were deparaffinized in three changes of xylene (three minutes in each)

Then the sectioned tissues were rehydrated through descending grades of alcohol as per following schedule.

- Absolute alcohol — three changes (three minutes for each)
- 95% alcohol - two minutes
- 80% alcohol - two minutes
- 70% alcohol - two minutes
- Dipping with distilled water for 10 minutes.
- The tissues were stained with Harris hematoxylin for 10 minutes.
- Washed in running tap water for 10-15 minutes.
- Then the tissues were dipped in ammonia water (few dips).
- Stained with eosin for one minute.
- Differentiated and dehydrated in ascending grade of alcohol.
- 95% alcohol — three changes (2-4 dips for each.)
- Absolute alcohol — three changes (2-3 minutes for each)
- Cleaned in xylene: three changes (five minutes each).

Tissues were mounted with cover slip by using DPX. The slides were dried at room temperature and examined under a low (10X) and high (40X, 100X) power objectives.



Figure 5: Histopathological examination of ovary

3.15 Microscopic examination

Samples were fixed, processed, sectioned, stained and observed under microscope using various objectives (X4, X10, and X40).



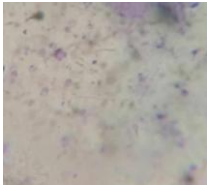
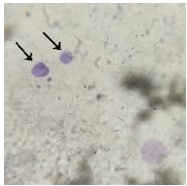
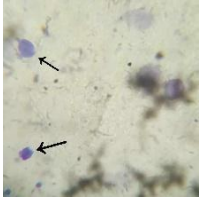
CHAPTER 4 **RESULTS**

CHAPTER IV

RESULTS

4.1 Result of laboratory identification of immune cell through Giemsa's stain

The identification of immune cell such as, t-lymphocytes, neutrophil, monocyte, basophiletc. presented in table no.2

Sample no.	Name of the uterine cyst	Immune cell	Name of cells
01	control	Absent 	
05	Luteal cyst	Present 	T-lymphocyte
06	Luteal cyst	Present 	T-lymphocyte

4.2 Results of Cultural Examination

The cultural characteristics of *E. coli*, *Staphylococcus aureus*, various media are presented in Table 3

Sample no.	Name of the uterine cyst	Name of the bacteria
01	control 	No specific bacteria found
02	Luteal cyst 	<i>Escherichia coli</i>
03	Luteal cyst 	<i>Staphylococcus aureus</i>



Fig : No bacteria growth in control sample



Fig : *Staphylococcus aureus*

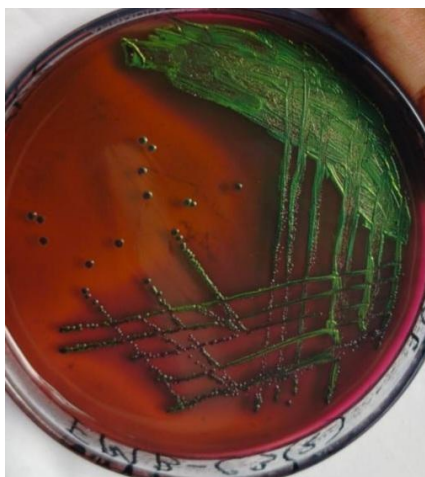


Fig: *E-coli* cell culture

Fig. 6: Bacteria found in cell culture

Table 4: Cultural characteristics of *E.coli*, *Staphylococcus aureus*, *klebsiella pneumoniae* and on various media

Name of bacteria	Staining characteristic	Name of Media	Colony characteristics
<i>E. coli</i>	Gram negative large rod shaped, pink color, arranged singly or in pair.	Nutrient Agar	Large, mucoid, white colony.
		Mac-Conkey Agar	Produce large mucoid rose-pink colony.
		EMB agar	Transmitted light blue-black center with a narrow, cleared edge. Bluegreen metallic sheen with reflected light.
<i>Staphylococcus aureus</i>	Spherical shape, grape like clusters	Nutrient Agar	Golden yellow and opaque colonies with smooth glistening surface, 1-2mm in diameter
		Mac-Conkey Agar	The colonies are small and pink in color
		Mannitol salt agar	Yellow color colonies
<i>Klebsiella pneumoniae</i>	Nonmotile, encapsulated, Rod-shaped bacillus	Nutrient agar	Colonies are large, thick, greyish white, mucoid, smooth and opaque
		Emb agar	Pink to purple colonies

Here, EMB Agar = Eosin Methylene Blue Agar

4.3 Results of antibiogram test

For antibiogram test common antibiotics such as amikacin, clotrimazole, ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, colistin, penicillin, mecillinam, rifampicin, streptomycin and levofloxacin were used for *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* 6 antibiotics (Neomycin, clotrimazole, ampicillin, penicillin, streptomycin, ciprofloxacin, penicillin, streptomycin, ciprofloxacin and) were used for antibiogram test.

4.3.1 Antimicrobial profile

Antibiogram test showed that isolates from every study area resistant to, clotrimazole, any antibiotics where isolates were sensitive to. Neomycin, ampicillin, streptomycin, ciprofloxacin, penicillin percentage of antibiotic resistance pattern for *bacterial samples* was presented in Table no. 12.

Table 5: Antimicrobial profile

Antimicrobial agent	Disc code	Potency (µg/Disc)	Result							
			Sample 01	Sample 02	Sample 03	Sample 04	Sample 05	Sample 06	Sample 07	Sample 08
Amikacin	AK	30	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)
Clotrimazole	COT	1.25/23.75	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(R)
Ampicillin	AMP	25	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)
Ceftriaxone	CTR	30	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)
Streptomycin	ST	30	(S)	(S)	(S)	(S)	(S)	(S)	(I)	(I)
Ciprofloxacin	CIP	5	(S)	(R)	(S)	(R)	(S)	(S)	(S)	(S)
Penicillin	PC	10	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)
Neomycin	NM	30	(S)	(I)	(S)	(S)	(S)	(S)	(S)	(I)
Tetracycline	TE	30	(S)	(S)	(R)	(R)	(S)	(S)	(R)	(S)

Source: CLSI document, 31th edition, 2021 [Note: S=Sensitive, R=Resistant, I=Intermediate]

4.4 Gross observation of ovary

35 blood and uterus samples are collected from Dinajpur district. Then blood samples are tested for determination of estrogen and progesterone hormone level. Some picture of gross observation are showed below which was cystic ovary



A. Normal ovary



B. Follicular Cystic ovary



C. Luteal Cystic ovary

Fig. 7 Gross observation of ovary

4.5 Histopathological examination

36 uterus samples were collected from the abattoir at Dinajpur district. Following that, the histopathology of the normal uterus and ovary (containing normal structure) individuals (Figure 8) and luteal cystic ovary and uterus (containing inflammation, fibrous tissue) individuals (Figure 9) is carried out.

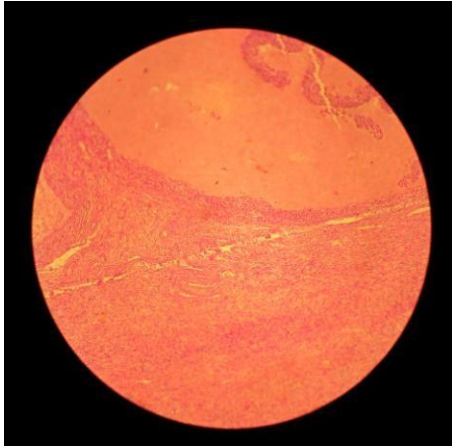


Fig 8: Histopathology of normal control samples

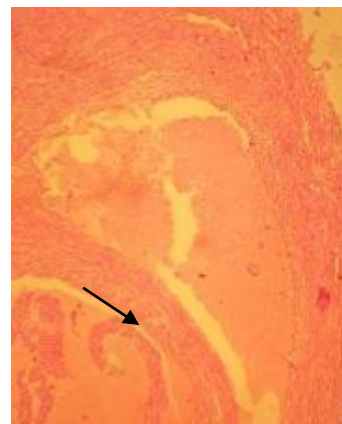
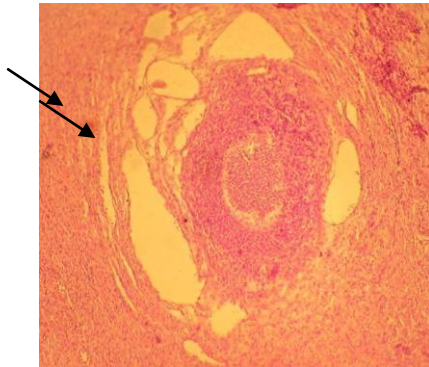


Fig 9: Histopathology of luteal cystic ovary

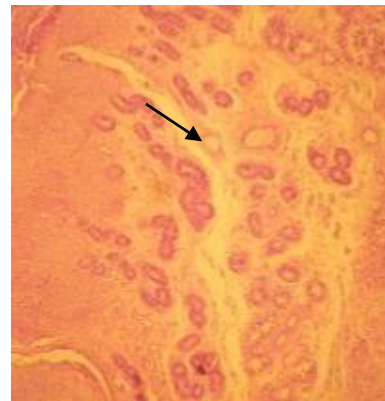
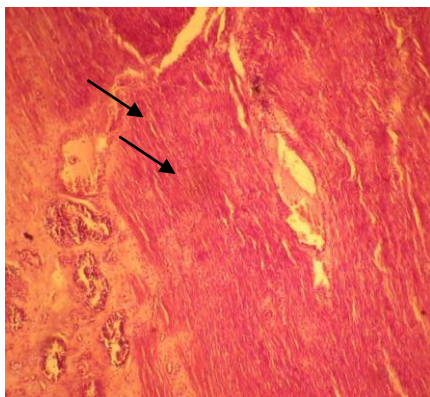


Fig 10. Histopathology of luteal cystic uterus



CHAPTER V

DISCUSSION

Well-furnished mucosal immune system in the uterus regulates the local immunity through the release of several molecules which influence the functions of underlying resident immune cells and thereby provide immunological protection against foreign particles. PMNs appears transiently in the uterine lumen during the follicular phase of the estrus cycle. PMNs availability in the uterine lumen is treated as pathological (Studer E et al, 1978). lymphocyte especially T- lymphocytes , B- lymphocytes and macrophage are present in the sub epithelial stroma of bovine endometrium (Leung ST et al., 2000)

The relation between luteal cystic ovary and immune cells presented in Figure 4. Immune cells are significantly related to the luteal cystic condition in uterus samples were collected and tested, It was found that immune cell such as lymphocytes and neutrophils are presence in luteal cystic condition whereas normal non cystic control animals were negative.

Previous study showed that as a central component of innate immunity, macrophages are known to perform several major functions during infection and inflammation, they engulf and breakdown pathogens during infection, and present antigens on their surface to helper T- cells *Biol report 2021,Mar; 104 (3):527-538*. An immunohistochemical study in the bovine ovary, using visually stages corpora lutea, described populations of immune cells within the corpus luteum. Spanel-Borowski *et al.* (1997). A significant increase in the total number of leucocytes was observed between the development and regression stages of the corpus luteum. However in contrast to our results, Spanel-Borowski failed to detect T-lymphocytes within luteal tissue.

The relation between luteal cystic condition uterus and presence of bacteria are presented in (table 3). Presence of bacteria significantly related to the clinical symptoms of dairy cow. Results indicate that *E.coli*, *Staphylococcus aureus* harmful bacteria present in the uterus and normal cyclic animals contain *klebsiella pneumonia in the uterus* .

Previous study showed uterine bacteria species are associate with greater endometrial inflammation and more severe clinical uterine disease. We have recently shown that other potential uterine pathogens or opportunistic contaminant bacteria in the uterine lumen do not have this same relationship. *Am J vet res.1994;45:22512255*. Uterine bacterial infections during the luteal cyst are associate with lower conception rates, increased intervals from

calving to first service or conception and more animals culled for failure to conceive many specific and non-specific pathogens of the genital tract affect cow infertility. To protect against these pathogens, mammals rely on the roles of the cervix and its secretions (Blandau and Moghissi, 1973)

Antimicrobial susceptibility testing methods that consistently provide reproducible and repeatable results. Disk diffusion testing is one of several phenotypic assays which can be utilized to determine the antimicrobial resistance profile (anti-biogramme) of an organism. Disk diffusion tests estimate in vitro susceptibility. The result showed that almost all the antibiotics were susceptible

From these studies, there is now an unprecedented opportunity to carefully and systematically understand the coordinated events that dictate luteal structure and function. Potential areas of research that would benefit from recent high-throughput omics approaches include defining the underlying initiators and effectors of luteal regression. Due to the dynamic and ephemeral nature of the corpus luteum, there are also opportunities to understand basic biological processes relevant to various pathologies and diseases.



CHAPTER 6

CONCLUSION

CHAPTER VI

CONCLUSION

From the present study it was concluded that the impact of luteal cyst in local immune regulation in uterus in dairy cow increased the progesterone level, affect clinical symptoms- anestrus, repeat breeding, conception failure and ovary health, cystic follicles. Histopathological evaluation indicates luteal cyst formation with accumulation of eosinophilic fluid and surrounded by one or two layer of fat cells, corpus hemorrhagicum surrounded by fibrous tissue. Bacterial cell culture identified some harmful pathogens such as *E. coli*, *Streptococcus aureus* and there was no antimicrobial resistance found. These results of the present study suggest that there is a impact on immune regulation in uterus that effect on reproductive performance in dairy cow.

Further study is recommended to immune related gene expression of epithelial cell determine the accurate therapy to overcome the effect of immune regulation on reproductive performance in dairy cows.



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