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Intan Nurfarzana Mohd Safini
Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia, intanfarzana15@gmail.com

Muhammad Iqbal Hafiz Saad
Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia, iqbalhafizsaad@gmail.com

Zuraifah Asrah Mohamad
Environmental Health Research Centre, Institutes for Medical Research, National Institutes of Health, Malaysia, asrahz@moh.gov.my

Siti Khairiyah Mohd Hatta
Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia, sitikhairiyah@uitm.edu.my

Aziyah Abd. Aziz
Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia, aziyah960@uitm.edu.my

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Authors

Intan Nurfarzana Mohd Safini, Muhammad Iqbal Hafiz Saad, Zuraifah Asrah Mohamad, Siti Khairiyah Mohd Hatta, Aziyah Abd. Aziz, Sophia Karen Bakon, and Norashirene Mohamad Jamil

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SPECIAL ISSUE ARTICLE

Patterns of Antibiotic Resistance in Staphylococci Associated with Bovine Subclinical Mastitis

Intan Nurfarzana Mohd Safini [1], Muhammad Iqbal Hafiz Saad [1], Zuraifah Asrah Mohamad [2], Siti Khairiyah Mohd Hatta [1], Aziyah Abd. Aziz [1], Sophia Karen Bakon [2], Norashirene Mohamad Jamil [1,*]

[1] Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia
[2] Environmental Health Research Centre, Institutes for Medical Research, National Institutes of Health, Malaysia

ABSTRACT

Bovine mastitis, particularly subclinical mastitis (SCM), is a major concern in dairy cattle due to its asymptomatic nature and significant economic impact. In Asia, *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) are key pathogens. The overuse of antibiotics has led to the emergence of multidrug-resistant (MDR) bacteria, posing risks to animal health and food safety. This study aimed to identify staphylococci associated with SCM and assess their antibiotic resistance (AR) profiles. Milk samples were collected from 20 cows at a dairy farm in Malacca, Malaysia, in August and October 2023. SCM was diagnosed using the California Mastitis Test (CMT), and bacterial isolates were cultured on Mannitol Salt Agar (MSA). Antibiotic susceptibility was assessed using the Kirby-Bauer disk diffusion method. CMT results indicated 56% of samples were SCM-positive. From 153 isolates, 84 were presumptive coagulase-positive staphylococci (CoPS) and 69 were CoNS. AR profiling revealed 52% resistance to penicillin, 12% to cefoxitin, and 5% to tetracycline; all isolates were susceptible to erythromycin. Kruskal-Wallis analysis showed significant differences in antibiotic susceptibility across both CoPS and CoNS ($P < 0.001$), indicating variable susceptibility patterns. Sequencing analysis of the *sodA* gene identified that 50% of isolates were *Staphylococcus*, 33% were *Macrococcus*, and 17% were *Macrococcoides*, highlighting their roles in mastitis and AR. This study highlights the importance of SCM and the need for monitoring AR in dairy cattle. These findings emphasize the importance of SCM surveillance and AR monitoring. The emergence of resistance is likely linked to recurrent antibiotic use, underscoring the need for prudent antimicrobial strategies in dairy herd management.

Keywords: Antibiotic resistance, Bovine mastitis, Milk, Subclinical mastitis, Staphylococci

Introduction

In cattle, inflammation of the mammary gland, known as bovine mastitis, is predominantly triggered by bacterial pathogens or physical injury.[1] Mastitis can be either clinical or subclinical based on symptoms.[1,2] Clinical mastitis (CM) is classified with visible alterations of the mastitic cattle milk, while subclinical mastitis (SCM) is characterized by invisible alterations of the mastitic cattle milk and udder.[3,4] Additionally, mastitis can be classified into contagious or environmental according to the primary reservoir and mode of transmission.[5,6] SCM is important due to its high prevalence, late diagnosis, and adverse effects on milk productivity and quality, despite the fact that the majority of cases are asymptomatic.[2] The high burden of SCM leads to the discarding of curdled and unqualified milk, increasing veterinary and treatment expenses, additional labor costs, and culling processes, thereby affecting

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* Corresponding authors.
E-mail addresses: intanfarzana15@gmail.com (I. N. Mohd Safini), iqbalhafizsaad@gmail.com (M. I. Hafiz Saad), asrahz@moh.gov.my (Z. A. Mohamad), sitikhairiyah@uitm.edu.my (S. K. Mohd Hatta), aziyah960@uitm.edu.my (A. Abd. Aziz), sophia.karen@moh.gov.my (S. K. Bakon), norashirene@uitm.edu.my (N. Mohamad Jamil).

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economic losses in fresh milk production and potentially interrupting dairy food consumption.[7]

Various aetiological agents, including bacteria, mycoplasma, and yeast, cause bovine mastitis. Among them, bacteria are the most commonly associated with mastitis, encompassing both Gram-positive and Gram-negative types such as staphylococci, streptococci, *Escherichia coli*, and *Mycoplasma* spp..[7–9] In Asia, staphylococci, particularly *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS), are mainly reported as pathogens associated with mastitis.[8,10,11] The introduction of aetiological agents in raw milk poses a risk to human health because raw or unpasteurized milk is a source of human food poisoning.[12] Therefore, consistent surveillance of bovine health is important to prevent the spread of infection within the herd and to minimize the risk of zoonotic transmission.[13]

Antibiotics are frequently used to treat animals by targeting and eliminating the bacteria responsible for infections and diseases. In bovine mastitis treatment, several antibiotics have been prescribed, including penicillin, sulfonamides, and aminoglycosides.[14,15] However, overprescription of antibiotics may lead to the development of antibiotic-resistant bacteria (ARB), making it more difficult to treat using the current antibiotics, as bacteria may acquire mechsms to resist and survive under antibiotic stress.[16,17] Furthermore, antibiotic-resistance genes (ARGs) can be transferred via mobile genetic elements and may spread to humans and the environment. This highlights the importance of consistency in monitoring and surveillance of antibiotic resistance in bacteria.

The relationship between local farming practices, antibiotic usage, and the emergence of resistant staphylococci remains underexplored, with only a few descriptive studies conducted on the east coast of Malaysia.[10,18] Therefore, this study aimed to isolate staphylococci from bovine milk associated with SCM and to determine their antibiotic resistance profiles. This study is crucial to present baseline data on mastitis cases at the local level, as well as their etiology, and to provide data on ARB by evaluating their antimicrobial susceptibility.

Materials and methods

Study area and design

The milk samples were collected from a dairy farm in Malacca (2.41109° N, 102.21742° E) in August 2023 and October 2023. The farm was selected as the sampling site due to its good housing management, milking and feeding practices, and its registration with the Department of Veterinary Services.

A total of 70 milk samples were screened *in-situ* for mastitis infection using the California mastitis test (CMT). The CMT is commonly used as a diagnostic tool due to its simplicity, rapid results, and cost-effectiveness in diagnosing mastitis in dairy cows. The test involves observing the coagulation formed and the color changes in the milk when the reagent is added. The thickness of gel formation indicates the severity of inflammation. The same volume of two mL of sample and CMT reagent was mixed in the CMT paddle for the reaction to coagulate. The reaction result was classified according to the degree of coagulation: negative (-), trace (T), weak positive (1), and distinct positive (2) according to the Mastitis Council, as in Table 1. The cows were considered associated with SCM if at least one quarter had a trace of CMT reaction.[19]

Sample collection

A composite sampling technique was applied to collect samples following the guidelines of the National Mastitis Council.[20] Approximately two mL from each quarter of bovines' milk were collected by trained milkers into a sterile 50 mL tube, as described by Reyher and Dohoo.[21] All the collected samples were stored at approximately 4°C in an ice box during transportation to the laboratory for analysis.

Bacterial isolation

The samples were processed by spreading onto Mannitol Salt Agar (MSA) using a sterile L-shaped spreader, followed by incubation at 37°C for 24 hours. The expected colonies in yellow resembled CoPS, while those in pink resembled CoNS. The colonies were then further reinoculated until single colonies were obtained. Then, the presumed *Staphylococci* colonies were tested using standard biochemical tests, including Gram staining and the catalase test.

Antibiotic susceptibility test

The Kirby-Bauer disc diffusion technique was employed to determine the antibiotic profiles of the presumed staphylococci isolates.[22] An overnight culture was prepared in Mueller-Hinton Broth (MHB) before proceeding to the next culture preparation of diluting 1:100 in MHB and shaking at 150 rpm, 3 hours at 37°C. The fresh culture was adjusted to a turbidity equivalent to a 0.5 McFarland standard, equivalent to 1 X 10^8 CFU/mL, and then was lawned onto Mueller-Hinton Agar (MHA). A total of six different antibiotic classes were tested using antibiotic discs obtained from Liofilchem®(Italy). The

Table 1. Interpretation of California mastitis test (CMT) scores corresponding to the reaction observed.

	Score	Meaning	Description of reaction	Individual Quarter Sample
	N	Negative	No gel formation was observed in the mixture.	No mastitis
	T	Trace	The mixture exhibited a slimy or gel-like consistency.	Trace of mastitis
	1	Weak Positive	The mixture distinctly transformed into a gel state	Mastitis
	2	Distinct Positive	The mixture thickened and immediately formed a gel	Mastitis

antibiotics included gentamicin (CN, 10 μg), tetracycline (TET, 30 μg), sulfamethoxazole-trimethoprim (SXT, 25 μg), cefoxitin (FOX, 30 μg), penicillin G (P, 10 μg), and erythromycin (E, 15 μg). The chosen antibiotics were commonly used in bovine mastitis treatment and veterinary practices in Malaysia.[10] Then, the results were measured after the incubation period of 24 hours at 37°C. The control strains of *S. aureus* ATCC 25923 (negative control) and *S. epidermidis* ATCC 35984 (positive control) were also tested.[23] The results were interpreted according to the Clinical and Laboratory Standards Institute guidelines.[24]

Amplification of the sodA gene

Amplification of the *sodA* gene was performed using the conventional polymerase chain reaction (PCR) for the resistant isolates. The primer sequences used in this study are d1 (5'-CCITAYICITAYGAYGCIYTIGARCC-'3) and d2 (5'-ARRTARTAIGCRTGYTCCCAIACRTC-'3), the degenerate primer.[25,26] Prior to PCR, bacterial genomic DNA was extracted using the HigherPurity ™ Bacterial Genomic DNA Isolation Kit following the manufacturer's standard protocol (Canvax, Spain). Conventional PCR was then carried out using

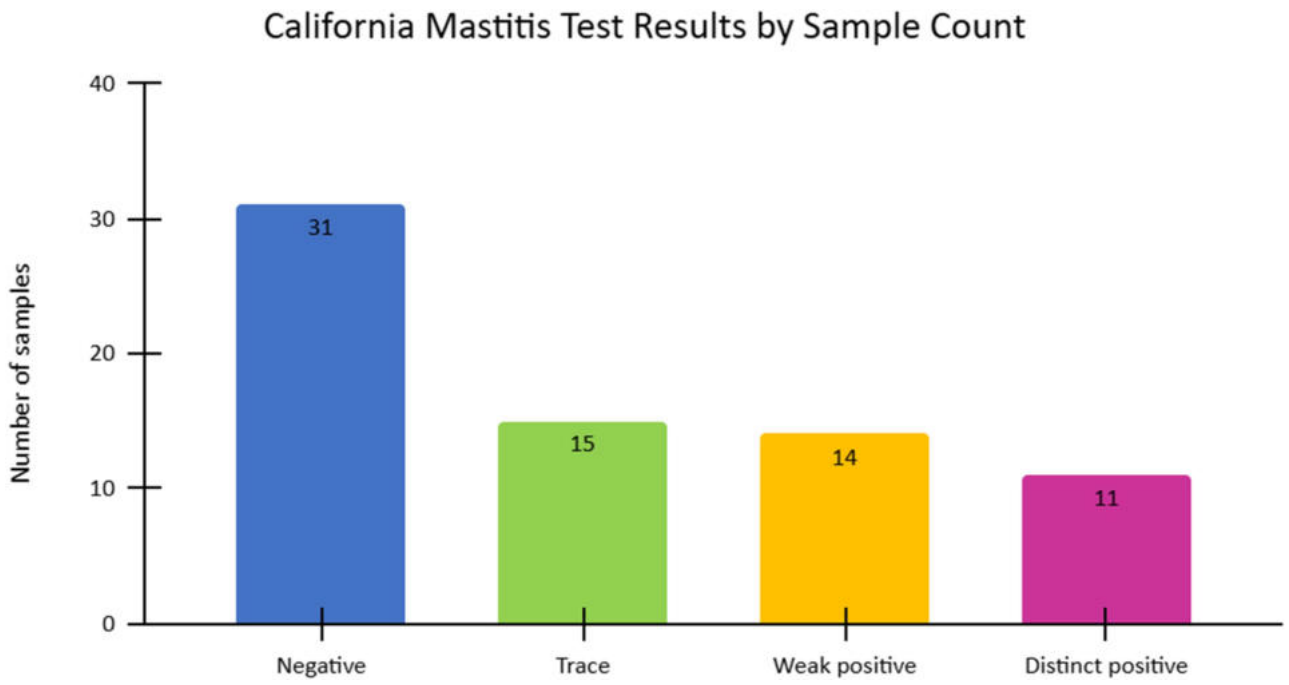

Fig. 1. The CMT score of the tested quarter milk samples (n=70).

MyTaq™ Red Mix (Bioline, USA) under the following conditions: initial denaturation at 95˚C for 3 minutes, followed by 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 37˚C for 60 seconds, and extension at 72˚C for 45 seconds. A final extension was carried out at 72˚C for 10 minutes to ensure complete elongation of the amplicons. The resulting PCR products were subjected to gel electrophoresis at 70V for 70 minutes, purified, and subsequently sequenced. The sequencing results were analyzed using the BLAST nucleotide search program (BLASTn).

Data analysis

The data were statistically analyzed using IBM SPSS Statistics version 20. A Kruskal-Wallis test was performed to assess significant differences in the zone of inhibition among the six tested antibiotics ($P < 0.001$). Post-hoc pairwise comparisons were also conducted to identify specific differences between individual antibiotic groups.

Results and discussion

In this study, 20 composite milk samples, representing 70 quarters, were collected. As shown in Fig. 1, the results revealed that 40% of the quarter milk samples tested positive, with scores ranging from trace (T) to 2, indicating varying degrees of infection severity. Specifically, 21% of the samples showed a trace reaction, suggesting possible early-stage infections, while 20% scored 1, reflecting mild infections, and 15% scored 2, indicating moderate infections. In contrast, 44% of the samples tested negative, demonstrating no signs of infection

Notably, a high percentage of 56% of quarter milks were positively associated with SCM in this study. This is in line with Bentayeb *et al.*[27] and Khasanah *et al.*[28] of 62.77% (59 out of 94 bovines) and 66.72% (395 out of 592 bovines) quarters had SCM, emphasizing the widespread nature of the condition. This relatively high percentage raises significant concern, as it indicates that more than half of the bovines in the sample were affected, albeit without visible clinical symptoms. This asymptomatic condition complicates the identification and management of mastitis because the infected animals may continue to produce milk that appears normal to the naked eye, thus allowing pathogens to remain undetected by consumers and producers alike.

The high mastitis rate observed in this study suggests potential gaps in herd management practices, including milking hygiene, udder health monitoring, and overall farm biosecurity measures. Poor hygiene during milking, inadequate cleaning of equipment, and improper housing conditions may contribute to the spread of mastitis-causing pathogens. Additionally, the lack of routine screening programs may delay the identification of infected animals, allowing the infection to persist and spread within the herd.

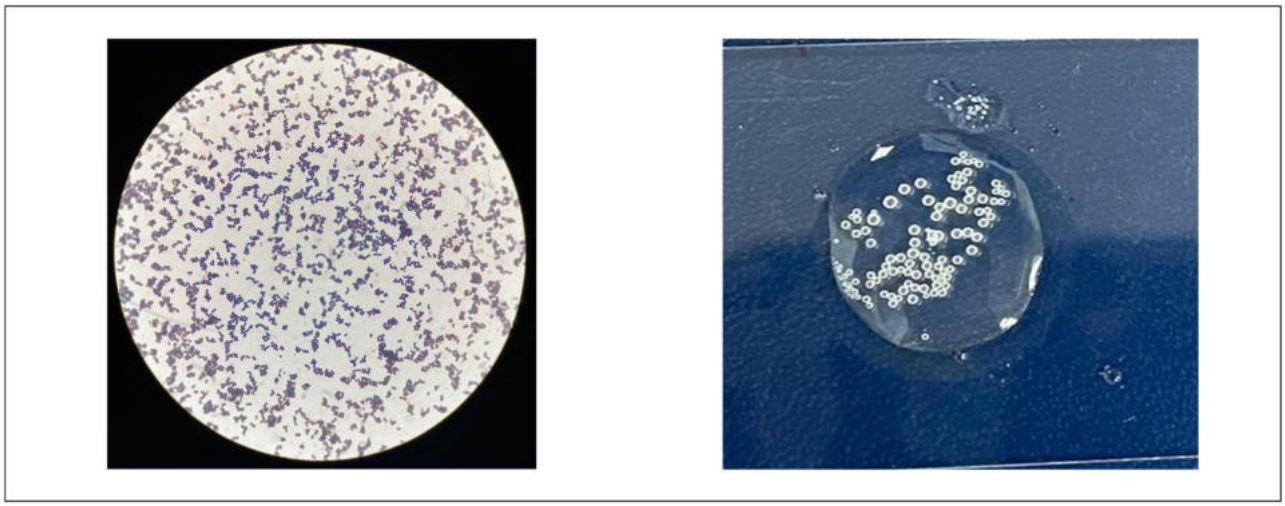

Fig. 2. A- The Gram staining of presumptive *Staphylococcus* Gram-positive isolates under a compound microscope of 100X magnification, and B- The result of a catalase positive reaction with bubble formation.

A total of 153 bacterial isolates were obtained and presumptively identified as *Staphylococcus* species based on their growth characteristics on selective and differential Mannitol Salt Agar (MSA). Staphylococci were intended to be isolated, as they are frequently reported as the causative agents of SCM and are known for being salt-tolerant and either mannitol fermenters or non-mannitol fermenters.[27] MSA contains 1% mannitol and 7.5% salt, which is selective for bacteria that can tolerate high salt levels, particularly *Staphylococcus* spp.. It can also differentiate mannitol fermenting *S. aureus,* causing a color change from red to yellow due to the acidic environment created by fermentation. In contrast, CoNS cannot ferment mannitol and does not cause this color change.[29] Among these isolates, 84 were presumed to be CoPS, which are often associated with pathogenic strains, while 69 were presumed to be CoNS, known to act as opportunistic pathogens. All isolates exhibited Gram-positive staining and tested catalase-positive, as shown in Fig. 2, confirming their identification as *Staphylococcus* spp..

Antibiotic resistance profiles of all presumed *Staphylococcus* isolates against six antibiotics revealed varying degrees of susceptibility, as illustrated in Figs. 3 and 4. Overall, penicillin shows the highest resistance rate of 52%, followed by cefoxitin, tetracycline, trimethoprim-sulfamethoxazole, and gentamicin at 12%, 5%, 3%, and 1%, respectively. Contrarily, all isolates were found to be sensitive to erythromycin, showing no resistance.

Antibiotic residues in milk bring concern due to the spread of ARB to the environment, hence making treatment of the disease ineffective. Additionally, the emergence of antibiotic resistance, driven by unrestricted use of antibiotics across various fields, has led to a significant challenge to veterinary medicine, agriculture, and the human health care system.[30] Notably, resistance against penicillin is quite common among staphylococci isolates.[31] In a previous study on SCM conducted in a few states on the East Coast of Malaysia, *S. aureus* exhibited the highest resistance to penicillin, with 46% (18 out of 39 sample isolates) showing resistance.[10] Similarly, a study conducted in Serdang, Selangor, reported that *S. aureus* isolated from three farms displayed significant resistance to penicillin, with a resistance rate of 38.9% (14 out of 36 isolates).[32] These findings support the results of our study, which also found a high resistance rate of 52% to penicillin. *Staphylococcus* spp. is commonly found to be resistant to penicillin due to the acquisition of *blaZ*, the gene encoding β-lactamase. This enzyme hydrolyzes the β-lactam ring, deactivating penicillin.[33]

Resistance against cefoxitin can occur due to the acquisition of *mecA* and *mecC* genes.[34] These genes encode the altered penicillin-binding protein (PBP2a) that has reduced affinity for β-lactam antibiotics such as cefoxitin and methicillin.[35] Furthermore, other mechanisms confer cefoxitin resistance in *Staphylococcus* spp, through efflux pump proteins that could reduce the intracellular concentration of drug compounds by the cell's active extrusion.[36] Additionally, cefoxitin resistance may result from mutations or alterations in regulatory genes that control the expression of penicillin-binding proteins or other components of the cell wall.[35] This includes alterations that increase the synthesis of proteins that confer

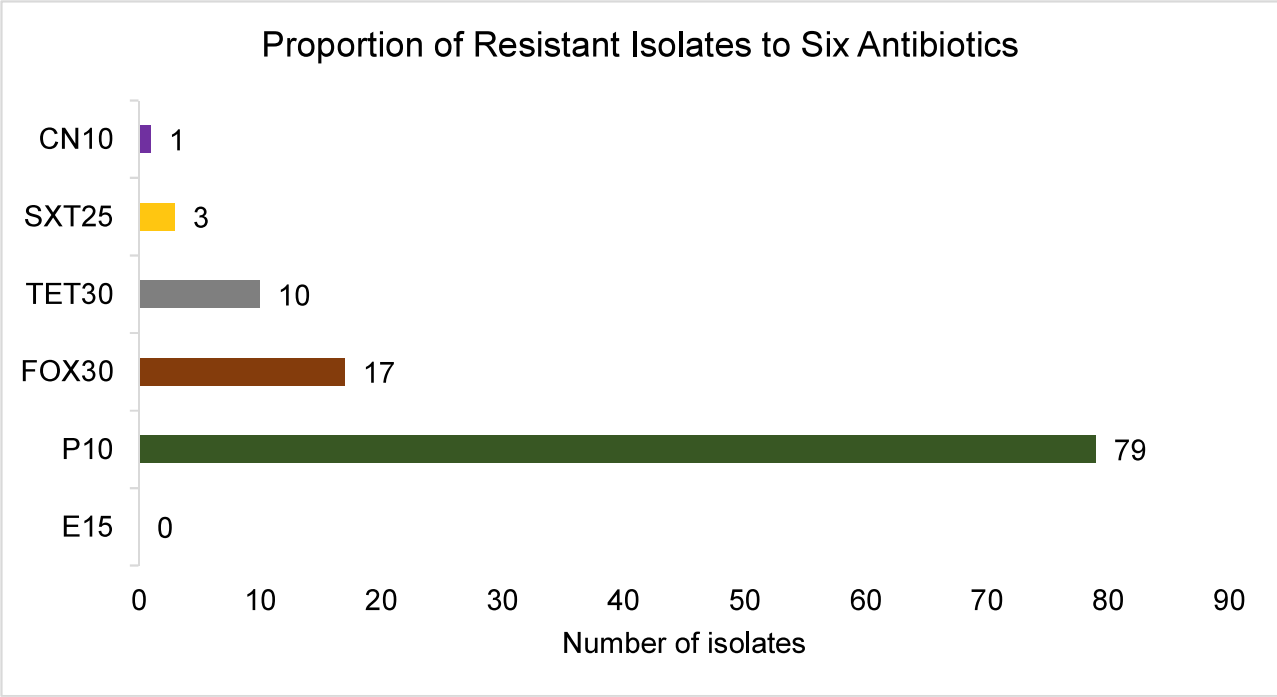

Fig. 3. Proportion of all presumed staphylococcal isolates showing resistance to six different antibiotics (n=153). The antibiotics tested include CN10 (Gentamicin, 10μg), SXT25 (Trimethoprim-sulfamethoxazole, 25μg), TET30 (Tetracycline, 30μg), FOX30 (Cefoxitin, 30μg), P10 (Penicillin, 10μg), and E15 (Erythromycin, 15μg).

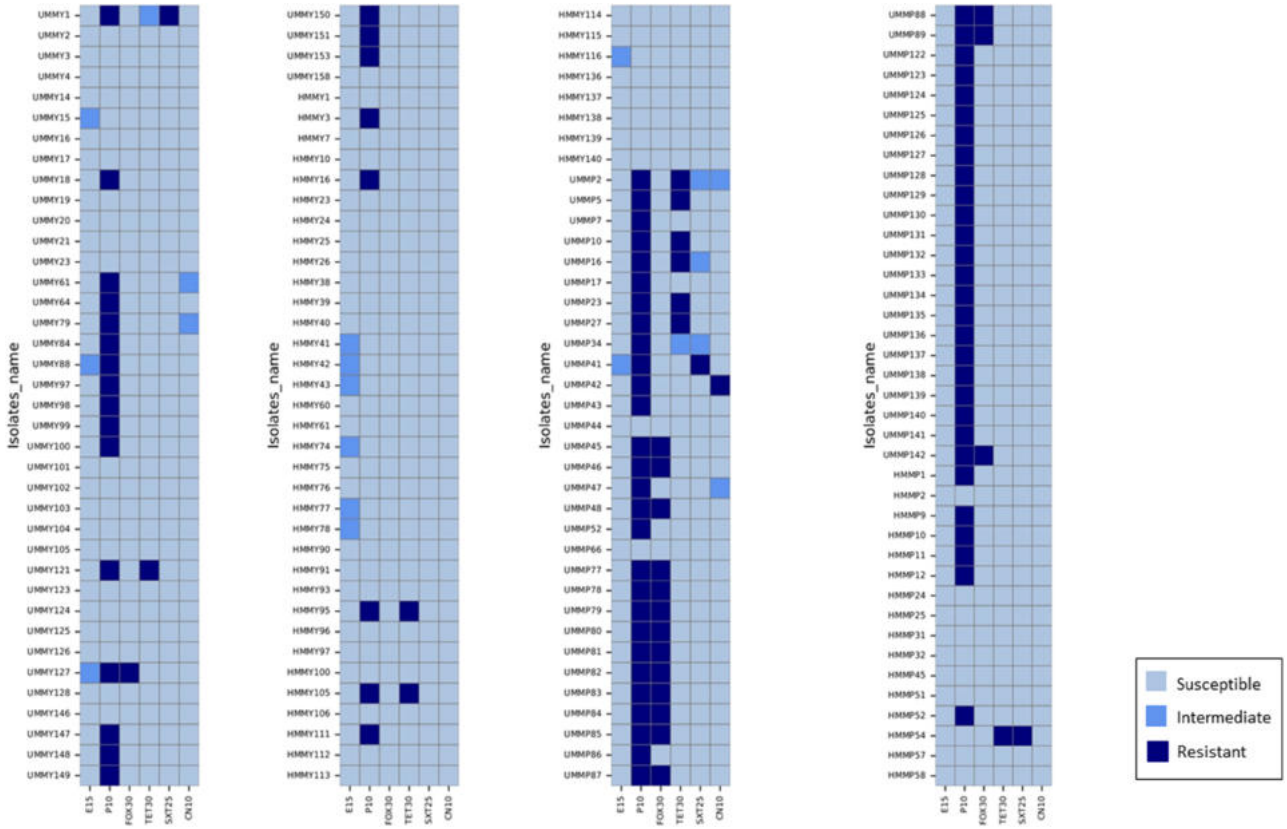

Fig. 4. Heatmap of the resistance profiles of presumed staphylococcal isolates (n = 153).

Table 2. Patterns of antibiotic resistance of CoPS in six different types of antibiotics. Values with different superscripts in a column differed significantly (Kruskal-Wallis pairwise test, P < 0.001).

Antibiotics	Means of zone of inhibition ± standard deviation (mm)	Range (mm)
Cefoxitin	29.38 ± 3.49[a]	44.87–19.00
Erythromycin	25.84 ± 2.86[b]	37.67–20.00
Gentamicin	22.19 ± 3.07[c]	36.27–12.80
Penicillin	31.63 ± 7.72[a]	50.00–13.43
Tetracycline	27.05 ± 5.13[bd]	49.63–10.50
Trimethoprim-sulfamethoxazole	28.22 ± 4.03[ad]	41.07–9.50

resistance or decrease the effectiveness of antibiotics' ability to bind.[37] Cefoxitin resistance in *Staphylococcus* spp. may also be caused by modifications to the peptidoglycan composition in the cell wall.[35] This mechanism makes it more difficult for β-lactam antibiotics to reach their target locations; these structural changes could reduce their effectiveness. In this study, 11% (17 isolates) were found to be resistant to cefoxitin. Resistance against cefoxitin is quite concerning, as resistance to cefoxitin is frequently utilized as an indicator of methicillin-resistant *S. aureus* (MRSA).[38] MRSA strains are well-known for resistance to numerous antibiotics, reducing treatment choices and heightening the risk of infection complications.[38] Furthermore, these resistant strains can also be disseminated from the dairy sector to the communities, posing a significant public health threat.[39] Infections caused by bacteria resistant to cefoxitin can pose greater challenges for treatment, potentially leading to extended hospital stays, heightened healthcare expenses, and increased rates of illness and death, especially among vulnerable patient groups.[40] Therefore, it is crucial to implement effective infection control measures and antibiotic stewardship to mitigate the spread of these resistant strains.

Resistance against tetracycline among staphylococci isolated from bovine milk associated with SCM has been highlighted in previous studies. Gebremedhin *et al.* reported that 72.72% (40 isolates) of staphylococci show high resistance to tetracycline.[41] Additionally, a similar trend of resistance rate against tetracycline was also discovered by Pascu *et al.,* with 62.5% (15 isolates).[42] In contrast to the current findings, 5% (seven isolates) showed resistance to tetracycline. The variability in resistance results could be attributed to the frequency of antibiotic usage in the study area. Staphylococci may acquire the genes *tetK* or *tetL* conferring tetracycline resistance, which is typically carried on a plasmid.[43–45] These genes encode membrane proteins that pump the antibiotics out before they enter the bacterial cell, reducing their intracellular concentration. On the other hand, the acquisition of genes *tetM* or *tetO* provides protection for ribosomes from binding to tetracycline.[33] These genes encode ribosomal protection proteins that prevent tetracycline from interfering with the ribosome, allowing protein synthesis to continue even in the presence of the antibiotic.

In the current study, the high sensitivity of trimethoprim-sulfamethoxazole and gentamicin isolates was determined at 98% and 99%, respectively. Similarly, other studies also reported the highest sensitivity of bacterial isolates toward trimethoprim-sulfamethoxazole (100%) and gentamicin (100%).[10,46] The present research aligns with studies in several states of Malaysia, where it showed sensitivity to trimethoprim-sulfamethoxazole (87.9%) and gentamicin (6.3%).[18] The consistently high sensitivity rate of these antibiotics makes them effective in the treatment. Trimethoprim-sulfamethoxazole, known as co-trimoxazole, acts synergistically by inhibiting the folic acid synthesis that is required for DNA and protein synthesis.[47] The trimethoprim inhibits dihydrofolate reductase required to activate tetrahydrofolate, the active form of folate in DNA and protein synthesis.[47] The sulfamethoxazole acts by inhibiting dihydropteroate synthase that is needed for the conversion of p-aminobenzoic acid (PABA) into dihydropteroic acid, a precursor of dihydrofolic acid in DNA synthesis.[47] Meanwhile, gentamicin disrupts protein synthesis at the 30S subunit, making gentamicin bactericidal and leading to bacterial cell death.[48]

Multidrug resistance (MDR) isolate refers to bacteria that resist at least three different classes of antimicrobial.[49] It is characterized by the insensitivity or resistance of a microorganism to antimicrobial drugs, even when these drugs have diverse molecular targets and are structurally unrelated.[50] In this study, one isolate (UMMP2) was classified as MDR as it exhibited resistance against penicillin and tetracycline. It was also observed that UMMP2 showed intermediate resistance against sulfamethoxazole-trimethoprim and gentamicin.

Table 2 shows the antibiotic resistance profiles of CoPS isolates against six commonly used antibiotics, based on the mean zone of inhibition. The Kruskal-Wallis test revealed statistically

Table 3. Patterns of antibiotic resistance of CoNS in six different types of antibiotics. Values with different superscripts in a column differed significantly (Kruskal-Wallis pairwise test, P < 0.001).

Antibiotics	Means of zone of inhibition ± standard deviation (mm)	Range (mm)
Cefoxitin	28.19 ± 9.10[a]	45.77–9.50
Erythromycin	28.51 ± 3.43[a]	38.83–22.00
Gentamicin	24.19 ± 4.06[b]	35.70–11.00
Penicillin	21.29 ± 7.40[b]	41.80–10.67
Tetracycline	29.24 ± 7.28[a]	48.77–12.00
Trimethoprim-sulfamethoxazole	28.70 ± 8.00[a]	43.03–0.00

significant differences between the antibiotics tested (P < 0.001), as indicated by the different superscripts. Penicillin recorded the highest mean zone of inhibition (31.63 ± 7.72 mm) and the widest range (50.00-13.43 mm), suggesting varied responses among isolates. This variety suggests that some bacterial isolates have developed resistance, possibly due to β-lactamase production, while some bacterial isolates remain highly susceptible. Penicillin resistance remains a concern due to persistent selective pressure either in clinical or agricultural settings.[51,52] Cefoxitin followed with a mean zone of 29.38 ± 3.49 mm (range: 44.87–19.00 mm), indicating intermediate susceptibility and possibly the lack of *mecA*-mediated methicillin resistance in certain isolates. Tetracycline and trimethoprim-sulfamethoxazole had moderate efficacy, with mean inhibition zones of 27.05 ± 5.13 mm and 28.22 ± 4.03 mm, respectively. The broad inhibition range observed for tetracycline (49.63–10.50 mm) further suggests variable sensitivity that is probably influenced by the existence of tetracycline resistance genes (*tet*).[53] Erythromycin and gentamicin exhibited the lowest mean inhibition zones at 25.84 ± 2.86 mm and 22.19 ± 3.07 mm, respectively. These findings could be the result of growing resistance, which could be caused by aminoglycoside-modifying enzymes (AMEs) or macrolide resistance genes (*erm*).[54,55] The AMEs such as acetyltransferases (*aac*), phosphotransferases (*aph*), or nucleotidyltransferases (*ant*), may attributed to aminoglycoside resistance which chemically inactivate the antibiotic.[54] The *erm* genes are responsible for the disruption of the antibiotic-targeted site, which modify the bacterial 23SrRNA, reducing the antibiotic binding and thereby leading to resistance.[56] These findings are concerning since both antibiotics are frequently used in clinical and veterinary settings, and emerging resistance may restrict available treatments.

Table 3 shows the antibiotic resistance profiles of CoNS isolates against six commonly used antibiotics, based on the mean zone of inhibition. The Kruskal-Wallis test revealed statistically significant differences between the antibiotics tested (P < 0.001), as indicated by the different superscripts. Tetracycline recorded the highest means of zone of inhibition and wide ranges (48.77-12.00 mm), highlighting inconsistent susceptibility, possibly due to the presence of resistance genes such as *tetK* or *tetM* in certain isolates.[57] Additionally, the diversity in response can be connected to the exposure to tetracycline in animal husbandry or healthcare settings. Cefoxitin and erythromycin showed high mean zones of inhibition, indicating that the CoNS isolates were comparatively susceptible to these antibiotics. Nonetheless, the wide range for cefoxitin (45.77–9.50 mm) indicates varied responses, suggesting that some isolates are still very vulnerable, others might have resistance mechanisms, such as methicillin-resistant *mecA* gene carriage. Trimethoprim-sulfamethoxazole showed mean inhibition zones (28.70 ± 8.00 mm) with wide ranges (up to complete resistance with a lower bound of 0.00 mm). This resistance may be attributed to the presence of *dfr* genes.[58] However, only a few report the *dfr* genes associated with sulfonamides and trimethoprim resistance in Asia.[58,59] Penicillin (21.29 ± 7.40 mm) and gentamicin (24.19 ± 4.06 mm) showed the lowest mean zones of inhibition, suggesting decreased efficacy. The wide range of penicillin inhibition (41.80–10.67 mm) highlights the high frequency of β-lactam resistance in CoNS isolates, which is frequently associated with changes in penicillin-binding proteins or β-lactamase synthesis. Often in CoNS, gentamicin resistance might be a result of aminoglycoside-modifying enzymes, including *aac*(6').[54]

This study was followed by a confirmatory test using the *sodA* gene through conventional PCR amplification, which produced amplicons of 480 bp, as shown in Fig. 5. The sodA primer was used because it is known to effectively identify staphylococci and it exhibits higher divergence than the 16S rDNA gene.[25] Additionally, the *sodA* gene encodes superoxide dismutase, an essential enzyme for bacterial oxidative stress defense, making it a reliable marker for identifying staphylococcal species at the molecular level. The sequencing analysis of the amplified *sodA* gene

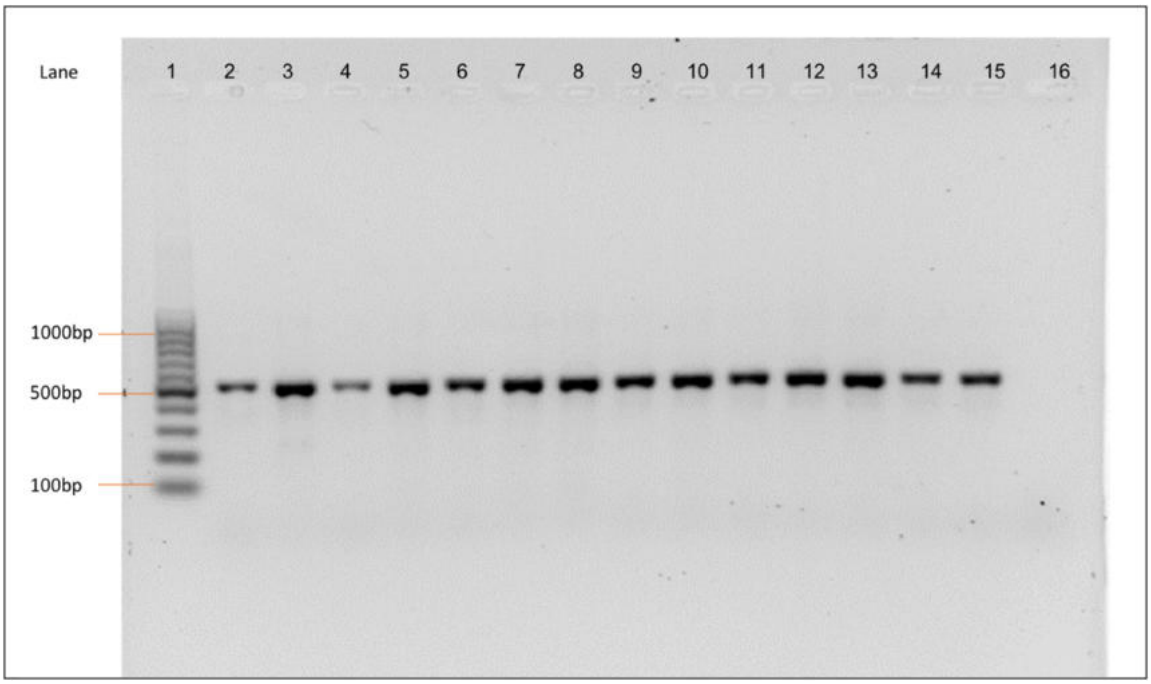

Fig. 5. The amplification of the *sodA* gene of presumptive staphylococci isolates. Lane 1: 100bp DNA ladder (Vivantis), Lane 2: *S. aureus* ATCC 29970, Lane 3: UMMY98, Lane 4: UMMP42, Lane 5: UMMP45, Lane 6: UMMP48, Lane 7: UMMP80, Lane 8: UMMP81, Lane 9: UMMP82, Lane 10: HMMY95, Lane 11: UMMP79, Lane 12: UMMP83, Lane 13: UMMP84, Lane 14: UMMP88, Lane 15: UMMP89, Lane 16: negative control.

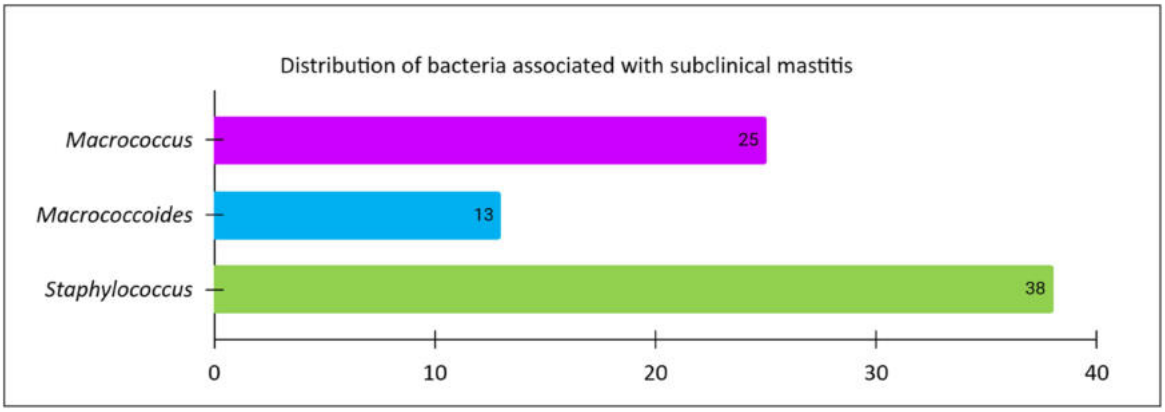

Fig. 6. Bacterial species isolated from bovine milk associated with SCM.

revealed similarity percentages ranging from 92.33% to 99.76%, confirming the identity of isolates. As in Fig. 6, the results portrayed that 50% of the isolates were identified as *Staphylococcus*, 33% as *Macrococcus*, and 17% as *Macrococcoides*.

Approximately 50% of the resistant isolates were identified as *Staphylococcus* species, aligning with their well-known association with bovine mastitis, particularly *S. aureus* and CoNS. These species are major pathogens in mastitis due to their ability to form biofilms, produce virulence factors, and resist antimicrobial treatments, posing challenges to dairy herd management.[31,60]

Interestingly, 33% of the isolates were identified as *Macrococcus* species. The present study agreed with the findings of Schwendener *et al.*[61], who found that 13 strains of *Macrococcus* were isolated from mastitis milk. Similarly, Machado *et al.*[62] identified 21.9% (14 out of 33 isolates) of *Macrococcus* in milk samples. Although *Macrococcus* is closely related to *Staphylococcus* and is generally considered less pathogenic, recent studies indicate that some strains may carry genes conferring antimicrobial resistance. This raises concerns about their potential role as emerging pathogens in mastitis or reservoirs of resistance genes.

Additionally, 17% of the isolates were classified as *Macrococcoides*. These bacteria are less commonly reported in bovine mastitis but may be opportunistic pathogens or contaminants. Their presence highlights the diversity of bacterial species that can colonize bovine udders, emphasizing the need for comprehensive bacterial profiling to guide effective treatment strategies.

Conclusion

The findings of the current study unveiled that *Staphylococcus*, *Macrococcus,* and *Macrococcoides* are the pathogens associated with SCM in bovines, each displaying varying antibiotic resistance profiles. The observed variability in resistance patterns suggests that antibiotic resistance genes play a crucial role in the emergence of resistance, potentially driving the spread of resistant strains. Therefore, identifying and characterizing the specific resistance genes involved is essential for understanding the mechanisms underlying antibiotic resistance and developing effective strategies.

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Authors' declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine/ours. Furthermore, any Figures and images that are not mine/ours have been included with the necessary permission for republication, which is attached to the manuscript.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at University Teknologi MARA Perlis Branch, Malaysia.

Authors' contributions statement

I. N. M. S., N. M. J., and A. A. A. designed the study. I. N. M. S., M. I. H. S., Z. A. M., S. K. B. and N. M. J. and carried out the field work and identification process. The study was analyzed by I. N. M. S., N. M. J., S. K. M. H., I. N. M. S. wrote the first draft of the manuscript. Z. A. M., S. K. B. and N. M. J. revised the final version of the manuscript.

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نماط مقاومة المضادات الحيوية في المكورات العنقودية المرتبطة بالتهاب الضرع تحت السريري في الأبقار

إنتان نورفرزانا محمد صافيني[1]، محمد إقبال حفيظ سعد[1]، زريفة أسرح محمد[2]، سيتي خيرية محمد حتى[1]، أزيه عبد العزيز[1]، صوفيا كارين باكون[2]، نوراشيرين محمد جميل[1]

[1] كلية العلوم التطبيقية، جامعة التكنولوجيا مارا، 40450 شاه علم، سيلانغور، ماليزيا.
[2] مركز أبحاث الصحة البيئية، معاهد البحوث الطبية، المعاهد الوطنية للصحة، ماليزيا.

الملخص

يُعدّ التهاب الضرع البقري، وخاصة التهاب الضرع تحت السريري، مشكلة رئيسية في ماشية الحليب نظرًا لطبيعته عديمة الأعراض وآثاره الاقتصادية الكبيرة. وفي آسيا، تُعد المكورة العنقودية الذهبية والمكورات العنقودية سالبة التخثر (CoNS) من أبرز الممرضات. وقد أدى الإفراط في استخدام المضادات الحيوية إلى ظهور بكتيريا متعددة المقاومة(MDR) ، مما يشكّل مخاطر على صحة الحيوان وسلامة الغذاء. هدفت هذه الدراسة إلى تحديد المكورات العنقودية المرتبطة بالتهاب الضرع تحت السريري وتقييم أنماط مقاومتها للمضادات الحيوية. جُمعت عينات الحليب من 20 بقرة في مزرعة ألبان بولاية ملقا، ماليزيا، خلال شهري أغسطس وأكتوبر 2023. وتم تشخيص التهاب الضرع تحت السريري باستخدام اختبار كاليفورنيا لالتهاب الضرع(CMT) ، وعُزلت البكتيريا على وسط مانيتول سولت آغار .(MSA) كما تم تقييم حساسية المضادات الحيوية باستخدام طريقة كيربي–باور للانتشار بالأقراص. أظهرت نتائج اختبار CMTأن 56% من العينات كانت إيجابية لالتهاب الضرع تحت السريري. ومن بين 153 عزلة بكتيرية، كانت 84 عزلة يُشتبه بأنها مكورات عنقودية موجبة التخثر(CoPS) ، بينما كانت 69 عزلة سالبة التخثر .(CoNS) كشفت أنماط المقاومة للمضادات الحيوية عن مقاومة بنسبة 52% للبنسلين، و12% للسيفوكسيتين، و5% للتتراسايكلين، في حين كانت جميع العزلات حساسة للإريثرومايسين. وأظهر تحليل كروسكال–واليس وجود فروق معنوية في حساسية المضادات الحيوية لكل من CoPS وCoNS (P < 0.001)، مما يشير إلى تباين أنماط الحساسية. وكشف تحليل تسلسل جين sodA أن 50% من العزلات تعود إلى جنسStaphylococcus ، و33% إلىMacrococcus ، و17% إلىMacrococcoides ، مما يؤكد دورها في التهاب الضرع ومقاومة المضادات. وتبرز هذه الدراسة أهمية التهاب الضرع تحت السريري وضرورة مراقبة مقاومة المضادات الحيوية في ماشية الحليب. وتشير النتائج إلى أنّ ظهور المقاومة يرتبط غالبًا بالاستخدام المتكرر للمضادات الحيوية، مما يؤكد الحاجة إلى تبنّي استراتيجيات رشيدة للاستخدام المضاد للميكروبات في إدارة قطعان الأبقار.

الكلمات المفتاحية: مقاومة المضادات الحيوية، التهاب الضرع البقري، الحليب، التهاب الضرع تحت السريري، المكورات العنقودية.