UNIVERSITI PUTRA MALAYSIA

PATHOGENS CAUSING BOVINE MASTITIS IN SELECTED FARMS IN LABIS, JOHOR

AYUNARNI BINTI S EFENDI

PATHOGENS CAUSING BOVINE MASTITIS IN SELECTED FARMS IN LABIS, JOHOR

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A project paper submitted to the

Faculty of Veterinary Medicine, University Putra Malaysia

In partial fulfilment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

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DEDICATION

This project is dedicated to:

- Dad (S.efendi B. S.Ahmad)
- Mum (Kapsiah bt. Bain)
- Brothers (Ridzuan & Hazrir)
- Sisters (Roziah, Ismadora, Hazira, Haryani, Aziani)
- Lecturers of Veterinary Faculty, UPM
- Special friend (Nazreen Usri)
- All my friends

It is hereby certified that we have read this project paper entitled "Pathogens Causing Bovine Mastitis in Selected Farms in Labis, Johor", by Ayunarni binti S Efendi and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD4999 – Final Year Project.

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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fullfilment of the course VPD 4999- Project

PATHOGENS CAUSING BOVINE MASTITIS IN SELECTED FARMS IN LABIS, JOHOR.

By

Ayunarni Bt. S.Efendi

2016

Supervisor: Assoc. Prof. Dr. Zunita Zakaria

This study was conducted to determine the prevalence and bacteriological assessment of subclinical mastitis and antimicrobial resistance of bacterial isolates from dairy cows in selected farms in Labis, Johor. A total of 128 samples were collected from four farms and tested using California Mastitis Test (CMT). Thirty four (26.56%) milk samples were found to be positive and subsequently subjected to bacterial culture and identification. A total of seven bacteria species were successfully isolated from the samples. The most prevalent bacteria was *Staphylococcus aureus* (41.67%), followed by *Staphylococcus intermedius* (27.10%) and *Streptococcus uberis* (10.40%), *Staphylococcus shleiferi* (8.33%) and *Aerococcus viridans 2* (8.33%). The other two bacteria are *Corynebacterium*

sp. and *Chromobacterium sp.*; both having 2.10% prevalence. In general, the antibiotic susceptibility test displayed variable susceptibility against tested antibiotics. *Staphylococcus aureus* showed highest resistance *at* 92.60%, 88.89%, 74.08%, 66.67% and 14.82% towards gentamycin, streptomycin, tetracyline, penicillin G and oxytetracycline respectively. *Staphylococcus intermedius* are 100% resistant against gentamycin, streptomycin, 84.62% against tetracycline, 54.85% against penicillin G and 30.77% against oxytetracycline. It was found that most of the isolated bacteria are sensitive towards oxytetracycline, therefore making the antibiotic the most effective among the five tested antibiotic. This study indicates the need for urgent and effective control measures to tackle the increase in prevalence of subclinical mastitis and their antimicrobial resistance in the study area.

Keywords: Mastitis, prevalence, antibiotic sensitivity test, cattle farms

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999- Projek

PATOGEN YANG MENYEBABKAN MASTITIS LEMBU TENUSU DI LADANG TERPILIH DI LABIS, JOHOR

Oleh

Ayunarni Bt. S Efendi

2016

Penyelia: Prof. Madya Dr. Zunita Zakaria

Kajian ini dijalankan untuk menentukan prevalens dan penilaian bakteriologi mastitis subklinikal dan rintangan antimikrob daripada pencilan bakteria daripada lembu tenusu di ladang terpilih di Labis, Johor. Sebanyak 128 sampel telah diambil dari empat ladang dan diuji menggunakan California Mastitis Test (CMT). Tiga puluh empat (26.56%) sampel susu didapati positif dan seterusnya spesies bakteria di tentukan. Sebanyak tujuh spesies bakteria telah berjaya diasingkan daripada sampel. Bakteria yang paling lazim adalah *Staphylococcus aureus* (41,67%), diikuti oleh *Staphylococcus intermedius* (27.10%) dan *Streptococcus uberis* (10.40%), *Staphylococcus shleiferi* (8.33%) dan *Aerococcus viridans 2* (8.33%).

Corynebacterium sp. dan *Chromobacterium sp.* kedua-duanya mempunyai 2.10% kelaziman. Secara umum, ujian kerentanan antibiotik menunjukkan kecenderungan berubah-ubah terhadap antibiotik yang diuji. *Staphylococcus aureus* menunjukkan rintangan tertinggi pada 92.60%, 88.89%, 74.08%, 66.67% dan 14.82% ke arah gentamycin, streptomycin, tetracyline, penicillin G dan oxytetracycline masing-masing. *Staphylococcus intermedius* adalah 100% tahan terhadap gentamycin dan streptomycin, 84.62% terhadap tetracycline, 54.85% terhadap penicillin G dan hanya tahan sebanyak 30.77% terhadap oxytetracycline. Kajian ini telah mendapati bahawa kebanyakan bakteria sensitif terhadap oxytetracycline, oleh itu menjadikan oxytetracycline sebagai antibiotik yang paling berkesan antara lima antibiotik yang di uji. Kajian ini menunjukkan keperluan untuk langkah-langkah kawalan segera dan berkesan untuk menangani peningkatan dalam kelaziman mastitis subklinikal dan rintangan antimikrob dalam kawasan kajian.

Kata kunci: Mastitis, kelaziman, ujian sensitiviti antibiotik, ladang lembu

1.0 INTRODUCTION

Mastitis is one of the important production diseases of dairy animals which directly or indirectly affects the economy of the farmers and ultimately affect the economy of the country (Sharma et. al., 2014). Mastitis is inflammation of the mammary gland affecting all species of domestic animals and is of great concern to the dairy industry. The disease is characterised by the inflammation of the mammary gland that is caused by bacterial infections. Economic losses associated with mastitis is derived mainly from a decrease in milk production and to a lesser extent, from the culling of chronically infected cows, cost of veterinary treatment, and penalties on milk quality (Seegers et al., 2003). Organisms responsible for causing mastitis are classified into environmental and contagious bacteria (Hamadani et al. 2013). Contagious mastitis infections are acquired by transmission of contagious bacteria from cow to cow during the milking process while for environmental infections it is acquired from bacteria in the environment. Contagious mastitis can be further categorised into subclinical, clinical, acute, acute gangrenous and chronic mastitis (Zoetis, 2013). In general, the most common organisms found in milk samples are *Staphylococcus aureus, Streptococcus uberis, Escherichia coli, Pseudomonas spp., Mycoplasma sp.* and coagulase-negative staphylococci. Researches on bovine mastitis have been carried out since the last seven decades but unfortunately, the problem remains especially for field veterinarians in treat and control (Sharma et. al., 2012).

Even though mastitis is common, up to date information is lacking in terms of the major causing bacteria causing mastitis. By collecting samples of the milk

produced by cows suffering from the symptoms of mastitis, and indeed samples of cows identified as having high Somatic Cell Counts (SCC), it enables to identify which pathogen or pathogens are to blame, in order to target effective treatment. Besides to target for the effective treatment, appropriate control measures can be implemented on the farm to reduce the incidence of the disease.

According to Aarestrup (2005), antimicrobials are routinely used for treatment of dairy cattle affected with clinical and subclinical infections. The use of antimicrobials have, over time, increased the number of antimicrobial-resistant microbes globally, and any use of these agents will to some extent benefit the development of resistant strains. Inappropriate usage of antimicrobials such as wrong dose, drug or duration may contribute the most to the increase in antimicrobial resistance (Williams, 2000).

This study aims to determine the types of pathogens that are currently causing mastitis at four selected farms in Labis, Johor. This information can be used in designing appropriate and effective treatment to the infected cows. This study also aims to determine the presence of antibiotic resistant organisms among the pathogens. Presence of resistant pathogens may become a challenge in treatment. The objectives of this study were:

1. to identify the most common pathogens causing mastitis in cows.
2. to determine the antibiotic susceptibility of the pathogens against antibiotics.

2.0 LITERATURE REVIEW

2.1 Mastitis

Mastitis (Mast: breast, itis: inflammation) is defined as an inflammation of the udder resulting in an inflamed quarter or quarters with a change in the appearance of the milk (Blowey, 1999). The main types are contagious mastitis and environmental mastitis. *Staph. aureus and Strep. agalactiae* are the main bacteria causing contagious mastitis. They mostly live inside udders or on teat skin and are spread either by splashes of infected milk or sprays during stripping, on milkers' hands or teatcup liners. Soil, manure, bedding, calving pads and water host bacteria are source of pathogens cause environmental mastitis. The main bacteria are *Strep. uberis* which can sometimes persist, and can spread at milking. The other culprit is *E. coli* which does not thrive in the lactating udder and often the infections do not persist.

Bovine mastitis is one of the important production diseases of dairy animals which directly or indirectly affect the economy of the farmers and ultimately affect the economy of the country. Reduced milk production, treatment cost, increased labor, milk withheld following treatment and premature culling in dairy cattle mastitis results in severe economic losses from (Miller et al., 1993).

2.2 Aetiological agents

There are several pathogens causing mastitis. Bacteria incriminated in bovine mastitis include *Streptococcus spp.* (*S. dysgalactia, S. uberis)*, *Staphylococcus spp.* (*S.*

aureus, coagulase-negative staphylococci (CNS)), *Klebsiella spp*., *Pseudomonas spp*., *Corynebacterium spp*., *Mycoplasma spp*. Fungi incriminated in bovine mastitis include *Cryptococcus spp.* and *Prototheca spp.*. The majority of cases are caused by direct infection by *Streptococcus spp., Staphylococcus spp.* and lactose fermenting coliforms such as *Escherichia coli. Streptococcus agalactiae* is a contagious mastitis bacterium, often associated with cases of subclinical mastitis (Akerstedt, 2012).

2.2.1 *Staphylococcus spp.*

Staphylococcus spp. are the most prevalent pathogens causing mastitis in ruminants (Buzzola et al., 2001). According to Honkanen-Buzalski (1990), coagulase-positive *Staph. aureus* is considered a major cause of bovine mastitis. Bovine mastitis due to *Staph. aureus* is generally chronic, not easily cured by antibiotic treatment (Sol et al., 1997). *Staphylococcus intermedius* and *Staphylococcus shleiferi* also can be found in CMT positive milk samples but as not common as *Staph. aureus.*

2.2.2 *Streptococcus uberis*

Streptococcus. uberis is the most common Streptococcus species isolated from cases of mastitis (Petersson-wolfe, 2012). *Streptococcus uberis* are environmental organisms commonly found in manure and other organic matter, including bedding. Increased in the risk of spreading *S. uberis* to uninfected cows are due to poor udder cleanliness, inadequate stall management, and damaged teat ends. New infections can occur at any time during lactation and may also occur during the dry period (Petersson-wolfe, 2012). Because of the nature of this bacteria, it is better to prevent the infections than treat the infections.

2.2.3 *Corynebacterium sp.*

Corynebacterium sp. are aerobic or facultative anaerobic, irregularly shaped, non-sporeforming, gram-positive rods. *Corynebacterium bovis* is the most frequently isolated species in milk from dairy cows with intramammary infections (IMI). *Corynebacterium bovis* can be found in milk from infected mammary glands and teat canals of cows (Schröder, J., 2012). It cause IMI with a slight increase in somatic cell counts and a small decrease in milk production in affected cows, therefore, it is considered a highly contagious mastitis pathogens (Watts, J. L., 2000). *Corynebacterium pseudotuberculosis* are less known as an exclusive agent of bovine mastitis without simultaneously occurring cutaneous lesions.

2.2.4 *Chromobacterium violaceum*

Chromobacterium violaceum is considered as an opportunistic pathogen of extreme virulence (Yang and Li, 2011). The organism belongs to the Family Neisseriacea of β-Proteobacteria. It is a Gram-negative, flagellated heterotroph which can be found in a variety of ecosystems in tropical and subtropical regions, including the soil and water. The colonies can be identified on conventional culture media by its striking deep purple pigment (Mahmud et al., 2009). There are reports of isolation of *C. violaceum* from raw milk, separated milk and separator drain in organized dairy plant (Reid, 1997). However they could not detect the organism in pasteurized milk.

2.3 Epidemiology of mastitis

Mastitis is a complex disease problem. It is a classic example of the interaction of microorganisms, host factors and the environment. Microorganism involve in mastitis are contagious and environmental pathogens. The most important environmental microorganisms involved are the Coliforms (*E.coli* and *Klebsiella spp*.) and the environmental streptococci (Smith et al. 1985). Common contagious pathogens are *Staphylococcus aureus* and *Strep. agalactiae.* Next is the host factors itself. Several host factors are important in determining the outcome of an infection. According to Lohuis 1989, most infection result in very little clinical signs, and host parameters like peripheral blood leukocyte activity, blood leukocyte count, and presence of antibodies partially predict the outcome of infection. Other factors such as age of the animal, its metabolic status (noticeably ketosis), mineral nutrition, periparturient stress and milk production level also affect the outcome of infection. Several factors in the environment affect the exposure of a cow to microorganisms. Sources of environmental exposure are manure, bedding, feeds, dirt, mud and water. A good example of this is *E. coli*, which is present in the environment of the cow.

The prevalence of subclinical mastitis in dairy cows is high and there is a significant difference ($p<0.05$) among the farms with a minimum of 70% and a maximum of 100% prevalence in all the farms visited (Marimuthu et al., 2014). As determined by the CMT, the overall prevalence of subclinical mastitis was found to be very high (81.7% or 49/60 cows) (Othman and Bahaman, 2005).

Bacterial identification from study done by Marimuthu et al., 2014 revealed *Staphylococcus sp.,* as the most prevalent bacteria followed by *Bacillus sp.* and

Corynebacterium sp. as the first 3 most prevalent bacterial pathogens with a prevalence of 55, 21 and 7%, respectively. Based on study done by Othman and Bahaman, 2005, *Staphylococcus aureus* was shown to be the predominant organism isolated and it made up between 23% (10/42) in Farm C to 35% (14/40) in Farm B, with a 28.6% overall prevalence of *S. aureus* in all three farms.

2.4 Treatment and control

When clinical mastitis is detected, the cow is milked out and then given an intramammary infusion of antibiotic. Clinical mastitis symptoms are most often are recognized by the milker from detection of clots or flakes in the milk, from a cow that has a quarter sensitive to the touch, a quarter that is swollen or hot to the touch. Prior to intramammary infusion, the teat is cleaned well and the tip of the teat is swabbed with an alchohol swab and allowed to dry for a number of seconds. The antibiotic comes in a plastic tube with a plastic infusion cannula on the end. After emptying the antibiotic tube, the teat is pinched off and the antibiotic fluid is palpated up into the gland. Cephapirin sodium is the example of intramammary infusion antibiotic that commercially being used. It is a cephalosporin which possesses a wide range of antimicrobial activity against gram-positive and gram-negative organisms. Common antibiotics such as from the penicillin group (penicillin G, ampicillin, erythromycin), the aminoglycosides (streptomycin, gentamycin, and the broad spectrum group such as chloramphenicol, tetracyclines, polymxin B, and sulfonamide compound were also being used.

Besides antibiotic treatment, oxytocin treatment also can be used. It is key contributing factor to duration of mastitis is the frequency and completeness of milk removal from the infected quarter. In some cases, cows are stripped between normal milking times, sometimes with injection of oxytocin to stimulate an effective milk let down. Clearly removal of the primary growth medium of the bacteria, the milk, more often should enhance rate of recovery from infection (Hillerton et al., 1999).

There are also non-responding cases. In this case, antibiotic are unable to elimiate the infection. These are often considered to be chronically infected cows, typically with *Staph. aureus*, and remain a constant source of infection for other cows. Culling of chronically infected cows sometimes is the only way to effectively control spread of mastitis in the herd (Østerås & Sølverød, 2009).

Treating infection with antimicrobials can, in conjunction with good farming practices, assist in this endeavor to eliminate, or at least decrease, the incidence of mastitis infection within a dairy herd.

Contagious mastitis can be effectively controlled through a rigorous program of teat dipping and dry cow antibiotic treatment (Whist et al., 2007). Cows with contagious mastitis should be milked last or a separate milking claw used for the infected cows. Milking claws should be flushed with hot water or germicide after milking infected cows (called backflushing). Environmental pathogens are more difficult to control than the contagious pathogens. Identification of the source and removal (bedding, ponds, mud) is the key to control. Only clean dry teats should be milked and the teat should be dipped after milking (Whist et al., 2007).

2.5 Multi-drug resistance (MDR) organisms in mastitis

Multidrug-resistant organisms are bacteria that have become resistant to certain antibiotics, and these antibiotics can no longer be used to control or kill the bacteria (Harrison et al., 1998). Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and certain gram-negative bacilli (GNB) are multidrug-resistant organisms (MDROs) (Siegel et al., 2006). The severity and extent of disease caused by these pathogens varies by the populations affected and by the institutions in which they are found. Multidrug-resistant organisms develop when antibiotics are taken longer than necessary or when they are not needed. At first, only a few bacteria may survive treatment with an antibiotic. The more often the antibiotics are used, the more likely it is that resistant bacteria will develop. These MDROs can go on to infect people. Multidrug-resistant organism infections are hard to treat because they do not respond to many common antibiotics (Siegel et at., 2006).

3.0 MATERIALS AND METHOD

3.1 Test animals

The study was carried out on 32 lactating cows from four cattle farms in Labis, Johor. The animals were mainly Friesian-Sahiwal crosses (85%) while a small percentage (15%) was made up of Jersey, Friesians and local Indian dairy crosses. In these four farms, majority of the animals are between the age of two to four years old. Most of the animals have had third parturition. Two farms implement combined milking method which are manually-milked and machined-milked while the other two farms use either manually-milked or machined-milked only. The animals were milked twice daily for two farms having combined milking method and once daily for the other two farms.

Demographic composition and dynamics of animal populations were determined. Farm A, B, C and D have 69, 132, 40 and less than 25 cattle respectively. The animals are managed semi-intensively. In Farm A and B, the animals are milked every morning by using combined method which are hand milking and machine milking. Farm C uses hand milking method only while farm D only use machine. The animals are released for grazing after milking. Supplements such as palm kernel cake (PKC), molasses and mineral block are usually given to the animals. The use of disinfectant in these farms is rare. Aseptic milking procedures are practiced.

3.2 California Mastitis Test (CMT)

The CMT paddle has four shallow cups marked A, B, C, and D to help identify the individual quarter from which the milk was obtained. Two millilitre of milk withdrawn from each quarter were placed in each well of the CMT plate and an equal amount of CMT reagent was introduced. They were mixed thoroughly by gentle swirling of the test plate. The results were read after a few seconds and positive results were indicated by fine thread-like streaks or when the test mixture became mucilaginous and jelly-like. The test was considered negative when the test mixture remained fluid and there was no change in appearance. Gross abnormalities of the milk were noted before subjecting the milk samples to the CMT for the presence of subclinical mastitis.

3.3 Samples collection

Milk was withdrawn from the four quarters into four test wells of the CMT plate. CMT was done and ten millilitres of CMT positive milk samples were aseptically collected to reduce contamination to the sample. The udder and the teats washed and thoroughly dried. Then, the teats were dipped using povidone iodine and allowed for contact time about 10 second before wiping off. After that, the teats were swabbed using 70% ethanol starting with the furthest away towards the closest teat, then allowed to dry. The first four to six squirts of milk was discarded, and then the teats swabbed with surgical spirit a second time and allowed to dry. The sample tube was held at an angle under the teats so that material cannot fall into the opening and the lid was opened with the thumb and forefinger and held as a shield to prevent any

contaminants from falling into the tube. Samples were packed in ice before being transported to the laboratory in UPM.

3.4 Bacteriological examination

3.4.1 Isolation and identification

The milk samples was inoculated onto blood agar and MacConkey agar for primary culture. MacConkey has been used specifically for the growth of the gram-negative bacteria. Bacterial inoculation was done by inoculating a loop of the milk samples onto the agar using four streak quadrant technique and it was incubated at 37°C for 24 hours.

3.4.2 Pure culture

After 24 hours, all cultures on blood agar and MacConkey agar were subcultured onto fresh media in order to obtain pure culture. The colonial and cell morphology were carried out for all pure cultures.

3.4.3 Biochemical tests

Following the preliminary classification of bacteria using colony and cell morphology, series of biochemical tests were carried out for the identification of bacteria species.

Biochemical tests for gram-positive bacteria

For gram-positive cocci bacteria, catalase test was performed. This test classifies the bacteria into Staphylococci and Streptococci group. Coagulase-positive

staphylococci are then subjected to hemolysis, acetoin production (Voges-Proskauer test), fermentation of maltose and mannitol and ADH (decarboxylation of the amino acid arginine by arginine dihydrolase) tests.

For gram-positive rods bacteria, tests carried out comprise of urease, glucose, nitrate reduction, sucrose, hemolysin, trehalose and motility tests.

The API (Analytical Profile Index) 20E Test System was used for streptococci group (Figure 1). It is a biochemical panel for identification and differentiation of members of the family Enterobacteriaceae. In API 20E, the plastic strip holds twenty mini-test chambers containing dehydrated media having chemically-defined compositions for each test. A single isolated colony from a pure culture was picked up and a suspension of it in sterile normal saline was made. Some drops of water was added in the tray to give a humid condition. The 20 compartments was filled up with the bacterial suspension using a pasteur pipette. Sterile oil was added into the ADH, LDC, ODC, H2S and URE compartments. The tray was incubated at 37°C for 18 to 24 hours.

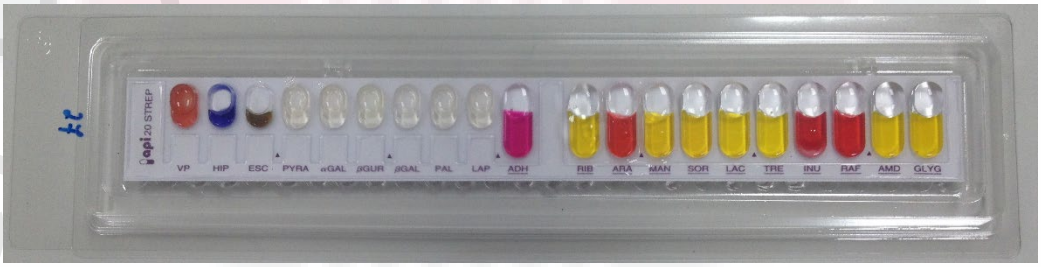

Figure 1: API test kit

Biochemical tests for gram-negative bacteria

Oxidase test

This test was done to determine the presence of bacterial cytochrome oxidase. Cytochrome oxidase is an enzyme found in some bacteria that transfers electrons to oxygen, the final electron accept or in some electron transport chains. Thus, the enzyme oxidizes reduced cytochrome C to make this transfer of energy. Presence of cytochrome oxidase can be detected through the use of an Oxidase Disk which acts as an electron donator to cytochrome oxidase. If the bacteria oxidize the disk (remove electrons) the disk will turn purple, indicating a positive test. No color change indicates a negative test.

Urease test

This test is used to determine the ability of an organism to produce the enzyme urease which hydrolyzes urea. Urease is an enzyme that breaks the carbon-nitrogen bond of amides to form carbon dioxide. Urease can be detected by plating bacteria onto an amide containing medium, specifically urea. When urea is broken down, ammonia is released and the pH of the medium increases (becomes more basic). This pH change is detected by a pH indicator that turns pink in a basic environment. A pink medium indicates a positive test for urease.

Triple Sugar Iron test

Triple sugar iron (TSI) agar is a medium used in the identification of Gram-negative enteric rods. The medium measures a bacterium's ability to utilize three sugars, glucose, sucrose and lactose, the concentrations of which are 0.1%, 1.0%, and

1.0%, respectively. A pH indicator included in the medium can detect acid production from fermentation of these carbohydrates.

Sulfide indole motility agar (SIM)

Purpose of this test is to differentiate between bacteria based on three tests which are sulphur reduction (cysteine desulfurase), indole production (tryptophanase), and motility. If a bacterium can produce the enzyme tryptohanase then it can use the amino acid tryptophan as a carbon and energy source (as pyruvate). One of the biproducts of this conversion is indole, which is detected with Kovac's reagent. If a bacterium posseses the enzyme cysteine desulfurase, sulphur containing amino acids will be broken down into pyruvate, ammonia and hydrogen sulfide. Iron in the medium reacts with hydrogen sulphide producing the characteristic black percipitate. Motility is observed as growth away from the stab line, but cannot be detected if hydrogen sulphide is formed.

Citrate test

The citrate test is used to determine the ability of a bacterium to utilize citrate as its only source of carbon. Bacteria can break the conjugate base salt of citrate into organic acids and carbon dioxide. The carbon dioxide can combine with the sodium from the conjugate base salt to form a basic compound, sodium carbonate. A pH indicator in the medium detects the presence of this compound by turning blue (a positive test).

3.5 Antibiotic sensitivity test

The antibiotic sensitivity test was performed according to the Kirby-Baeur disc diffusion method on Mueller-Hinton (MH) agar (Baeur et al., 1966). Fresh colonies of each isolate were grown onto the blood agar a day before antibiotic sensitivity test was performed. In order to prepare the inoculum, four or five isolated colonies of the test organism to be tested will be suspended into 2 ml of sterile saline and was vortexed to create smooth suspension. The turbidity of this suspension was adjusted to the turbidity of 0.5 McFarland standard. Inoculation of the MH plate as done thereafter. Firstly, a sterile swab was dipped into the inoculum tube. The swab was then rotated against the side of the tube (above the fluid level) using firm pressure, to remove excess fluid. The dried surface of a MH agar plate was inoculated by streaking the swab three times over the entire agar surface and the plate was rotated approximately 60 degrees each time to ensure an even distribution of the inoculum. Lastly, the swab was discarded into an appropriate container containing disinfectant. The plate was allowed to sit at room temperature about 3 to 5 minutes for the surface of the agar plate to dry. The next step was placement of antibiotic disks by using multidisc dispenser. The antibiotics tested were penicillin G (10 µg), streptomycin (10 µg), gentamycin (10 µg), oxytetracyclines (30 µg) and tetracyclines (30 µg). The choice of antibiotics was in accordance with current antibiotics used in therapy. Then, the plates was incubated at 37°C for 24 hours. The diameter of the zone of inhibition was measured using a pair of callipers (Figure 2). The results for each antibiotic was determined based on the Kirby-Baeur interpretation chart (CLSI 2010).

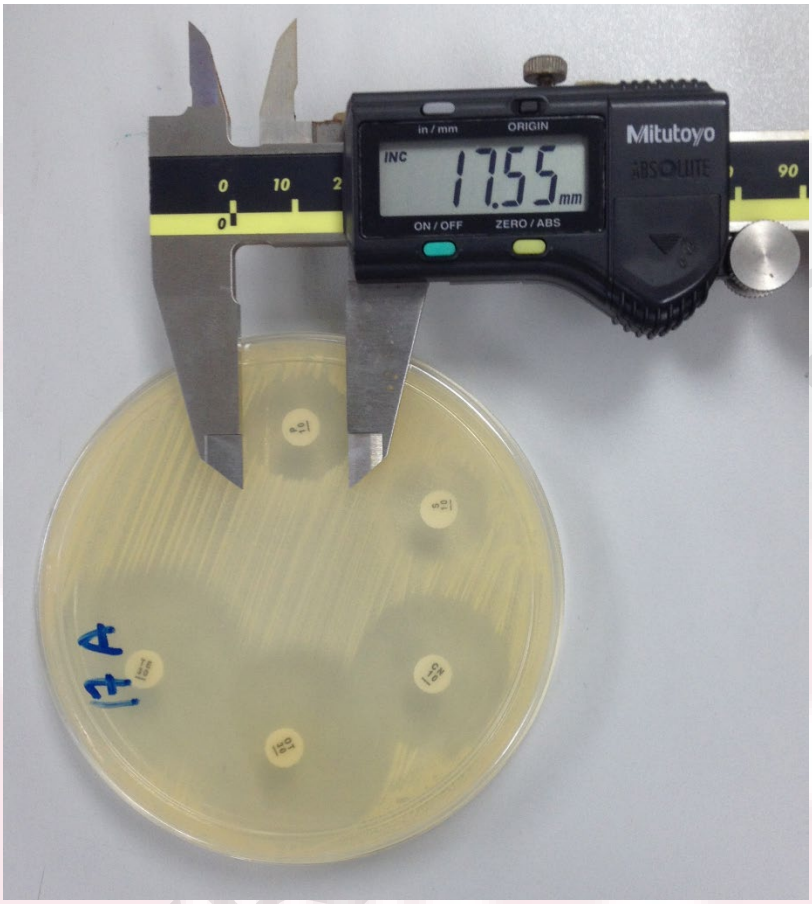

Figure 2: Measuring zone of inhibition using a pair of callipers

4.0 RESULTS

4.1 California Mastitis Test (CMT)

Samples collected were tested using CMT test. Those producing positive results in CMT, milk were collected and transported to laboratory for further tests (Table 1). As determined by the CMT, the overall prevalence of subclinical mastitis in the selected farms was found to be quite high at 56.25 % (18/32 cows). There was variation in prevalence between the four farms which ranged from 37.5 to 75.0 %. Farm B and Farm C showed high prevalence with 75% of CMT positive for both farms. Farm A and Farm D showed 37.5 % of their animals having CMT positive (Table 1).

A total 34 samples were taken for further investigation comprising four samples from Farm A, 15 samples from Farm B, 12 samples from Farm C and three samples from Farm D (Table 1).

Table 1: Prevalence of subclinical mastitis in the four studied farms as determined by the California Mastitis Test

FARMS	No. of animals examined	CMT positives (Animals)	% CMT positives (Animals)	No. of quarters examined	CMT positives (Quarters)/ No. of Samples	% CMT positives (Quarters)
A	8	3	37.5	32	4	11.76
B	8	6	75.0	32	15	44.12
C	8	6	75.0	32	12	35.30
D	8	3	37.5	32	3	8.82
Total	32	18	56.25	128	34	100

4.2 Isolation and identification

A total of 55 bacterial isolates were obtained from the 34 milk samples. Altogether seven bacterial species were identified which are *Staphylococcus aureus, Staphylococcus intermedius, Staphylococcus shleiferi, Streptococcus uberis, Aerococcus viridans 2, Corynebacterium sp.* and *Chromobacterium sp*.. Two of the main species identified were the *Staphylococcus aureus* and *Staphylococcus intermedius*. *Staphylococcus aureus* was shown to be the predominant organism isolated in all four farms. It made up between 41.67 % (10/24) in Farm B to 66.67 % (6/9) in Farm A, with a 49.10 % overall prevalence of *S. aureus* in all four farms (Table 2). *Staphylococcus intermedius* made up from 8.33% (2/24) in Farm B to 46.15% (2/9) in farm C. Only one species of gram-negative bacteria was isolated which is *Chromobacterium sp.* and this species is commonly found in contaminated water.

Table 2: Bacteria isolated from milk samples obtained from the four studied farms

Organisms	Farm A	Farm B	Farm C	Farm D	Total no.
Staphylococcus aureus	6 (66.67%)	10 (41.67%)	6 (46.15%)	5 (55.56%)	27 (49.1%)
Staphylococcus intermedius	3 (33.33%)	2 (8.33%)	6 (46.15%)	2 (22.22%)	13 (23.64%)
Staphylococcus shleiferi	0	4 (16.67%)	0	0	4 (7.27%)
Streptococcus uberis	0	4 (16.67%)	0	1 (11.11%)	5 (9.10%)
Aerococcus viridans 2	0	3 (12.5%)	1 (7.69%)	0	4 (7.27%)
Corynebacterium sp.	0	0	0	1 (11.11%)	1 (1.81%)
Chromobacterium sp.	0	1 (4.17%)	0	0	1 (1.81%)
Total no. of isolates	9	24	13	9	55 (100%)

4.3 Antibiotic sensitivity test (AST)

Overall AST results showed that *Staphylococcus aureus* have 14.81% resistant towards Oxytetracycline and Tetracyline, 11.11% resistant towards Penicillin G, and 7.41 % towards Gentamycin. *Staphylococcus intermedius* shows same percentage of resistancy toward Penicillin G, Streptomycin and Gentamycin which is 23.07% and 15.38% towards Oxytetracyline and Tetracycline. Based on this study, *Staphylococcus*

shleiferi and *Chromobacterium sp.* have no resistance towards any of antimicrobial tested in this study (Table 3). *Streptococcus uberis* isolated in this study was resistant to Streptomycin. *Aerococcus viridans 2* resistant to Penicillin G, Streptomycin and Tetracycline. Only one sample have *Corynebacterium sp.* isolated and it is resistant to Penicillin G.

Table 3: The antibiotic resistance pattern of the major bacteria isolated from the milk samples

	No. of isolates	Penicillin G	Strepto-mycin	Genta-mycin	Oxytetra-cycline	Tetra-cycline
Staph. aureus	27	66.67%	88.89%	92.60%	14.82%	74.08%
Staph. intermedius	13	54.85%	100.00%	100.00%	30.77%	84.62%
Staph. shleiferi	4	0.00%	100.00%	100.00%	0.00%	35.00%
Strep. uberis	5	0.00%	100.00%	100.00%	0.00%	100.00%
Aerococcus viridans 2	4	50.00%	100.00%	100.00%	50.00%	100.00%
Corynebacterium sp.	1	100.00%	100.00%	100.00%	0.00%	100.00%
Chromobacterium sp.	1	100.00%	100.00%	100.00%	100.00%	100.00%

Staphyloccus aureus is having high susceptibility towards oxytetracyline which is 85.18% (Table 4) although some of the isolates are resistance to oxytetracycline. It is as same as *Staph. intermidius* which have 69.23% susceptibility

towards oxytetracyline. Most of the isolates are more susceptible to penicillin G and oxytetracycline compared to the other three antibiotic as in Table 4.

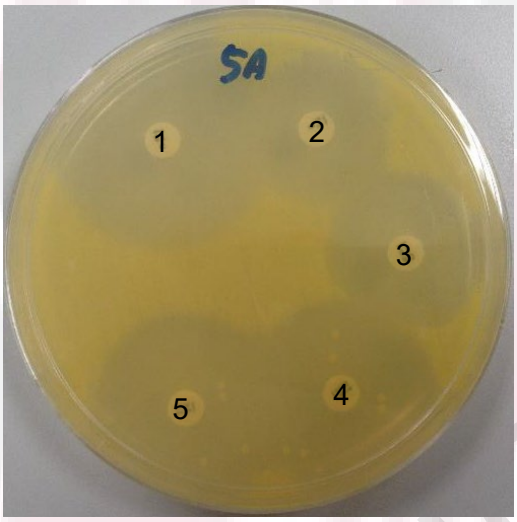

Plate 1: *Staphylococcus sp.*

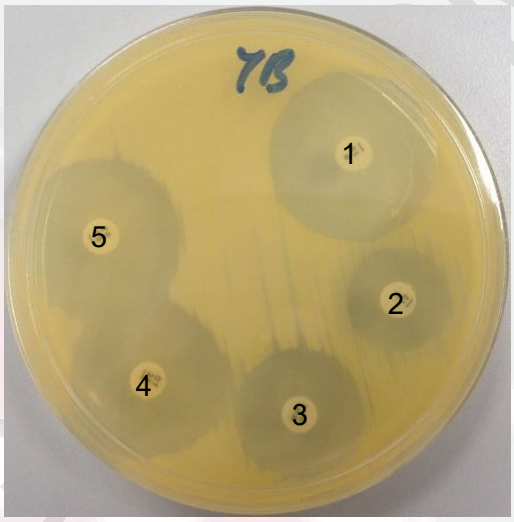

Plate 2: *Staphylococcus sp.*

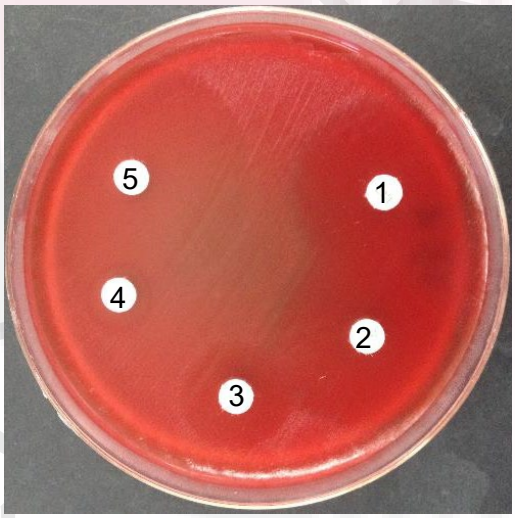

Plate 3: *Streptococcus sp.*

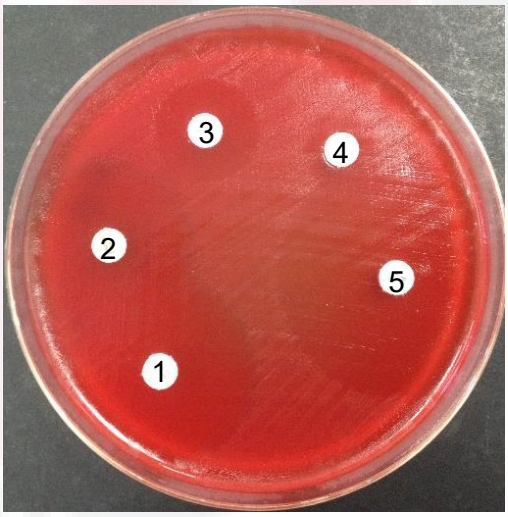

Plate 4: *Streptococcus sp.*

Figure 3: Antibiotic sensitivity test plates of bacteria against five antibiotics

1=Penicillin G; 2=Streptomycin; 3=Gentamycin; 4=Oxytetracycline; 5=Tetracycline

Table 4: Responses to application of antimicrobial disks (susceptibility in %)

Isolates	No.	Responses to application of antimicrobial disks (susceptibility in %)				
		PEN	STR	GEN	OXYTE	TETRA
Staph. aureus	27	33.33%	11.11%	7.40%	85.18%	25.92%
Staph. intermidius	13	45.15%	0%	0%	69.23%	15.38%
Staph. shleiferi	4	100%	0%	0%	100%	75%
Str. uberis	5	100%	0%	0%	100%	0%
Aerococcus viridans 2	4	50%	0%	0%	50%	0%
Corynebacterium sp.	1	0%	0%	0%	100%	0%
Chromobacterium sp.	1	0%	0%	0%	0%	0%

No.=number of observations; PEN=Penicillin G; STR=streptomycin; GEN=Gentamycin; OXYTE=Oxytetracycline; TETRA=Tetracycline

5.0 DISCUSSION

The overall prevalence of subclinical mastitis in the four farms was 56.25 % and this is relatively lower than the earlier prevalence reported Othman and Bahaman, 2005 at 81.7% and 70-100% by Marimuthu et al.,. *Staphylococcus aureus* was the predominant organism isolated from the samples. Of the 55 organisms isolated, 27 (49.10 %) were *S. aureus*, which was higher than the findings of Othman and Bahaman, 2005 which was 28.3%. Staphylococci are the most common cause of mastitis and this is not surprising as their habitats are the skin, mouth, upper respiratory tract and the mammary gland itself. Infection can be easily spread by milking, licking and suckling of the mammary gland. Jain (1979) had suggested that as *S. aureus* had the capacity to penetrate tissues producing deep seated foci, intramammary antibiotic therapy quite often fail to eradicate staphylococcal mastitis. All these factors may possibly contribute to the high prevalence of *S. aureus* infection in these farms. The predominance of *Staphylococcus sp.* as the most prevalent bacteria in subclinical mastitis is higher in this study which is 80.01%, compared to 55% from the report by Marimuthu et al., 2014. Coagulase-negative staphylococci clearly predominated (53.5% positive samples) followed by streptococci and enterococci (both occurring in 16.1% samples) in Czech Republic (Cervinkova, 2013). Another study done in Iran by Hashemi (2011), the most prevalent isolated bacteria were coagulase positive staphylococci followed by streptococci, *Escherichia coli* and coagulase negative staphylococci.

The prevalences of mastitis between the farms were different (Table 1). The inconsistency observed could be due to the different environment and management practices in each farm as some of the farms use combined milking methods. Other major organisms isolated in this study were *Staphylococcus intermedius*, *Streptococcus spp*., and *Corynebacterium spp*. These Gram-positive organisms made up almost 98% (54/55) of the isolates obtained and were the main cause of mastitis. This is not surprising as this group of bacteria is usually found in the animal's environment where they can easily infect mammary glands and cause mastitis. Most of the organisms isolated from this study have also been reported as either contagious or environmental pathogens that are found in raw milk from different parts of the world (Waller et al., 2011; Hogan et al., 2011; Santman-Berends et al., 2012; Hill et al., 2012; Oliver and Muranda, 2012; Chaudhary and Payasi, 2013).

The presence of some bacteria in milk is an indication of contamination. Inappropriate human practice like poor personal hygiene can lead to contamination of water by human waste products (Chaudhary and Payasi, 2013; Hassan and Alkafagi, 2013). *Chromobacterium sp*. is not commonly found in milk, however, there are reports of isolation of *C. violaceum* from raw milk, separated milk and separator drain in organized dairy plant (Reid, 1997).

The antibiotic sensitivity of isolates were determined in this study. It was observed that a significant number of isolates tested was highly susceptible to oxytetracycline. Oxytetracycline has not been used in farm practices as reported in previous studies (Table 4). Bacteria are found to be most resistant to streptomycin and gentamycin. The susceptibility pattern of the tested bacteria differs considerably with

previous studies. Based on study done by Othman and Bahaman in 2005, Itwas observed that a significant number of S. *aureus* tested was resistant to penicillin, chloramphenicol, tetracycline and the sulphonamides. Antimicrobial sensitivity tests of *Staphylococcus aureus* as seen in study done by Marimuthu et al. in 2014, was resistance to penicillin and ampicillin. It is might be due the different systems of production, such as comparing those dairies that use little to no antimicrobials and those that use antimicrobials in all categories of animals in the farm as stated in study done by Marimuthu et al., 2014. It was also found that none of the bacteria isolated are multi drug resistant as all bacteria are resistant to less than three classes of antibiotic.

6.0 CONCLUSION

Prevalence of subclinical mastitis is high at 56.25% in the selected farms around Labis. *Staphylococcus aureus* was the predominant organism isolated followed by *Staphylococcus intermedius*, *Streptococcus uberis*, *Staphylococcus shleiferi, Aerococcus viridans 2, Corynebacterium sp.* and *Chromobacterium sp.*. Isolated microorganism have high susceptibility towards Oxytetracycline, and have high resistance towards Streptomycin and Gentamycin. It is recommended that training and guidance should be given to farmers and animal handlers to prevent or reduce the occurrence of mastitis, farmers should practice more hygienic method in their productions because most of the pathogens causing mastitis are environmental pathogens and also it is usually being transmitted by the workers.

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