



UNIVERSITI PUTRA MALAYSIA

**RELATIONSHIP OF MILKING PRACTICES AND MILK MICROBIAL
DIVERSITY WITH PREVALENCE OF SUBCLINICAL MASTITIS IN
LACTATING DAIRY BUFFALOES IN SELANGOR, MALAYSIA**

RUSYAIDI SHAH BIN RAMDAN

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DIVERSITY WITH PREVALENCE OF SUBCLINICAL MASTITIS IN
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RUSYAIID SHAH BIN RAMDAN

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Faculty of Veterinary Medicine, Universiti Putra Malaysia

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DEGREE OF DOCTOR OF VETERINARY MEDICINE

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CERTIFICATION

It is hereby certified that we have read this project paper entitled “Relationship of Milking Practices and Milk Microbial Diversity With Prevalence Of Subclinical Mastitis In Lactating Dairy Buffaloes In Selangor, Malaysia”, Rusyaidi Shah Bin Ramdan and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 - Project.

ASSOC. PROF. DR. ROZAIHAN MANSOR

DVM (UPM), PhD (University of Glasgow)

Associate Professor

Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor
(Supervisor)

PROFESOR DR. MD. ZUKI ABU BAKAR

DVM (UPM), PhD (University of Glasgow)

Professor

Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor
(Co-Supervisor)

DR. SHARINA OMAR

DVM (UPM), MVS (Massey University), PhD (University of Leicester)

Senior Lecturer

Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor

(Co-Supervisor)

DR. MOHAMMED BABATUNDE SADIQ

MVSc (UPM), PhD (UPM)

Senior Lecturer

Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor

(Co-Supervisor)

DEDICATION

This project paper is dedicated to:

The One Almighty God, Allah S.W.T., who had made all things possible.

My father and mother's persistent love, encouragement, and genuine prayers have been the guiding light that has enabled me to traverse this difficult road.

All my dedicated lecturers, who have wholeheartedly committed themselves to the noble cause of education, deserve admiration and appreciation.

To my best friends, who have been the foundation of my existence with their constant support, limitless inspiration, and boundless pleasure."

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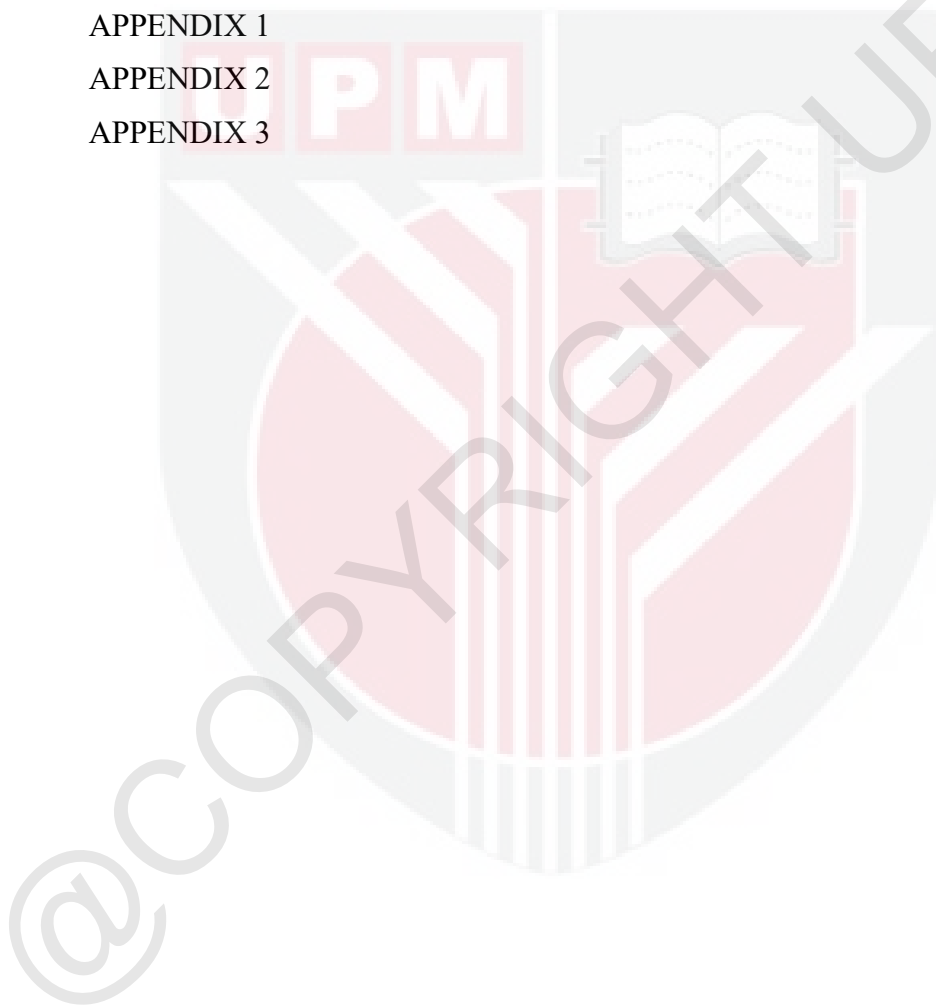
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LIST OF ABBREVIATIONS

California Mastitis Test	CMT
Somatic Cell Count	SCC
Subclinical Mastitis	SM
Clinical Mastitis	CM
Coagulase negative staphylococci	CoNS
<i>Escherichia coli</i>	<i>E. Coli</i>
Universiti Putra Malaysia	UPM
et al. (abbr. Latin)	<i>et al</i>
Milliliter	ml
Percentage	%
White Blood Cell	WBC
Deoxyribonucleic acid	DNA

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ABSTRAK

HUBUNGAN ANTARA AMALAN PEMERAHAN SUSU DAN KEPELBAGAIAN MIKROB SUSU DENGAN PREVALENS MASTITIS SUBKLINIKAL DALAM KERBAU TENUSU DI SELANGOR, MALAYSIA

Oleh

Rusyaidi Shah bin Ramdan

2023

Penyelia: Prof. Madya Dr. Rozaihan Mansor

Penyelia Bersama: Prof. Dr Md Zuki Bin Abu Bakar, Dr. Sharina Omar,

Dr. Mohammed Babatunde Sadiq

Mastitis adalah salah satu penyakit yang paling serius yang menjejaskan pengeluaran susu di seluruh dunia. Walaupun dalam spesies kerbau, mastitis tetap menjadi penyakit paling mahal dalam industri tenusu, walaupun kepercayaan tradisional bahawa kerbau kurang terdedah kepada mastitis daripada lembu. Mastitis subklinikal dibezakan oleh peningkatan dalam kiraan sel somatik dan bakteria dalam susu, tetapi tiada keabnormalan fizikal pada ambing atau dalam susu. Kajian ini bertujuan untuk menilai hubungan amalan pemerahan susu dan profil bakteria dengan prevalens mastitis

subklinikal kerbau tenusu di Selangor, Malaysia. Kajian ini dijalankan di enam ladang kerbau separa intensif di Selangor, dengan 46 kerbau menyusu dikenal pasti. Ujian Mastitis California (CMT) digunakan untuk mengenal pasti kerbau dengan mastitis subklinikal. Lapan belas daripada 46 kerbau menyusu, bersamaan dengan (39.13%), didapati mempunyai mastitis subklinikal. Semua sampel positif telah menjalani pengasingan dan pengenalpastian bakteria, menghasilkan sejumlah 69 mikroorganisma, dengan 19 jenis bakteria dikenal pasti menggunakan ujian biokimia. Bakteria yang paling banyak ditemui ialah stafilokokus koagulase-negatif (CoNS) (27.54%), diikuti oleh *Staphylococcus intermedius* (17.39%), *Streptococcus agalactiae* (10.14%), *Staphylococcus aureus* (8.70%), *Corynebacterium diptheriae* (7.25%), dan *Corynebacterium spp.* (4.35%). Bakteria lain yang dikesan, seperti *Streptococcus dysgalactiae*, *Klebsiella spp.*, *Acinetobacter iwoffii*, dan *Enterococcus spp.*, dikenal pasti kurang daripada dua peratus. Di antara keempat-empat kriteria amalan pemerah susu yang baik yang diperhatikan di semua ladang, teknik pemerah susu menunjukkan hubungan signifikan yang kuat dengan jumlah bakteria yang terdapat didalam susu kerbau subklinikal mastitis dan prevalens mastitis subklinikal di setiap ladang. Sebagai penutup, CoNS adalah bakteria yang paling biasa ditemui yang menyebabkan mastitis subklinikal dan kebanyakan kerbau tenusu menyusu mempunyai kelaziman mastitis subklinikal yang tinggi. Hubungan signifikan yang diperhatikan antara teknik pemerah susu dengan kelaziman mastitis subklinikal dan bilangan bakteria yang diasingkan dalam susu menekankan kepentingan kritikal menggunakan amalan pemerah susu yang betul untuk melindungi kesihatan ambing kerbau dan mengekalkan kualiti susu yang tinggi.

Kata kunci: kerbau, mastitis subklinikal, bakteria, Ujian mastitis California, stafilokokus koagulase-negatif (CoNS), amalan memerah susu



ABSTRACT

RELATIONSHIP OF MILKING PRACTICES AND MILK MICROBIAL DIVERSITY WITH PREVALENCE OF SUBCLINICAL MASTITIS IN LACTATING DAIRY BUFFALOES IN SELANGOR, MALAYSIA

By

Rusyaidi Shah bin Ramdan

2023

Supervisor: Assoc. Prof. Dr. Rozaihan Mansor

Co-Supervisor: Prof. Dr Md Zuki Bin Abu Bakar, Dr. Sharina Omar,

Dr. Mohammed Babatunde Sadiq

Mastitis is one of the most serious diseases affecting dairy producers globally. Even for buffalo species, mastitis remains the dairy industry's most expensive disease, despite the traditional belief that buffaloes are less susceptible to mastitis than cattle. Subclinical mastitis is distinguished by an increase in somatic cell count and presence of bacteria in milk, but no physical abnormalities on the udder or in the milk. The purpose of this study is to assess the relationship of milking practices and bacterial profiles with the prevalence of subclinical mastitis of dairy buffaloes in Selangor,

Malaysia. This study was conducted at six semi-intensive buffalo farms in Selangor, with 46 lactating buffaloes identified. The California Mastitis Test (CMT) was used to identify buffaloes with subclinical mastitis. Eighteen out of 46 lactating buffaloes, equivalent to (39.13%), were found to have subclinical mastitis. All positive samples were subjected to bacterial isolation and identification, yielding a total of 69 microorganisms, with 19 types of bacteria identified using a biochemical test. The most common bacteria discovered were coagulase negative staphylococci (CoNS) (27.54%), followed by *Staphylococcus intermedius* (17.39%), *Streptococcus agalactiae* (10.14%), *Staphylococcus aureus* (8.70%), *Corynebacterium diphtheriae* (7.25%), and *Corynebacterium spp.* (4.35%). Other bacteria detected, such as *Streptococcus dysgalactiae*, *Klebsiella spp.*, *Acinetobacter iwoffii*, and *Enterococcus spp.*, were determined to be less than two percent. Among all four criteria of the good milking practices observed in all farms, milking technique showed a strong significant relationship with the number of pathogenic bacteria isolated in subclinical mastitis and prevalence of subclinical mastitis on each farm. Conclusively, CoNS is the most common pathogens causing subclinical mastitis and most lactating dairy buffaloes had high prevalence of subclinical mastitis. The significant relationship observed between milking technique with prevalence of subclinical mastitis and number of bacteria isolated in the milk highlights the critical importance of employing proper milking practices to safeguard buffalo udder health and maintain high milk quality.

Keywords: buffaloes, subclinical mastitis, bacteria, California Mastitis Test, coagulase negative staphylococci (CoNS), milking practice

1.0 INTRODUCTION

A frequent and costly illness that affects dairy animals worldwide is mastitis (Seegers et al., 2003). The most essential activity in a dairy farm is milk production, and mastitis is the most notable disease. It is an infection of the mammary gland that may also be caused by improper milking techniques, environmental factors, and bacterial infections (Cheng & Han, 2020). Mastitis can directly affect the quality of milk by altering both its chemical and physical characteristics. The most noticeable alterations in milk include an increase in the number of somatic cells, the presence of clumps, change in colour of the milk (Izquierdo et al., 2017).

Mastitis can be categorised into sub-clinical, clinical mastitis and chronic mastitis. Subclinical mastitis (SM) is the most frequent type of mastitis, a non-symptomatic form of intramammary inflammation that usually affects 20-50 percent of cows in a herd (Forsbäck et al., 2009). Clinical mastitis (CM) is distinguished by the presence of increased volume, colour (redness), and discomfort in the mammary gland, which is characterised by a rise in temperature and is sometimes followed by symptoms such as loss of appetite, fever, and deterioration (Izquierdo et al., 2017). Other than that, the presence of flakes and clots in milk may be seen with the naked eye. Subclinical mastitis is a more serious of the two since it is asymptomatic, which means that low-quality milk may enter the bulk tank without the farmer's awareness and lead to reduced bulk-tank milk quality (Barbano et al., 2007). Chronic mastitis in cows is defined by a persistent infection of the udder, and the major symptoms of chronic mastitis include inflammation of the udder, which turns into a red, hot, and hard mass.

Bacteria is the most common and significant cause of mastitis in dairy cows. The bacteria that induced mastitis in cows were classified notably as contagious, environmental, and opportunistic (Hawari and Hassawi, 2008). Contagious microorganisms' dwell on the udder and are spread during milking from infected to uninfected teats. *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Mycoplasma bovis* are the most common. Environmental pathogens, on the other hand, are frequently connected with diseases that originate in the cow's surroundings, such as the bedding, flooring, pasture, and the milking equipment. Coliforms, *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Arcanobacterium pyogenes* are the most common environmental pathogens. Opportunistic infections which are the Coagulase-negative staphylococci have extremely strong adhesion qualities, allowing them to infiltrate the gland's inner lining and colonize the teat skin lining (Ashraf & Imran, 2020).

Mastitis is typically transmitted from cow to cow during milking if the milking practice standards are not met. Good milking practices are critical for preserving mammary gland health and avoiding mastitis in dairy cows. It is generally established that milking processes and practices used by milkers have a significant impact on the occurrence of mastitis in herds as well as the entire milk quality (Schukken et al., 2003). Proper milking procedures, in conjunction with a well-designed and maintained milking equipment, and environmental conditions that allow cows to remain clean, dry, and comfortable can help to reduce the spread of infectious mastitis-causing bacteria and environmental infections (Bushnell, 2017). Proper pre-milking, milking,

and post-milking techniques, such as examining foremilk for symptoms of mastitis, stimulating the teats, and periodically sanitising hands and gloves, are also suggested.

The incidence of subclinical mastitis in dairy buffaloes can be influenced by various factors, including milking practices and milk microbial diversity. However, there is limited information on the relationship between milking practices and milk microbial diversity with the incidence of subclinical mastitis in lactating dairy buffaloes. This study aims to contribute to the understanding of the factors influencing the incidence of subclinical mastitis in dairy buffaloes and provide insights into the development of effective prevention and control strategies.

2.0 LITERATURE REVIEW

2.1 Subclinical Mastitis in Dairy Buffaloes

Subclinical mastitis (SCM) is a serious disease that causes production losses, poor milk quality, and high somatic cell count (SCC) in dairy cows (Gruet et al., 2001). This illness is crucial for consumers and processors as it can cause farmers to suffer enormous financial losses (Bilal et al., 2007). Subclinical mastitis is a non-symptomatic form of intramammary inflammation that affects 20-50 percent of cows in a herd and is the most common kind of mastitis (Forsbäck et al., 2009). Even though there are no apparent indications of infection, subclinical mastitis can impair milk output and quality. As a result, subclinical mastitis is far more difficult to diagnose than clinical mastitis since the teat may seem normal. One of the acceptable diagnostic procedures for detecting subclinical mastitis is the California Mastitis Test (CMT). The California mastitis test is an excellent diagnostic tool for early detection of SCM (Ahmed et al., 2018). A study reported a high prevalence of subclinical mastitis (56.76%) in Selangor (Iqbal, 2022). Other studies in India have found a greater prevalence (59-78 percent) (Bhanot et al., 2012; Charaya et al., 2015).

2.2 California Mastitis Test (CMT)

The California mastitis test (CMT) is one approach for detecting mastitis at low levels of abnormalities (subclinical). This test is simple and effective in detecting mastitis (Surjowardojo et al. 2008). This test provides qualitative measurement of SCC that normally applied as a screening test for subclinical mastitis. This is advantageous

since the amounts of DNA and white blood cells (WBCs) in milk are exactly proportional (Marini & Yeager, 2018). According to Sardarwanto (1998), CMT has a high sensitivity (over 90%) for identifying mastitis. The milk was mixed with CMT reagent, which caused a reaction by rupturing any cells in the milk with cell membranes, allowing the DNA in those cells to interface with the teat reagent and create gel forming like. The CMT score zero was deemed to be the best cut-off value for diagnosing mastitis and for selecting animals for subsequent microbiological analysis on their milk. Thus, milk quarter samples with a CMT score greater than zero which is trace, +1, +2 and +3; are likely to be infected.

2.3 Bacterial Isolation and Identification

Bacteria is the most common and significant cause of mastitis in dairy cows. The bacteria that induced mastitis in cows were classified notably as contagious, environmental, and opportunistic (Hawari and Hassawi, 2008). Thus, the most frequent diagnostic technique for detecting mastitis pathogens is bacterial isolation and identification of mastitis-causing organisms. Positive samples of subclinical mastitis were primarily cultured in the blood agar and incubated for 24 hours. According to Quinn et al. (2011), suspected colonies were recognised by their morphological characteristics and haemolytic activity, followed by Gram staining and motility tests before being put onto semisolid or slope agar for further identification. As needed, biochemical assays such as indole generation, methyl red test, urease production, citrate utilisation, triple sugar iron agar (TSI), and sorbitol were carried out. The haemolytic reaction on blood agar is valuable in determining the

Streptococcus agalactiae through colonies clearing. Catalase and coagulase test can distinguish between *Streptococcus* species and the Coagulase Negative *Staphylococcus* (CoNS). As needed, biochemical assays such as indole generation, methyl red test, urease production, citrate utilisation, triple sugar iron agar (TSI), and sorbitol were carried out.

2.4 Prevalence of Subclinical Mastitis in Buffaloes

In Malaysia, a study conducted by Othman et al, in 2003 on the prevalence of mastitis in dairy cows in Malaysia. This research was conducted on three farms in Serdang, Selangor, and Kluang, Johor. In these farms, their study found a significant frequency of subclinical mastitis (81.7%). Another research, done on selected dairy farms in Kelantan and Pahang by Saeed et al., (2022), revealed the incidence of subclinical bovine mastitis (31.4%).

There was a study by Krishnamoorthy et al., (2021) on systematic review and meta-analysis found that the global prevalence of subclinical and clinical mastitis in buffaloes was 42% and 15%, respectively. Different countries in the world reported different percentages of subclinical mastitis prevalence in dairy buffaloes. One study in northwest Pakistan conducted by Ali et al., (2021) reported that prevalence of subclinical mastitis was higher in buffaloes (66%) than cattle (53%). Another study in Egypt conducted by Ahmed et al., (2018) stated that from 174 dairy buffaloes, (44.3%) were reported positive for subclinical mastitis. Another study in Nueva Ecija,

Philippines conducted by Salvador et al., (2012) reported that prevalence of subclinical mastitis of water buffalo was (42.76%).

2.5 Pathogens Causing Subclinical Mastitis

The most prevalent and major cause of mastitis in dairy cows is bacteria. Many studies conducted to determine the bacteria that lead to subclinical mastitis in dairy buffaloes. For example, one study conducted by Preethirani et al., (2015) the most frequent staphylococci were discovered to be coagulase-negative staphylococci (64.8%) bacteria, followed by *Streptococci spp.* (18.1%), *Escherichia coli* (9.8%) and *Staphylococcus aureus* (7.3%). In another study conducted by Moroni et al., (2006), they found that the most common bacteria are coagulase-negative *Staphylococcus*, with *Streptococcus* accounting for 15% infecting the lactating buffaloes in northern districts at Italy. Another study conducted by Dhakal I.P. (2006) found that the most predominant bacteria in this investigation were coagulase-negative *Staphylococcus epidermidis*. in Nepalese Murrah buffaloes. Moreover, according to a study conducted by Vásquez et al., (2017), they reported that the microorganisms most often isolated were *Staphylococcus epidermidis* (17%), *Staphylococcus aureus* (15%), *Bacillus spp.* (14%), and *Acinetobacter spp.* (12.5%) among dairy buffaloes in (state country). Another study reported in Iraq conducted by Sadoon, (2021) showed that *Staphylococcus chromogens* (14.61%), *Staphylococcus xylosus* (12.78%), *Streptococcus agalactiae* (11.87%), *Streptococcus dysagalactiae* (11.41%), *Streptococcus uberis* (10.04%), and *Klebsiella pneumoniae* (9.58%), *Escherichia coli* (8.21%), *Corynebacterium bovis* (14.11%), *Escherichia coli* (10.58%), *Pseudomonas*

aeruginosa (7.05%), and *Pasteurella multocida* (7.05%) were among the most isolated bacteria causing subclinical mastitis cases in buffaloes.

2.6 Milking Practices in Dairy Buffaloes Farm

Many risk factors can lead to development of subclinical mastitis in dairy animals such as host factor, pathogen factor, environmental factor, and milking practice (Cheng & Han, 2020). According to Toledo, (2021), the first step in establishing and understanding a regular milking schedule is necessary for a successful milking management programme and minimizing mastitis infections. A study conducted in India by Sabapara & Kharadi, (2021) reported that improved milking procedures increase productivity, help to keep the udder and teat in good shape, and contribute to the production of clean milk. Hence good milking practice is essential in reducing the risk of mastitis in dairy farms and better udder health.

Mastitis cases increase because of poor milking practices applied in the farm. Good milking practices can start with proper animal identification as farms should have protocols in place to manage animals that require special handling during milking, such as sick or diseased animals, or animals that produce milk that is unfit for human consumption (FAO, 2011). Next, the animal should be restrained using methods that will not cause pain and stress. Proper restraint technique is important to prevent injury to the udder that can lead to mastitis. Cows that are stressed may disrupt regular milk let-down that later prolonged or diminished milk let-down increases machine-on time and may have an impact on teat health (NMC, 2013).

Milk handling employees (milkers) may directly introduce numerous organisms, including pathogens, to milk when they are careless, misinformed, or wilfully irresponsible (Nangamso, 2006). The hygiene of the milker is important in milking practice of dairy animals. Their hands must be clean and dried before they start milking the animal. A study conducted by Bekuma & Galmessa, (2018) mentioned that wet milking by the milkers should be avoided because germs from the milker's hands can enter the milking instrument, contaminating the milk and causing spoiling. One of the most critical hygiene activities is cleaning cow udders before milking (Yilma, 2010). The udders of milking cows may come into close touch with the ground, urine, faeces, and feed refusals. Cleaning and removing dirt particles, bedding material, and manure from the udder and flanks is required to prevent several forms of germs from entering the milk (Connor 1995).

Most of the farmers used calves suckling to promote milk letdown. According to a study in dairy buffaloes' farms in India by Sabapara & Kharadi, (2021) it was reported that 37 farmers out of 50 (74%) allowed the calves for suckling before milking. Another study by Gupta et al., (2008) reported that more than (91%) buffaloes farmers let the calves to suckle before starting to milk. According to Mbabazi (2005), most farmers do not wash their cows' udders before milking as they believe that letting the calf to suck before milking is enough to clean the teats. This can lead to mastitis as the calf's saliva and uncleaned teats increase the bacterial levels in the milk, causing spoiling (Bekuma & Galmessa, 2018). Hence cleaning of the udder and teat is important to prevent mastitis. One of the methods to remove germs from teat ends before milking is pre-dipping with a sanitising solution. Pre-dipping teats in an

effective disinfectant before milking lowers new intramammary infections caused by coliforms during lactation (Hogan & Smith, 2012). A study conducted by Pankey, (1989) pre-dipping has been found to lower the occurrence of clinical mastitis by 50% in herds with low levels of infectious mastitis. Use of antiseptics at post-milking is also important to pre-milking. Both play a major role in reducing the risk of new intramammary infections. According to Deb et al., (2013) mentioned that the use of antiseptics after milking at the teat opening minimises the likelihood of microbial infiltration and as an effective management approach for lowering the load of infectious pathogens. Despite its efficiency in avoiding mastitis, teat dip is neglected in most dairy buffalo management. According to a study in India by Sabapara & Kharadi, (2021) they reported that teat dipping after milking was not practiced in 50 selected buffaloes' farms.

The teats need to be dried thoroughly using individual cloth towels. Drying of the teats is one of the most critical parts in good milking practices. A single cloth or paper towel should be used to dry and remove any debris, dirt, and pre-dip solution residue from the teats using a downward twisting motion (Toledo, 2021). By creating a wet environment for bacteria to thrive and reproduce, washing the udder without drying can increase the number of germs near the teat end and can increase the risk of mastitis because germs might enter the teat canal during milking (Garcia, 2004).

Next, the equipment and the milking area need to be clean and well maintained. The challenge of eliminating milk residues and deposits from milk contact surfaces makes achieving low bacterial counts on milking equipment problematic. According to Food and Organization (FAO), bacteria proliferate on these surfaces between

milking, especially in warm, wet weather, resulting in significant quantities even when the equipment seems superficially clean. A comprehensive cleaning and disinfection programme is vital for ensuring sanitary conditions while minimising effort and expenses. Yeasts and *Prototheca zopfii* are common microorganisms found in the environment, as well as on milking equipment and cow teats and these microorganisms can easily penetrate the mammary glands, especially if teats are not properly cleansed after repeated intramammary treatments (Costa et al., 2011). Furthermore, a clean environment is vital for preventing mastitis induced by the environment (Toledo, 2021). Hence measures to decrease bacterial exposure can be used (Klaas & Zadoks, 2017). According to a study conducted by Barrett et al., (2005) reported that the prevalence of *Staphylococcus aureus* is greater in inadequate milking parlour hygiene rather than adequate milking parlour hygiene. According to Ricchi et al., (2013) reported an outbreak of bovine mastitis as the milking parlour appears to be a significant site in the control of *Prototheca* outbreaks, emphasising the necessity of appropriate sanitary conditions.

3.0 MATERIALS AND METHODS

3.1 Dairy Buffalo Farms Selection and Management

Six dairy buffalo farms in Selangor, Malaysia, were chosen to participate based on the Department of Veterinary Services Malaysia database. These dairy buffalo farms were managed on a semi-intensive basis. Three of the six farms employ milking machines, while the others practiced hand milking.

3.2 Animal Selection

Ten percent (10%) from lactating buffaloes from each farm with no systemic clinical signs such as fever, inappetence, lethargy, reduced milk production or abnormalities of the mammary glands or milk will be tested for subclinical mastitis using the California Mastitis Test (CMT). Most of the buffaloes are *Bubalus bubalis* in various phases of lactation. Milk from buffaloes with subclinical mastitis infection (CMT results of trace, +1, +2, and +3) were sampled aseptically for bacterial isolation and identification.

3.3 California Mastitis Test

Approximately two (2) mL of milk were sampled from each quarter onto a CMT paddle. To test for the existence of a gel or slime reaction, an equivalent volume of CMT reagent was added to the milk and carefully stirred. The CMT score was

determined, and positive CMT scores of traces, +1, +2, and +3 were recorded for subsequent analyses.

3.4 Aseptic Composite Milk Sampling

The teats were cleansed with 2% chlorhexidine gluconate and pre-dipped in germicidal teat dip. After that, the teats were cleaned with cotton wool soaked in 70% ethyl alcohol. After discarding the first two milk strips, 2 ml of milk was collected from each quarter and placed in a sterile cup. These milk samples were then taken to the Bacteriology Laboratory, Faculty of Veterinary Medicine, University Putra Malaysia for bacterial isolation and identification. To preserve the milk and minimise cross-contamination, the milk samples are kept in an ice box right after sampling.

3.5 Milking Practice Observation

Observation on the milking practices prior to milking based on four criteria which were animal preparation, milking technique, equipment, and milking area hygiene was done on each farm.

3.6 Bacteria Isolation and Identification

A loop of milk samples which declared positive for subclinical mastitis were primarily cultured on blood agar. The plates were then incubated for 24 hours in aerobic conditions at 37°C. If more than one growth was found on the plate, it was classified

as a mixed growth. If there was no growth, the plates were incubated for another 24 hours. To obtain the pure culture, the bacteria colonies were subcultured on blood agar. Based on colony appearance, haemolytic properties, gram staining, and biochemical features, pure bacterial cultures were found (catalase, coagulase, and oxidase test). Other biochemical tests were performed on gram negative bacteria include triple sugar iron (TSI), citrate, sulphur, indole, motility media (SIM), urea test, MacConkey agar growth, and haemolysis test. Further tests for streptococcus identification include haemolysis, 6.5% NaCl, bile esculin, lactose, sorbitol, trehalose, and Lancefield grouping. Further tests for Staphylococcus species identification include haemolysis, maltose, mannitol, arginine dihydrolase (ADH), and Voges-Proskauer (VP). The following tests were performed to further identify rod bacteria: haemolysis, SIM (motility only), nitrate, sucrose, and trehalose. The whole test reaction was observed and recorded.

3.7 Data Analysis

Data analysis was performed using bivariate correlations namely the correlation coefficient. This analysis was done to assess the strength and direction of the linear relationship between two variables.

4.0 RESULT

4.1 Farm Practices

Based on Table 1, six farms were examined, all farms practiced semi-intensive (100.0%). Most farms reared river buffaloes (83.3%), with one farm having a mixed breed (16.7%). All six farms maintain their buffaloes in groups (100%). The feeding system was also consistent, with no farms using feedlot or grazing systems, and all six farms using a mixed feeding system (100.0%). Regarding milking practices, multiple milkers are utilised in all farms (100.0%), and there was an even distribution between full hand milking (50.0%) and machine milking (50.0%) methods. Milking was commonly conducted at the same location as buffalo housing (83.3%), while one farm practiced separate and dry milking area (16.7%). Milking was performed twice daily (100%), with all farms enabling calves to suckle for milk letdown (100%). Additionally, three farms utilised oxytocin injections to assist with milk letdown (50.0%). Three farms providing pre-milking feed (50.0%) and three farms did not offer feed before milking (50.0%).

Table 1: Descriptive statistic of management from six dairy buffalo farms, Selangor, Malaysia

General management (N=6)	n	(%)
Rearing system		
• Extensive system	0	0.0
• Intensive system	0	0.0
• Semi-intensive system	6	100.0
Types of buffaloes		
• River buffaloes	5	83.3

• Swamp buffaloes	0	0.0
• Mixed	1	16.7
<hr/>		
Buffaloes' placement		
• Individually	0	0.0
• In group	6	100.0
<hr/>		
Type of feeding system		
• Feedlot	0	0.0
• Grazing	0	0.0
• Mixed	6	100.0
<hr/>		
Number of milkers		
• Single	0	0.0
• Multiple	6	100.0
<hr/>		
Method of milking		
• Full hand	3	50.0
• Knuckling	0	0.0
• Stripping	0	0.0
• Machine	3	50.0
<hr/>		
Place of milking		
• Milking at the same place	5	83.3
• Separate and dry place	1	16.7
<hr/>		
Frequency of milking		
• One time	0	0.0
• Two times	6	100.0
• Three times	0	0.0
<hr/>		
Practice for milk letdown		
• Allow calf to suckle	6	100.0
• Feeding concentrates and massaging the udder	0	0.0
• Oxytocin injection	3	50.0
<hr/>		
Is there any feed offered before milking		
• Yes	3	50.0
• No	3	50.0
<hr/>		

4.2 Milking Practices

Based on Table 2, milking practices were observed in six farms. Only one of the six farms (16.7 %) identify animals individually. Udder and teat examinations were performed on five out of six farms (83.3%) to detect signs of mastitis. None of the farms (0.0%) observed abnormalities in foremilk. All six farms had their animals gently restrained during milking (100%). Proper restraint is critical for both the animal's and the milker's safety. The farms were evenly divided between those that use milking machines and those that use manual milking methods (50%) each. Five out of six farms observed that the milkers had clean and dry hands (83.3%) prior to milking. None of the observed farm practiced clean and dry teat before milking (0.0%). Five out of six farms handled teats gently (83.3%), which is reduce any unnecessary pressure, sudden movements, or excessive force used to the teats during milking. Next, all the farms did not practice disinfecting the teats after milking. Half of the observed farms had their milkers followed basic hygiene practices (50.0%). Next, only two out of six farms properly installed and maintained milking equipment (33.3%). Lastly, four out of six farms kept the milking area clean and tidy (66.7%).

Table 2: Descriptive statistic of milking practices of six dairy buffalo farms, Selangor, Malaysia

Milking practices (N=6)	n	(%)
Individual animals are identifiable		
• Yes	1	16.7
• No	5	83.3
Udder and teat inspections to identify abnormalities		
• Yes	5	83.3
• No	1	16.7

Foremilk is extracted and assessed for abnormalities.		
• Yes	0	0.0
• No	6	100.0
Restrain the animal gently for milking.		
• Yes	6	100.0
• No	0	0.0
Milking method		
• Machine	3	50.0
• Manual	3	50.0
Milker's hands must be clean and dry.		
• Yes	5	83.3
• No	1	16.7
Clean and dry teats before milking		
• Yes	0	0.0
• No	6	100.0
Handle teats gently		
• Yes	5	83.3
• No	1	16.7
Disinfect teats after milking		
• Yes	0	0.0
• No	6	100.0
Milkers follow basic hygiene practices		
• Yes	3	50.0
• No	3	50.0
Milking equipment installed and maintained properly		
• Yes	2	33.3
• No	4	66.7
Milking area kept clean and tidy		
• Yes	4	66.7
• No	2	33.3

4.3 Prevalence of subclinical mastitis

The prevalence of subclinical mastitis in buffaloes from six (6) farms in Selangor, Malaysia, is shown in Table 3.

Table 3: Prevalence of Subclinical Mastitis in Buffaloes

Farm	Total number of lactating buffaloes	Sample taken (10% from the lactating buffaloes)	Animal with positive SCM	Prevalence percentage%
FARM A	50	5	3	100.00
FARM B	30	3	2	66.67
FARM C	200	20	6	30.00
FARM D	10	1	0	0.00
FARM E	110	11	5	45.45
FARM F	60	6	2	33.33
Total	460	46	18	39.13

Twenty-five (25) of the 46 buffaloes tested positive for CMT, equating to 39.13%. FARM A had the highest incidence (100.00%), followed by FARM B (66.67%), FARM E (45.45%), FARM F (33.33%) and FARM C (30.00%). FARM D however had the lowest frequency of SCM (0.00%).

4.4 Bacterial profile

A total of 25 positive CMT milk samples were sent to the Bacteriology Laboratory, Faculty of Veterinary Medicine, UPM for bacterial isolation and

identification. All positive samples grew bacteria on agar, and Table 4 displays the isolated bacteria from each farm.

Table 4: Various Bacteria Genera Isolated from Milk Sample of Each Farm

Name of Bacteria	Farms					
	FARM A	FARM B	FARM C	FARM D	FARM E	FARM F
<i>Coagulase negative staphylococci (CoNS)</i>	5	2	7	0	4	1
<i>Staphylococcus intermedius</i>	5	2	1	0	2	2
<i>Staphylococcus aureus</i>	2	0	1	0	2	1
<i>Streptococcus agalactiae</i>	0	1	5	0	1	0
<i>Streptococcus dysgalactiae</i>	0	0	0	0	0	1
<i>Streptococcus equi</i>	0	1	1	0	0	0
<i>Streptococcus viridans</i>	0	0	1	0	1	0
<i>Streptococcus spp.</i>	0	0	1	0	0	0
<i>Enterococcus spp.</i>	0	0	0	0	1	0
<i>Corynebacterium diphtheriae</i>	1	0	2	0	2	0
<i>Corynebacterium bovis</i>	0	0	1	0	0	0
<i>Corynebacterium spp.</i>	0	0	1	0	2	0
<i>Moraxella bovis</i>	0	0	1	0	0	1
<i>Klebsiella spp.</i>	1	0	0	0	0	0
<i>Klebsiella pneumoniae</i>	1	1	0	0	0	0
<i>Chryseobacterium</i>	0	0	0	0	0	1
<i>Acinetobacter iwoffii</i>	0	0	1	0	0	0
<i>Pasteurella hemolytica</i>	0	0	1	0	0	0
<i>Sphingo.</i>	0	0	0	0	1	0
Total	15	7	24	0	16	7

Table 5 indicates the proportion of bacteria detected following the biochemical test. The percentage is derived by dividing the number of the specified bacterium by the overall number of bacteria isolated (n=73).

Table 5: Percentage of The Bacteria Identified

No.	Name of Bacteria	Total isolation	Percentage (%)
1	<i>Coagulase negative staphylococci (CoNS)</i>	19	27.54
2	<i>Staphylococcus intermedius</i>	12	17.39
3	<i>Staphylococcus aureus</i>	6	8.70
4	<i>Streptococcus agalactiae</i>	7	10.14
5	<i>Streptococcus dysgalactiae</i>	1	1.45
6	<i>Streptococcus equi</i>	2	2.90
7	<i>Streptococcus viridans</i>	2	2.90
8	<i>Streptococcus spp.</i>	1	1.45
9	<i>Enterococcus spp.</i>	1	1.45
10	<i>Corynebacterium diphtheriae</i>	5	7.25
11	<i>Corynebacterium bovis</i>	1	1.45
12	<i>Corynebacterium spp.</i>	3	4.35
13	<i>Moraxella bovis</i>	2	2.90
14	<i>Klebsiella spp.</i>	1	1.45
15	<i>Klebsiella pneumoniae</i>	2	2.90
16	<i>Chryseobacterium</i>	1	1.45
17	<i>Acinetobacter iwoffii</i>	1	1.45
18	<i>Pasteurella hemolytica</i>	1	1.45
19	<i>Sphingo.</i>	1	1.45
Total:		69	100.00

Table 4 and Table 5, respectively, show a total of 21 types of bacteria found in positive SCM buffaloes farms in Selangor. Coagulase negative staphylococci (CoNS) were the most prevalent microorganisms found in the data, accounting for (26.03%) of all isolates. The next most common bacteria found were *Staphylococcus intermedius* (16.4%), *Staphylococcus aureus* (8.2%), *Streptococcus agalactiae* (9.5%), and *Corynebacterium diphtheriae* (6.8%). These five bacteria account for

more than three-quarters (77.13%) of all isolates. The other microorganisms found in the data are in lesser numbers, accounting for fewer than (5%) of all isolates. These include *Streptococcus dysgalactiae*, *Streptococcus equi*, *Streptococcus viridans*, *Streptococcus spp.*, *Enterococcus spp.*, *Corynebacterium bovis*, *Corynebacterium spp.*, *Moraxella bovis*, *Klebsiella spp.*, *Klebsiella pneumoniae*, *Chryseobacterium*, *Acinetobacter iwoffii*, *Pasteurella hemolytica*, *Sphingo.*, *Trichosporon asahii* and *Candida rugosa*. Overall, the data shows that CoNS, *Staphylococcus intermedius*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Corynebacterium diphtheriae* are the most prevalent microbes found in the samples.

4.5 Statistical analysis

Bivariate correlation test was carried out to determine any significant relationship between variables (milking practices) and number of bacteria found in subclinical mastitis buffaloes. Each category of milking practices in each farm were summed up to determine the relationship with the number of bacteria found in subclinical mastitis buffalo. Based on the results shown in Table 6, there was strong correlation and significant between pathogenic bacteria in the subclinical mastitis buffaloes' milk and milking technique ($r=-0.859$, $p=0.028$) on the sample taken at six farms. While there was no significant correlation between pathogenic bacteria in the subclinical mastitis buffaloes on the sample taken at six farms with other milking practices.

Table 6: Relationship between milking practices applied on six farms and population of pathogenic bacteria.

Variables	Bivariate model	
	Pearson Correlation	<i>P</i> -value
Animal Preparation	-0.558	0.25
Milking Technique	-0.859	0.028
Milking Equipment	0.229	0.662
Housing and Milking Area	0.006	0.991

Bivariate correlation test was carried out to determine if there is any significant relationship between variables (milking practices) and prevalence of subclinical mastitis. Based on the results shown in Table 7, there was strong correlation and significant between prevalence of subclinical mastitis and milking technique ($r=-0.859$, $p=0.028$) applied in the farms. While there was no significant correlation between prevalence of subclinical mastitis with other milking practices.

Table 7: Relationship between milking practices applied on six farms and prevalence of subclinical mastitis.

Variables	Bivariate model	
	Pearson Correlation	<i>P</i> -value
Animal Preparation	-0.487	0.327
Milking Technique	-0.835	0.039
Milking Equipment	-0.644	0.167
Housing and Milking Area	0.102	0.847

5.0 DISCUSSION

Mastitis is a major economic concern for milk producers since it has a detrimental influence on various areas of cow and herd performance. This disease imposes significant financial burdens on farmers, both in terms of direct and indirect costs. Direct costs include veterinary treatments and the disposal of wasted milk, whereas indirect losses include increase in risks of culling and mortality. Subclinical mastitis is significantly more difficult to identify than clinical mastitis as the udder may appear normal. Hence California Mastitis Test (CMT) is one of the diagnostic tests that can be applied to diagnose subclinical mastitis.

The study was done on six dairy buffalo farms located in Selangor, Malaysia, and the findings showed the calculated prevalence across these six farms. Based on Table 3, the results showed prevalence of subclinical mastitis (39.13%) from these six farms. This shows that a significant proportion of the lactating cow herd, about more than (50%), had subclinical mastitis. Subclinical mastitis in buffaloes can reduce milk quality by increasing bacterial content, resulting in a decrease in both quantity and quality of milk. Based on the previous study in Malaysia by Othman et al. (2003), they reported that high prevalence in dairy farms at Selangor and Johor which is (81.7%). Meanwhile a study by Saeed et al., (2022), revealed the incidence of subclinical bovine mastitis (31.4%). According to Krishnamoorthy et al., (2021), a comprehensive review and meta-analysis of the global prevalence of subclinical and clinical mastitis in buffaloes was found to be 42% and 15%, respectively. Various nations have reported various percentages of subclinical mastitis prevalence in dairy buffaloes. For example,

Ali et al. (2021) discovered a greater frequency of subclinical mastitis in buffaloes (66%) than in cattle in research done in northwest Pakistan (53%). Similarly, Ahmed et al. (2018) discovered that 44.3% of 174 dairy buffaloes tested positive for subclinical mastitis in Egypt. Furthermore, Salvador et al. (2012) discovered 42.76% frequency of subclinical mastitis in water buffalo in Nueva Ecija, Philippines. According to this report, the prevalence of subclinical mastitis in dairy buffaloes varies significantly among different countries and most of the research reported that the prevalence surpasses (40%).

Bacterial infection is the most common and leading cause of mastitis in dairy cows. According to Galiero (2002) mentioned that mastitis in buffalo is usually often caused by bacterial infections. Numerous investigations have been conducted to identify common bacteria that cause subclinical mastitis in dairy buffaloes. Based on this study, Table 5 showed that the highest percentage of bacteria identified which caused subclinical mastitis in Selangor, Malaysia was the coagulase-negative staphylococci (CoNS) (27.54%), followed by *Staphylococcus intermedius* (17.39%), *Streptococcus agalactiae* (10.14%), *Staphylococcus aureus* (8.70%), *Corynebacterium diptheriae* (7.25%), and *Corynebacterium spp.* (4.35%). Other bacteria detected, such as *Streptococcus dysgalactiae*, *Klebsiella spp.*, *Acinetobacter iwoffii*, and *Enterococcus spp.*, were determined to be less than two percent.

Several studies in other countries have been conducted to determine the prevalence of various bacteria in dairy buffaloes. For example, coagulase-negative staphylococci were the most prevalent (64.8%) in study done by Preethirani et al. (2015), followed by Streptococci spp. (18.1%), *Escherichia coli* (9.8%), and *Staphylococcus aureus* (7.3%). Similarly, a study by Moroni et al., (2006) discovered that coagulase-negative Staphylococcus was the most common pathogen in dairy buffalo from northern areas, with Streptococcus accounting for 15%. Another study by Dhakal I.P. (2006) examined normal somatic cell count (SCC) and subclinical mastitis in Nepalese Murrah buffaloes with coagulase-negative *Staphylococcus epidermidis* was found to be the most common bacterium. In another study by Vásquez et al. (2017) reported that the most common bacteria identify was *Staphylococcus epidermidis* (17%), *Staphylococcus aureus* (15%), *Bacillus spp.* (14%), and *Acinetobacter spp.* (12.5%).

Based on the studies across different places, it indicated that different studies isolated different types of bacteria that causing subclinical mastitis in dairy buffalo. Based on the comparison of this study and other studies reported that the highest prevalence of bacteria causing subclinical mastitis in buffaloes is (CoNS). (CoNS) is bacterial pathogens that normally inhabit teat skin can become opportunistic and produce intramammary infections. These bacteria may enter the udder through the streak canal which subsequently establish infection in the udder tissue. This microbe may have originated in the animal's environment, such as flooring and bedding. Improper milking procedures, such as insufficient hygiene precautions for equipment

and the milker's hands, may therefore encourage disease transmission. Environmental mastitis is caused by opportunistic microorganisms that present in the cow's surroundings and can enter the udder through teat orifices if adequate milking practices are not employed (Suleiman et al., 2017).

Many risk factors can lead to the development of subclinical mastitis in dairy animals especially the milking practices applied in the farms. According to Toledo, (2021), the first step in establishing and understanding a regular milking schedule is necessary for a successful milking management programme and minimizing mastitis infections. This study involved the observation and categorisation of milking practices on six farms, which were classified into four distinct criteria of milking practice which were animal preparation, milking technique, equipment, and milking area hygiene. The observed correlation across six farms revealed a significant association between milking procedures and the incidence of subclinical mastitis in buffalo milk as evidenced by a P-value < 0.05 ($P = 0.028$) for the number of bacteria and ($P = 0.039$) for subclinical mastitis prevalence. The data highlighted the link between inadequate milking practices with higher bacterial count in subclinical mastitis buffalo milk, as well as an increased prevalence of subclinical mastitis. A study by Azevedo et al. (2016) supported these results as they found that herds with inadequate milking practices are more likely to have many bacteria detected. Furthermore, Miyama et al., (2020) observed that proper milking practices can minimise teat damage and the prevalence of subclinical mastitis in buffaloes in Uganda. Collectively, these studies highlight the critical importance of milking procedures, demonstrating their

considerable effect on bacterial count and the occurrence of subclinical mastitis in buffalo milk.

Mastitis prevention is critical for dairy producers to avoid economic losses, and numerous strategies may be implemented to reduce its occurrence on the farm. Emphasising proper milking practices is important in this effort. Despite studies, such as the one by Jingar et al., (2014), suggesting that buffaloes exhibit more resistance to mastitis compared to cows, the significance of maintaining excellent milking techniques remains crucial in preventing mastitis in dairy buffalo farms. This emphasises the necessity of proactive measures and continuous development to protect the health of dairy animals and preserve the farm's economic viability. Good milking practices are critical for minimising mastitis in buffaloes. This includes proper animal preparation, using effective milking technique, maintaining equipment hygiene, and keeping the milking environment clean. By following these guidelines, dairy producers may considerably contribute to mastitis prevention, thereby improving the overall health and productivity of their buffalo herds.

6.0 CONCLUSION

In conclusion, this study showed prevalence of subclinical mastitis (SCM) in dairy buffalo farms at Selangor Malaysia is (39.13%). The Coagulase-Negative Staphylococcus (CNS) were the most predominant agents causing SCM contributing (27.54%) bacteria found in the milk of subclinical mastitis across 6 farms. Based on

the observation, good milking techniques were not applied in most of the dairy buffalo farms and there was a relationship between milking practices with the number of bacteria isolated from buffalo and prevalence of SCM.

7.0 RECOMMENDATION

It is advised that the sample size and farms included be increased to strengthen the robustness and generalizability of our study on the link between milking practices and the prevalence of subclinical mastitis. This means completing more study in several states, addressing regional variances. Furthermore, extending the study's duration would allow for a more thorough evaluation of historical patterns and fluctuations. Furthermore, a concentrated attempt to enhance milking practices on dairy buffalo farms is recommended, as this might give useful insights into lowering the number of bacteria isolated and the overall prevalence of subclinical mastitis. These multiple proposals are intended to broaden the scope and depth of future research, contributing to a more nuanced understanding of the intricate dynamics between milking practices and mastitis outcomes in buffalo herds.

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9.0 APPENDICES

APPENDIX 1

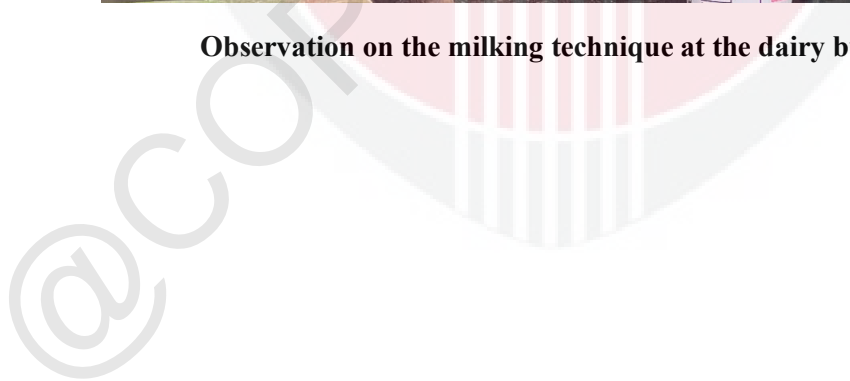


Mix of milk with CMT reagent after 30 seconds

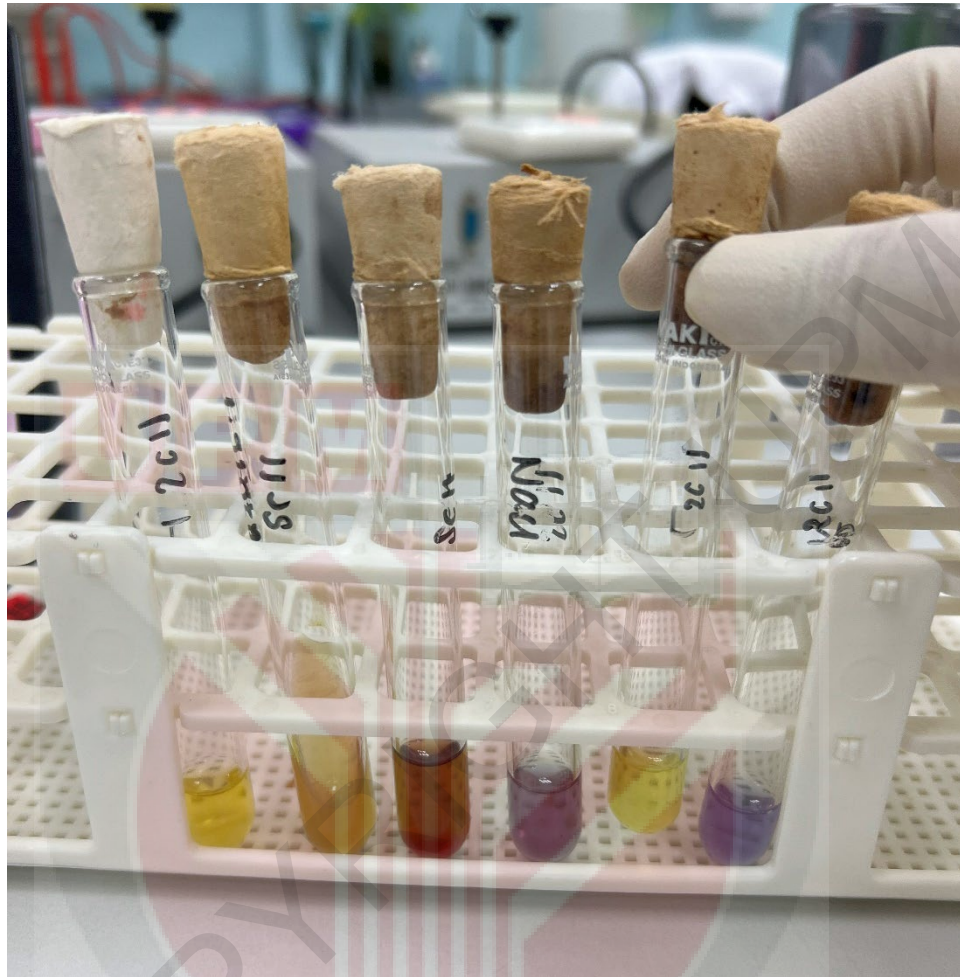
APPENDIX 2



Observation on the milking technique at the dairy buffalo's farm



APPENDIX 3



Biochemical tests for identifying Streptococcus species.

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