UNIVERSITI PUTRA MALAYSIA

THE PREVALENCE OF SUBCLINICAL MASTITIS IN DAIRY BUFFALOES IN SELANGOR, MALAYSIA

MUHAMMAD IQBAL FARIZ BIN ROSSLAN

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THE PREVALENCE OF SUBCLINICAL MASTITIS IN DAIRY BUFFALOES IN SELANGOR, MALAYSIA

MUHAMMAD IQBAL FARIZ BIN ROSSLAN

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "The Prevalence of Subclinical Mastitis and Its Pathogens in Dairy Buffaloes in Selangor, Malaysia", Muhammad Iqbal Fariz bin Rosslan and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 - Project.

ASSOC. PROF. DR. ROZAIHAN MANSOR
DVM (UPM), PhD (University of Glasgow)
Senior Lecturer,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
Serdang, Selangor
(Supervisor)

DR. SHARINA OMAR
DVM (UPM), MVS (Massey), PhD (Leicester)
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 my mother whose affection, love, encouragement and prays of day and night make me able to go through this challenging journey.

To my best friends who have become my pillar of strength, inspiration and source of happiness.

All my lecturers who have committed themselves towards the noble cause of education

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CONTENTS

LIST OF ABBREVIATIONS

California Mastitis Test	**CMT**
Somatic Cell Count	**SCC**
N-acetyl-β-D-glucosaminidase	**NAGase**
Subclinical Mastitis	**SM**
Clinical Mastitis	**CM**
Coagulase negative staphylococci	**CoNS**
Escherichia coli	***E. Coli***
Universiti Putra Malaysia	**UPM**
et al. (abbr. Latin)	***et al***
Milliliter	**ml**
Percentage	**%**

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ABSTRAK

PREVALENS MASTITIS SUBKLINIKAL DAN PATOGEN DALAM KERBAU DI SELANGOR, MALAYSIA

Oleh

Muhammad Iqbal Fariz bin Rosslan

2022

Penyelia: Prof. Madya Dr. Rozaihan Mansor

Penyelia Bersama: Dr. Sharina Omar

Mastitis adalah salah satu penyakit penting yang memberi kesan besar kepada penternak tenusu di seluruh dunia. Mastitis subklinikal dapat didefinisikan sebagai peningkatan bilangan sel somatik dan mikroorganisma dalam susu tanpa keabnormalan fizikal pada kelenjar mama dan dalam susu. Kajian ini bertujuan untuk menentukan prevalens mastitis subklinikal dan mengenal pasti patogen yang menyebabkan mastitis subklinikal pada kerbau di Selangor, Malaysia. Kajian ini dijalankan di enam ladang kerbau yang dilaksanakan separa intensif di Selangor dengan jumlah 74 ekor kerbau menyusu dikenal pasti. Kerbau dengan mastitis subklinikal telah didiagnosis menggunakan Ujian Mastitis California (California

Mastitis Test, CMT). Empat puluh dua daripada 74 kerbau menyusu, bersamaan dengan 56.76%, didapati menghidap mastitis subklinikal. Kesemua sampel positif tertakluk kepada pengasingan dan pengenalpastian bakteria dan sejumlah 163 bakteria telah diasingkan dengan 35 jenis bakteria dikenal pasti menggunakan Indeks Profil Analitik (Analytical Profile Index, API). stafilokokus koagulase-negatif (CoNS) (39.88%) adalah bakteria paling lazim yang dikenal pasti diikuti oleh *Streptococcus agalactiae* (9.82%), *Staphylococcus aureus* (6.13%), dan *Micrococcus spp*. (6.13%). Bakteria lain yang dikenal pasti seperti *Acinobacter spp.*, *Photobacterium spp.*, *Kluyvera spp.*, *Ochrobactum spp.*, dan *Enterococcus spp*., didapati kurang daripada satu peratus. Prevalens mastitis subklinikal yang tinggi dalam kalangan kerbau tenusu di Selangor yang disebabkan oleh stafilokokus koagulase-negatif (CoNS) boleh menyumbang kepada penentu rintangan antibiotik yang mungkin menimbulkan masalah kesihatan awam. Sebagai penutup, CoNS adalah patogen yang paling biasa menyebabkan mastitis subklinikal di ladang ini dan majoriti kerbau tenusu yang menyusu mempunyai prevalens tinggi mastitis subklinikal.

Kata kunci: Kerbau, Mastitis subklinikal, Patogen, Ujian Mastitis California, stafilokokus koagulase-negatif (CoNS)

ABSTRACT

THE PREVALENCE OF SUBCLINICAL MASTITIS AND ITS PATHOGENS IN DAIRY BUFFALOES IN SELANGOR MALAYSIA

By

Muhammad Iqbal Fariz bin Rosslan

2022

Supervisor: Assoc. Prof. Dr. Rozaihan Mansor

Co-Supervisor: Dr. Sharina Omar

Mastitis is one of the important diseases that greatly impact dairy farmers worldwide. Subclinical mastitis is characterised by increased somatic cell count and microorganisms in the milk with no physical abnormalities on udder and in milk. This study aims to determine the prevalence of subclinical mastitis and to identify the pathogens causing the subclinical mastitis in buffaloes in Selangor, Malaysia. This study was carried out at six semi-intensively buffaloes' farms in Selangor with a total of 74 lactating buffaloes identified. Buffaloes with subclinical mastitis were diagnosed using California Mastitis Test (CMT). Forty two out of 74 lactating buffaloes, equivalent to 56.76%, were found to have subclinical mastitis. All positive samples

were subjected to bacterial isolation and identification and a total of 163 bacteria were being isolated with 35 types of bacteria were identified using Analytical Profile Index (API). Coagulase negative staphylococci (CoNS) (39.88%) was the most prevalent bacteria identified followed by *Streptococcus agalactiae* (9.82%), *Staphylococcus aureus* (6.13%), and *Micrococcus spp.* (6.13%). Other identified bacteria for instance *Acinobacter spp., Photobacterium spp., Kluyvera spp., Ochrobactum spp.,* and *Enterococcus spp*., were found to be less than one percent. High prevalence of subclinical mastitis among dairy buffaloes in Selangor caused by coagulase negative staphylococci (CoNS) can contribute to acquiring antibiotic resistance determinants which may pose a public health problem. Conclusively, CoNS is the most common pathogens causing subclinical mastitis in these farms and the majority of lactating dairy buffaloes had high prevalence of subclinical mastitis.

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

1.0 INTRODUCTION

Mastitis is a disease that affects dairy cow productivity globally (FAO, 2014). Mastitis is also a major illness in the buffalo population, causing economic losses, a decrease in milk output production, higher treatment costs, and a higher culling process (Dhakal & Thapa, 2002; Singh & Bansal, 2004). Many control measures can prevent mastitis from happening on the farm, for instance, pre-milking udder cleanliness, post-milking teat dipping, dry cow therapy with long-acting antibiotics, segregation and culling strategies for animals with persistent infections, and environmental management throughout the dry cow and calving periods (Radostits *et al*., 1994). Mastitis could spread from animals to humans through consumption of raw milk which may carry harmful and zoonotic pathogens that, put public health in danger (Cobirka *et al*., 2020). It is not recommended to consume raw milk directly because of the high likelihood of contamination with germs from the cow, pasture, milking equipment, and containers.

There are three main components causing mastitis: host resistance, bacterial agents, and environmental factors (Gera & Guha, 2011). Mastitis may be categorised into sub-clinical, clinical, and chronic. These categories are determined by the causative organisms, the animal's breed, age, immunity, and lactation stage (Krishnamoorthy *et al.*, 2021). According to Krishnamoorthy *et al.*, (2021), subclinical mastitis (SCM) can be defined by absence of any apparent changes in milk and significant reducing milk production. Common clinical findings of clinical mastitis (CM) include udder enlargement, presence of clots and flakes in milk, and watery

milk. Although uncommon, chronic mastitis causes mammary glands to remain persistently inflamed.

Mastitis can also be categorised according to their causative agents namely contagious and environmental. Contagious mastitis is transmitted by transmitting bacteria from another infected udder to a non-infected udder during milking. This type of mastitis is primarily due to the unhygienic practice of milkers' hands. In contrast, environmental mastitis is caused by pathogens found from the surrounding environment such as dirty bedding, flooring and equipments. These pathogens gain entry into the mammary tissue via teat canal which may stay open for 1 to 2 hours after milking without post-dipping teat disinfection. Example of contagious pathogens are *Staphylococcus aureus* and *Streptococcus agalactiae* while environmental pathogens include *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae, Enterobacter aerogenes,* and *Streptococcus uberis* (Moroni *et. al*, 2018).

Controlling mastitis is very important. Dairy farmers should adhere to a mastitis control programme in order to reduce the incidence of mastitis. Cobirka *et al*., (2020) stated that the mastitis control programme is essential for mastitis protection and prevention. However, its effective implementation requires farmer's detailed understanding and accurate categorisation of mastitis-causing agents. In field situations, it is frequently challenging to determine the source and transmission mode. However, by continuously monitoring and improving milking hygiene practices, treating milking cows properly and managing dry cow therapy effectively, the

prevalence of clinical and acute mastitis cases in a herd can be reduced to minimal levels.

In the present study, the prevalence of subclinical mastitis was assessed in dairy buffaloes kept under different farm managemental conditions in Selangor, Malaysia. Therefore, this study was also conducted to identify the most common pathogens causing subclinical mastitis in the buffaloes' farms in Selangor, Malaysia.

2.0 LITERATURE REVIEW

2.1 Subclinical Mastitis in Buffalo

Subclinical mastitis is a severe condition affecting dairy cows and has become a significant factor in reducing milk output worldwide (Sumon *et al.*,2017). This illness is crucial for consumers and processors, causing farmers to suffer enormous financial losses (Bilal *et al.*, 2007). Subclinical mastitis is characterised by the presence of microorganisms in milk and an increase in somatic cell counts (SCC) in the absence of any signs of clinical problems. Since the milk seems normal but typically has a higher somatic cell count, the diagnosis of subclinical mastitis is much more challenging than that of clinical mastitis as the teat may appear normal. The California Mastitis Test (CMT) is one of the appropriate diagnostic methods for detecting subclinical mastitis. Subclinical mastitis results in two-thirds of the milk production losses compared to clinical mastitis (Radostits *et al.*, 2007).

2.2 California Mastitis Test (CMT)

Cases of subclinical mastitis are frequently not recognised, so their origin is seldom investigated. In order to confirm the diagnosis, a number of diagnostic techniques, such as electrical conductivity, bacterial agent identification, California Mastitis Test (CMT), Somatic Cell Count (SCC), N-acetyl-β-D-glucosaminidase (NAGase) can be performed (Fagiolo & Lai, 2007). California Mastitis Test (CMT) is very convenient in detecting subclinical mastitis for early detection as part of the udder health management program. This test is a quick, straightforward, and cheap cow-side

indication of the milk's somatic cell count. The mixture of milk with CMT reagent causes reaction by rupturing any cells in the milk sample with cell membranes, enabling the DNA in those cells to interact with the test reagent and forming gel. Therefore, this test offers a practical method for identifying subclinical mastitis patients.

The number of white blood cells in the milk sample may be estimated using the degree of gel formation. The test is interpreted as negative, trace, +1, +2, and +3; these results correspond well to somatic cell levels. The higher the degree of gel formation, the higher the score on the CMT. The higher the CMT score, the higher the milk's number of white blood cells. CMT helps in determining cows with subclinically infected quarters which contribute to significant numbers of somatic cells to the bulk tank milk in herds.

2.3 Bacterial Isolation & Identification

Bacterial isolation and identification of mastitis causing organisms is the most common diagnostic test in identifying mastitis pathogens. According to Sears *et al*., (2003), Gram-positive cocci, gram-negative bacteria (coliforms), *Corynebacterium spp.*, *Mycoplasma spp*., and others, which include *Nocardia spp.*, *Prototheca spp*., and yeasts, are the five types of organisms that cause bovine mastitis. Further identification of bacteria using phenotypic and genotypic through evaluation of bacterial morphology and growth characteristics, ability of bacteria to metabolise substrates via

biochemical tests and testing the antimicrobial sensitivity are very crucial to determine the effective drugs against the bacterial isolated (Audayra *et. al*, 2021).

Majority of organisms can grow on blood agar. The importance of haemolytic patterns calls for observation. Haemolysis could be valuable in detecting *Streptococcus agalactiae* through the colonies' clearings (beta haemolysis). On blood agar, the catalase test aids in the distinction between staphylococci and streptococci. A colony of staphylococci submerged in hydrogen peroxide on a glass plate exhibits a positive catalase response (bubbles). Moreover, the coagulase test allows for confirmation of *Staphylococcus aureus*.

2.4 Prevalence of Subclinical Mastitis in Buffaloes

In Malaysia, only one study was conducted by Othman *et al*., (2003). This study was done in three selected farms in Serdang, Selangor, and Kluang, Johor. The author mentioned a high prevalence of subclinical mastitis in these farms, ranging from 75% to 95%.

Different countries in the world, Asia or Europe, showed different percentages of the prevalence of subclinical mastitis, specifically in buffaloes. This can be shown based on several studies conducted in many countries. Based on the study conducted by Patel *et al.* in 2019, the author mentioned that 21 out of 92 buffaloes, which equivalent to 22.82%, were positive for subclinical mastitis in Jafarabadi, India.

However, in Bhola, Bangladesh, Aluil *et al.*, in 2020, the author stated that out of 200 total buffaloes, only 21 buffaloes (10.50%) were detected with subclinical mastitis. In Iran, 70 of 201 buffaloes (34.82%) were positive for subclinical mastitis (Beheshti *et al.*, 2011). Moreover, in Holeta, Ethiopia, 41.02%, equivalent to 224 out of 546 buffaloes, were diagnosed with subclinical mastitis (Ayano *et al*., 2013). These studies showed that different countries come out with a different percentage of buffaloes suffering from subclinical mastitis. Thus, this might contribute to many risk factors leading to subclinical mastitis.

2.5 Pathogens Causing Subclinical Mastitis in Buffaloes in Other Countries

Many pathogens can cause subclinical mastitis and many studies have been conducted to determine the bacteria which led to subclinical mastitis in other countries. For example, in Central Java, Indonesia, *Streptococcus spp*. was the most common bacteria causing subclinical mastitis, which is equivalent to 73.30%, followed by coagulase negative staphylococci (CoNS), which is equivalent to 26.50% (Harjanti *et al.*, 2018). However, in Punjab, Pakistan, the most common bacteria causing subclinical mastitis was Staphylococcus spp. (73.30%), followed by *Escherichia coli* (16.18%), *Pseudomonas spp.* (13.29%) and *Bacillus spp*. (12.42%) (Ali *et al*., 2011). One study was conducted in Dakahlia, Egypt, in 2015 by El. Naker *et al*., mentioned that *Escherichia coli* was the most common bacteria (30.32%), followed by *Streptococcus agalactiae* (20.49%) and *Staphylococcus aureus* (19.67%). Lastly, in Brandenburg, Germany, *Corynebacterium bovis* (9.1%) is the highest bacteria identified, followed by coagulase negative staphylococci (CoNS) (7.3%).

2.6 Risk Factors of Subclinical Mastitis in Buffaloes

Bacterial pathogens cause most cases of subclinical mastitis. The most reported environmental organisms are coagulase negative staphylococci. (CoNS) (Bal *et al*., 2010), *Streptococcus uberis* (Jayarao et al., 1999), *Klebsiella spp.*, (Newman, 1975) and *E. coli* (Hill, 1994).

Many risk factors can lead to subclinical mastitis such as handlers or workers, the milking machine, the environment such as bedding, or the buffaloes themselves. According to Salvador *et al.*, (2011), there was very little information available for water buffaloes, and almost all the published data regarding the risk factors for mastitis applied to dairy breeds of cattle. Hence, there is a good chance that similar risk factors will also be present in these species, it is yet unclear how much of an impact they will have.

Mastitis rises because of negligent milking behavior (Bilal *et al*., 2007). The worker might not be as cautious as the owner of the farm. The workers also might not clean their hands before milking, which introduces bacterial pathogens into the teat duct. As preventive ways, maintaining proper hygiene when milking is crucial, including using pre- and post-dip disinfectants, wearing gloves, wiping with separate towels, and creating uniform milking protocols, spread throughout a herd can be reduced (Moroni *et al*., 2011).

When the type of bedding was considered as an additional risk factor, it was discovered that sand flooring had a lower incidence of mastitis than concrete did and that the incidence was higher when the soil was used as the bedding material, which was supported by Bartlett *et al*., (1990). Additionally, filthy floor and hard or stone flooring may predispose to mastitis (Bilal *et al*., 2004). It is crucial to ensure the environment around the buffaloes to be kept as clean as possible by washing the floor with a running water pipe with detergent to remove dirt and feces twice daily.

Another recent study showed that milking can also lead to mastitis. When fisting instead of stripping was used as the method of milking, the incidence of clinical and sub-clinical mastitis was high (Kavitha *et al*., 2009). Furthermore, an unhygienic milking machine also can introduce bacterial pathogens to the udder leading to subclinical mastitis. Untreated subclinical mastitis can lead to clinical mastitis (Stuhr & Aulrich, 2010). A fundamental understanding of the milking machine and associated equipment is crucial when assessing mastitis or milk quality issues on a dairy. Teat ends can be permanently damaged by malfunctioning or poorly maintained milking equipment, which can also harm teat ends because of significant irregular vacuum variations, such as those that result from liner slips. Increased post-pasteurization and high bulk tank bacterial counts may be caused by improperly cleaned equipment (Moroni *et. al*, 2018).

3.0 MATERIALS AND METHODS

3.1 Dairy Buffalo Farms Selection and Management

Six (6) dairy buffalo farms located in Selangor, Malaysia, were selected to participate based on the database obtained from the Department of Veterinary Services, Malaysia. These dairy buffalo farms were managed semi-intensively. One of the six farms uses a milking machine, and the other farm uses hand milking.

3.2 Animal Selection

Lactating buffaloes from each farm with no systemic signs and abnormalities of mammary glands or milk will be subjected to California Mastitis Test (CMT) to diagnose subclinical mastitis. Most of the buffaloes are Bubalus bubalis and at various lactation stages. Those buffaloes with subclinical mastitis infection (CMT results of trace, +1, +2, and +3) will be subjected to aseptic milk sampling for bacterial isolation and identification.

3.3 Assessment of The Mammary System

All lactating buffaloes were subjected to clinical examination of the mammary gland by observing the symmetry of the mammary gland. Next, each teat was palpated to determine any enlargement, inflammation, or abnormalities present. Lastly, the lymph node was palpated to detect any enlargement.

3.4 California Mastitis Test

Approximately two (2) ml milk were drawn from each quarter onto a CMT paddle. An equal volume of CMT reagent was added to the milk and swirled gently to examine the presence of a gel or slime reaction. Score of CMT was determined and positive CMT score of trace, +1, +2 and +3 were recorded for further analysis.

3.5 Aseptic Composite Milk Sampling

The teats were cleaned with diluted chlorhexidine gluconate (2%) and pre-dipped with germicidal teat dip. The teats were then disinfected with cotton wool soaked in 70% ethyl alcohol. After discarding the first two milk strips, approximately 2 ml of milks were taken from every quarter into a sterile cup. These milk samples were then transported to the Bacteriology Laboratory, Faculty of Veterinary Medicine, UPM, for bacterial isolation and identification. The milk samples are stored in the ice box to preserve the milk and prevent cross-contamination.

3.6 Bacteria Isolation and Identification

The milk samples which declared positive were primarily cultured on blood agar and MacConkey agar using a 'spread out technique.' Then, the plates were incubated for 24 hours at 37°C in aerobic conditions. Mixed growths were determined if more than one growth was observed on the plate. If no growth was observed, the plates were incubated for another 24 hours. The bacteria colonies were sub-cultured on the blood agar to get the pure culture. Pure bacterial cultures were identified based on colony

morphology, hemolytic characteristics, gram staining, and biochemical characteristics (catalase, coagulase, and oxidase test). Next, Analytical Profile Index (API) was done to identify the bacteria. API STAPH and API 20STREP tests were conducted for cocci shaped bacteria whereas API 20NE and API 20E tests were performed for cocco-bacilli or rod-shaped bacteria. Catalase and oxidase tests were done on Gram-positive and Gram-negative bacteria, respectively. Isolates that showed positive reaction for catalase test were subjected to API STAPH test, whereas negative reaction was subjected to API 20STREP test. On the other hand, API 20NE tests were performed on isolates that showed positive reaction for oxidase test were continued while isolates with negative reaction for oxidase test were subjected to API 20E test.

3.7 Data Analysis

The tabulation of results followed by data calculation and analysis are all done using Microsoft Excel Spreadsheet.

4.0 RESULT

A total number of lactating cows were collected from six (6) semi-intensive farms in Selangor, Malaysia, and subjected for California Mastitis Test (CMT) to diagnose subclinical mastitis. Only FARM F practiced machine milking while the other five (5) dairy buffalo farms practiced hand milking. Table 1 shows the prevalence of subclinical mastitis in buffaloes from six (6) six farms in Selangor, Malaysia.

Table 1: Prevalence of Subclinical Mastitis in Buffaloes

	Farmers	**Total number of lactating buffaloes**	**Animals positive with subclinical mastitis**	**Prevalence percentage (%)**
1.	FARM A	17	8	47.06
2.	FARM B	7	4	57.14
3.	FARM C	10	5	50.00
4.	FARM D	10	5	50.00
5.	FARM E	2	1	50.00
6.	FARM F	28	19	67.86
	Total:	74	42	56.76

Forty-two (42) out of the 74 buffaloes showed positive results of CMT, which is equivalent to 56.76%. The highest prevalence was recorded at FARM F (67.86%), followed by FARM B (57.14%). Three farms showed the same percentage of the

prevalence of subclinical mastitis (50.00%), which are FARM C, FARM D, and FARM D. FARM A however showed the lowest prevalence of subclinical mastitis (47.06%).

A total of 42 positive CMT positive milk samples were brought to the Bacteriology Laboratory, Faculty of Veterinary Medicine, UPM for bacterial isolation and identification. All positive samples showed bacterial growth on the agar, and Table 2 shows the isolated bacteria found on each farm.

Table 2: 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**
Coagulase negative staphylococci (CoNS)	9	10	6	3	1	36
Streptococcus agalactiae	1	0	2	0	0	13
Staphylococcus aureus	3	1	2	2	0	02
Streptococcus spp.	0	0	0	3	0	6
Micrococcus spp.	2	4	0	1	0	3
Kocuria spp.	1	1	0	1	0	4
Aeromonas spp.	0	2	2	0	0	1
Enterobacter spp.	0	0	1	0	0	4
Brevundimonas spp.	0	0	0	1	0	4
E. Coli	0	0	0	0	0	3
Klebsiella	0	1	0	0	0	0

Pseudomonas	1	0	0	0	0	1
Bacillus spp.	2	0	0	1	0	0
Enterococcus spp.	1	0	0	0	0	1
Ochrobactum spp	1	0	0	0	0	1
Moraxella spp.	1	0	0	0	0	0
Rhizobium spp.	0	1	0	0	0	0
Chryseobacterium spp.	0	1	0	0	0	2
Vibrio spp.	0	1	0	0	0	0
Lactococcus spp.	0	0	1	0	0	1
Weeksella spp	0	0	1	0	0	0
Aerococcus spp.	0	0	0	1	0	2
Globicatella spp.	0	0	0	1	0	0
Bukholderia spp	0	0	0	0	0	1
Acinobacter spp.	0	0	0	0	0	1
Kluyvera spp.	0	0	0	0	0	1
Photobacterium spp.	0	0	0	0	0	1
Total	21	22	15	14	01	90

Table 3 shows the percentage of the bacteria that have been identified after the API Test. The percentages are calculated by dividing the number of the specific bacteria with the total bacteria (n=163) that were isolated.

Table 3: Percentage of The Bacteria Identified.

No.	Name of Bacteria	Total Isolation	Percentage (%)
1.	*Coagulase negative staphylococci (CoNS)*	65	39.88
2.	*Staphylococcus aureus*	10	6.13
3.	*Streptococcus agalactiae*	16	9.82
4.	*Streptococcus spp.*	09	5.52
5.	*Micrococcus spp.*	10	6.13
6.	*Kocuria spp.*	7	4.29
7.	*Aeromonas spp.*	5	3.07
8.	*Enterobacter spp.*	5	3.07
9.	*Brevundimonas spp.*	5	3.07
10.	*E. Coli*	3	1.84
11.	*Klebsiella*	1	0.61
12.	*Pseudomonas*	1	0.61
13.	*Bacillus spp.*	3	1.84
14.	*Enterococcus spp.*	1	0.61
15.	*Ochrobactum spp*	2	1.23
16.	*Moraxella spp.*	1	0.61
17.	*Rhizobium spp.*	1	0.61
18.	*Chryseobacterium spp.*	3	1.84
19.	*Vibrio spp.*	1	0.61
20.	*Lactococcus spp.*	2	1.23
21.	*Weeksella spp.*	1	0.61
22.	*Aerococcus spp.*	3	1.84
23.	*Globicatella spp.*	1	0.61
24.	*Bukholderia spp*	1	0.61
25.	*Acinobacter spp.*	1	0.61
26.	*Kluyvera spp.*	1	0.61
27.	*Photobacterium spp.*	1	0.61
	Total:	163	100

Table 2 and Table 3, respectively, show a total of 27 types of bacteria found in buffaloes farms in Selangor. Coagulase negative staphylococci (CoNS) showed the highest number of bacteria identified in milk samples of buffaloes with subclinical mastitis in Selangor, which was 65 out of 163; equivalent to 39.88%. The second highest percentage of bacteria that have been identified was *Streptococcus agalactiae*

(7.36%), followed by *Staphylococcus aureus* (6.13%), *Micrococcus spp*. (6.13%), and *Streptococcus spp*. (5.52%). Meanwhile, *Klebsiella spp., Pseudomonas spp., Enterococcus spp., Moraxella spp., Rhizobium spp., Vibrio spp., Weeksella spp., Globicatella spp., Bukholderia spp., Acinobacter spp., Kluyvera spp*., and *Photobacterium spp*. were the bacteria that have been identified the least (0.61%) compared to other bacteria.

5.0 DISCUSSION

Buffaloes' mastitis is a crucial disease that must be prevented as it can lead to economic loss for the farmers. This shows that controlling the bacteria from invading the teat canal is essential. California Mastitis Test (CMT) is one of the diagnostic methods which is very useful to farmers in the early detection of subclinical mastitis. CMT can also be implied in the udder health management in the farm as the diagnostic tools are inexpensive, especially for the smallholder. Comprehensive knowledge of mastitis disease in every farmer is also fundamental to control the disease, such as increased biosecurity, culling of the infected cows, improved hygiene during pre-milking and post-milking. However, the management system, for instance, feeding management, milking management, bedding, temperature, and humidity, might differ in the pathogens that cause subclinical mastitis in buffaloes on each farm in Selangor, Malaysia.

In this study, the prevalence of subclinical mastitis in buffaloes on six farms in Selangor was calculated. Based on Table 1, the results showed a high prevalence of subclinical mastitis (56.76%) from these six farms. This shows that half of the population of the lactating cows were subclinical mastitis. Buffaloes with subclinical mastitis cause reduced milk quality due to the bacteria content in the milk and therefore, decrease the quantity of the milk. According to Portolano *et al.*, (2007), the teat canal is the source way of entry for the pathogen to invade the mammary gland. Without post-dipping of the teat, the teat canal will remain open leading to invasion of the pathogens infecting the mammary gland.

The high prevalence of subclinical mastitis may be contributed to many reasons. Ahmed *et al*., (2018) stated that, one of the primary reasons for the high number of subclinical mastitis cases in farms was due to poor animal management practices such as poor bedding, poor feeding management, and overcrowding. Dirty bedding with manure can be a source of environmental pathogens. Furthermore, the procedures during pre-milking, milking, and post-milking are not being followed by the workers can also lead to subclinical mastitis in buffaloes. Moreover, keeping the buffaloes with one quarter infected with a low immune system without culling can also increase the probability of subclinical mastitis in the herd. Nevertheless, the dairy farmers should be focusing on preventing mastitis by implementing a mastitis control program.

Determining the source of the pathogens is the key to controlling mastitis. Many pathogens can lead to mastitis. Different pathogens come from different sources. However, identifying the pathogens and their sources is time consuming. Many studies have been conducted in many countries, and the results vary. Different geographical regions, such as tropical and temperate countries managed to identify different types of aetiological agents. Based on this study, the highest percentage of etiological agents that have been found which cause subclinical mastitis in Selangor, Malaysia, is coagulase negative staphylococci (CoNS) (39.88%), followed by *Streptococcus agalactiae* (9.82%), *Staphylococcus aureus* (6.13%), *Micrococcus spp*. (6.13%), and *Streptococcus spp*. (5.52%) respectively. All six farms had the highest prevalence of coagulase negative staphylococci (CoNS).

Based on the article by Vásquez-García *et al.,* (2017), the highest prevalence causing subclinical mastitis in the central region of São Paulo State, Brazil, was *Staphylococcus epidermidis* (17%), followed by *Staphylococcus aureus* (15%), and *Bacillus spp*. (14%). In another study conducted by Harjanti *et al.,* (2017), the highest percentage of prevalence in subclinical mastitis in Central Java, Indonesia, was *Streptococcus spp*. (73.30%) and coagulase negative staphylocci (CoNS) (26.50%). In Egypt, one study by Ahmed *et al.,* (2018) stated that *E. coli* was the main aetiological agent causing subclinical mastitis in buffaloes. Meanwhile, in Punjab, Pakistan, a research led by Ali *et al*., (2011) found that *Staphylococcus spp*. (28.32%), *E. coli* (16.18%), *Streptococcus spp*. (7.51%), and *Pseudomonas spp*. (13.29%) were among the most common pathogens found in subclinical mastitis, respectively. Additionally, a research from Germany conducted by Tenhagen *et al.*, (2006) found that *Corynebacterium bovis* (9.1%) is the highest bacteria identified, followed by coagulase negative staphylococci (CoNS) (7.3%).

Based on the comparison between this study and other studies, different countries isolated different types of pathogens causing subclinical mastitis. This might be due to the geographical factors other than the temperature and the humidity. These factors influence the survivability of the pathogens to invade the mammary gland (Sordillo *et. al,* 2005). In this study, coagulase negative staphylococci (CoNS) is the main etiological agent causing subclinical mastitis. From Table 3, 65 out of 163 samples are coagulase negative staphylococci (CoNS). The microorganism might originate from the animal environment such as manure and bedding as the pathogen is

an environmental pathogen. The pathogen might spread via the flies, the towel, or even the milker's hand. Improper procedure during milking might lead to the invasion of the bacteria into the mammary gland (Moroni *et. al*, 2018).

Preventing mastitis is very important to prevent economic loss to dairy farmers. Udder health management and mastitis control programme are examples of the practices that can be implemented among dairy farmers. Improvement of hygiene is one of the ways to eliminate pathogens. Cleaning the barn to remove the manure twice daily with continuous water can be practiced as manure is the primary source of environmental pathogens. The equipment of the farm, especially the milking machine, must also be cleaned before the milking procedure. During milking, the workers must follow proper procedures to maintain hygiene. Iodine and chlorhexidine are recommended to use in the milking procedure. Culling the infected quarter must be done even though one quarter is affected. According to Cobirka *et. al*, (2020), most developing countries, such as India and Pakistan, did not practice culling which subsequently led to the increasing prevalence of subclinical mastitis cases in the region. Culling is significant in preventing contagious mastitis. Moreover, dairy farmers have always practiced dry cow therapy for a long time. However, the use of antimicrobials is now questionable and controversial as it might contribute to Antimicrobial Resistance (AMR) (Cobirka *et. al*, 2020).

This study shows that these risk factors have influenced the prevalence of the subclinical mastitis in these six buffaloes' farms.

6.0 CONCLUSION

In conclusion, this study showed a high prevalence of subclinical mastitis in all six (6) dairy buffalo farms in Selangor, Malaysia which is 56.76%. Moreover, coagulase negative staphylococci (CoNS) is the most common pathogen causing subclinical mastitis in these farms.

7.0 RECOMMENDATION

In order to diagnose mastitis in Malaysia, specifically in buffaloes, more research on determining the prevalence of the subclinical mastitis and its pathogens should be written in the future. More research can therefore be conducted in other Malaysian states. Additionally, comparison studies are advised for various farm management models, such as smallholder farms or commercial farms. On occasion, it is necessary to investigate the risk variables to find the common element that can cause a high incidence of subclinical mastitis. It is recommended that the lactating buffaloes be divided based on lactating stages during the diagnosis so that the prevalence of subclinical mastitis may be determined based on the stages. Moreover, other diagnostic techniques should be included in diagnosing subclinical mastitis, such as determining the Somatic Cell Count (SCC) to confirm the disease.

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9.0 APPENDICES

APPENDIX 1

Normal appearance of the quarters

APPENDIX 2

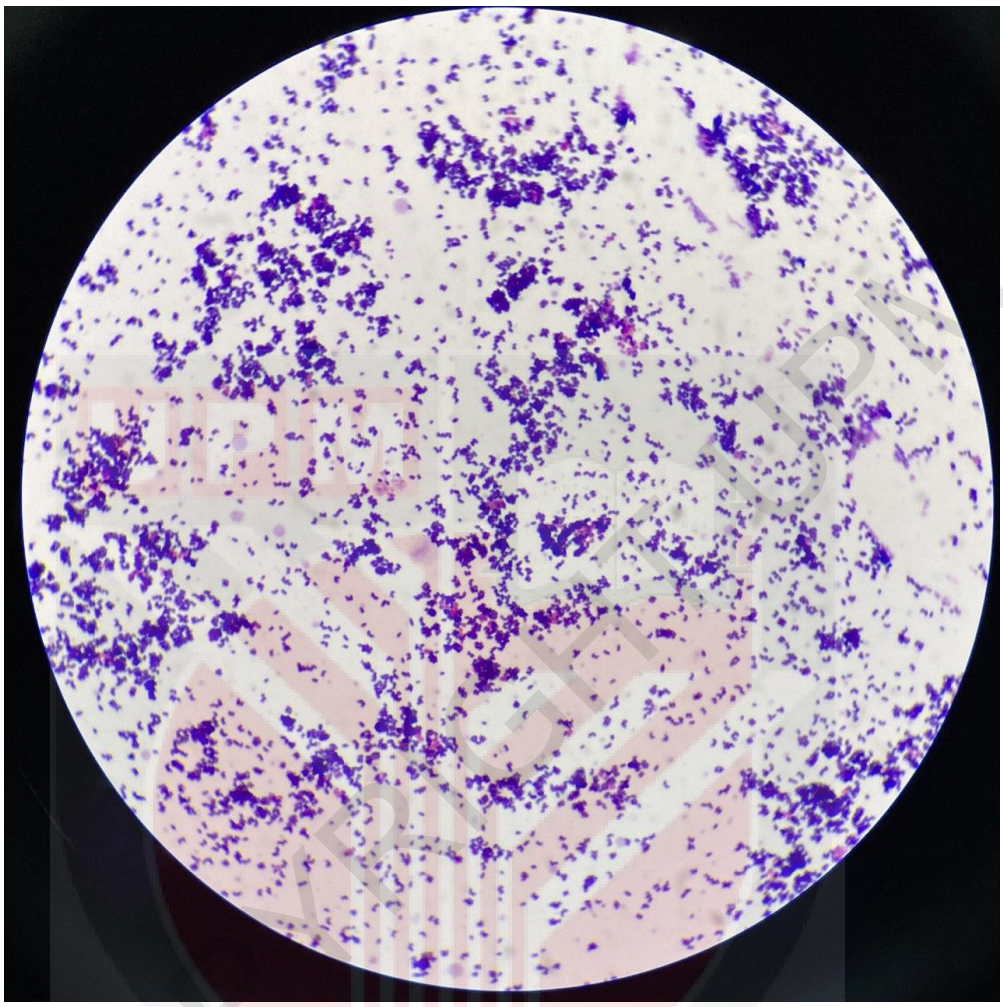
Mix of milk with CMT reagent after 30 seconds

APPENDIX 3

	Score	Meaning	Description of reaction	Individual Quarter Sample	Bucket Milk Sample
	N	**Negative**	Mixture remains liquid. No slime or gel form. It can drip out of the paddle well.	No Mastitis	No Mastitis
	T	**Trace**	Mixture becomes slimy or gel like. It's seen to best advantage by tipping paddle back and forth, observing mixture as it flows over the bottom of cups.	Trace of mastitis	Mastitis in one or more quarters
	1	**Weak Positive**	Mixture distinctly forms a gel.	Mastitis	Define mastitis - Check quarters
	2	**Distinct Positive**	Mixture thickens immediately, tends to form jelly. Swirling cup moves mixture in toward center exposing outer edges of the cup.	Mastitis	Serious Mastitis – Check quarters

CMT result and interpretation

APPENDIX 4



Gram positive cocci

APPENDIX 5

Animal ID	114			Animal ID	147			Animal ID	127			Animal ID	116		
Result		L	R	Result		L	R	Result		L	R	Result		L	R
	F	-	-		F	++	+++		F	-	+++		F	N/A	-
	H	-	-		H	+	+		H	++	-		H	++	N/A
Animal ID	113			Animal ID	121			Animal ID	120			Animal ID	135		
Result		L	R	Result		L	R	Result		L	R	Result		L	R
	F	-	+		F	-	-		F	-	-		F	+	++
	H	+	+		H	-	-		H	-	-		H	+	+
Animal ID	128			Animal ID	124			Animal ID	112			Animal ID	250		
Result		L	R	Result		L	R	Result		L	R	Result		L	R
	F	-	N/A		F	-	-		F	-	-		F	-	+
	H	+	N/A		H	+	-		H	-	+		H	-	-
Animal ID	119			Animal ID	138			Animal ID	133			Animal ID	122		
Result		L	R	Result		L	R	Result		L	R	Result		L	R
	F	-	-		F	-	++		F	++	+		F	-	-
	H	-	+++		H	++	++		H	++	+		H	-	-
Animal ID	118			Animal ID	136			Animal ID	107			Animal ID	115		
Result		L	R	Result		L	R	Result		L	R	Result		L	R
	F	+	N/A		F	+	+		F	+	+		F	-	-
	H	++	-		H	-	+		H	++	-		H	-	-
Animal ID	150			Animal ID	129			Animal ID	108			Animal ID	146		
Result		L	R	Result		L	R	Result		L	R	Result		L	R
	F	+	+++		F	-	-		F	-	-		F	-	+
	H	++	+++		H	-	-		H	N/A	-		H	-	-
Animal ID	106			Animal ID	123			Animal ID	214			Animal ID	203		
Result		L	R	Result		L	R	Result		L	R	Result		L	R
	F	+	-		F	-	-		F	++	+		F	-	-
	H	+	++		H	-	-		H	-	-		H	-	-

The infected quarters were recorded into Microsoft Excel