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# Occurrence of Methicillin resistant *Staphylococcus aureus* (MRSA) in Dairy Farms of Wayanad District of Kerala

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**ABSTRACT: *Staphylococcus aureus* is an ubiquitous bacterial pathogen causing food borne diseases. Indiscriminate use of antibiotics has led to the emergence of antimicrobial resistant pathogens in the environment. One among these antimicrobial resistant pathogens being the methicillin resistant *S. aureus* (MRSA), posing serious threat to the lives of people and animals. Therefore, an experimental study was conducted in randomly selected dairy farms of Wayanad district of Kerala, India from January 2023 to March 2023. The objectives of the study was to determine the occurrence of *S. aureus* and MRSA in raw milk of cow and its environment namely, udder washings, and hand washings. The study also attempted to evaluate the *S. aureus* isolates' antibiotic susceptibility patterns. A total of 60 samples, including 20 each of milk, udder washings, and milkers' hands, were collected and analysed. The isolates' susceptibility to various types of antibiotics was evaluated using the disc diffusion technique. The data were analysed using descriptive statistics. The result indicated that 63.33% (38/60) of the samples were positive for *S. aureus*. The occurrence of *S. aureus* was 20%, 80%, and 90% in raw milk, udder washings, and hand washings respectively. Among the 38 *S. aureus* isolates obtained from various samples, 42.11% isolates were resistant to oxacillin followed by 7.89% isolates resistant to cefoxitin. The occurrence of MRSA from the recovered *S. aureus* isolates was 7.89% and these isolates were resistant to cefoxitin. The presence of MRSA indicates possible public health problems, necessitating training and surveillance programmes, as well as severe efforts to curtail indiscriminate antibiotic usage, in order to avoid the disastrous impacts of the Antimicrobial Resistance silent pandemic.**

**Keywords:** AMR, Milk, MRSA, Hand washings, *S. aureus*, Udder washings.

## INTRODUCTION

The discovery of first antibiotic 'Penicillin' by Alexander Fleming in 1928 marked a remarkable history in the field of science that saved a number of people from death and infections. But a century later, the indiscriminate use of antibiotics has raised a global health concern of antimicrobial resistance (AMR). The intensity of this global threat has resulted in the inclusion of AMR as a specific indicator of Good Health and Wellbeing, under Goal 3 of the United Nations 2030 Sustainable Development Goals (Bryan, 2016). The indiscriminate use of the antibiotics led to the emergence and spread of antibiotic resistant microbes in the environment. The frequent use of antimicrobial agents in the livestock industry also contributes towards the spread of the AMR (Economou and Gousia 2015). It is predicted that by 2050, around 10 million people will succumb to death in each year as a result of antibiotic-resistant infections (Clifford *et al*., 2018).

India ranks the world's highest livestock owner with 535.78 million population. Kerala, a small state in southern India has reported a 6.34% rise in state livestock population in the latest 20$^{th}$ Livestock census carried out by Animal Husbandry Statistics Division under the Ministry of Agriculture & Farmers Welfare.

The livestock population went to 2.9 million (2019) from 2.7 million (2022) (PIB, New Delhi). The global demand for the livestock-derived food is expected to rise by 14% per person and 38% overall in between 2020 and 2050 (Komarek *et al*., 2021). In India, with increasing population and household income, there has been a tremendous increase in the demand for the livestock derived food. Livestock population has increased at a rapid pace to meet the demand for the livestock derived foods (Wright, 2022) along with a steep climb in the indiscriminate use of antibiotics. The AMR bacteria found in livestock derived food products may reduce the efficacy of antimicrobial therapies in humans or even in animals.

Methicillin was first introduced as a novel beta-lactam antibiotic to combat *S. aureus* in 1950. Ten years later, presence of Methicillin-resistant *Staphylococcus aureus* (MRSA)was detected in the United Kingdom (Harkins *et al*., 2017).It possessed virulence and fitness characteristics and can survive the selective pressure of many antibiotics. There is an emerging problem of MRSA colonization in food-producing animals due to the widespread improper use of antibiotics in the veterinary sector and their zoonotic transmission to people in contact with livestock (Algammal *et al*., 2020). As a result, the goal of the current study is to evaluate the occurrence of *S. aureus* in dairy farms in the Wayanad district as well as to identify methicillin resistance in recovered isolates.

## MATERIALS AND METHODS

*A. Study area, Duration, and Sampling*

The study area comprised of 20 dairy farms from the Wayanad district of Kerala and duration was for a period of 3 months (from January to March 2023). The samples were collected from apparently healthy crossbred-lactating dairy cows from these selected farms. A total of 60 samples from dairy farms were collected, which comprised of 20 each of milk samples, udder washings, and hand washings. Hand washings were collected from the concerned milkers of these farms.

*B. Transportation of samples*

Udder washings and hand washing samples were collected in the 0.1% Peptone water transport medium. Milk samples along with the udder washings and hand washings were properly labelled and immediately transferred to laboratory under insulated chilled conditions. The collected samples were processed in the laboratory for *S. aureus* isolation and molecular detection.

*C. Isolation, identification and molecular confirmation of S. aureus by culture method*

*S. aureus* isolation and identification were carried out in the method reported by Agarwal *et al*. (2003). The samples were enriched in Tryptone soya broth with 10% NaCl (Hi Media Laboratories Pvt. Ltd, Mumbai) and cultured at 37°C for 24 hours to isolate *S. aureus*. Enriched samples were streaked on Baird parker agar and incubated at 37°C for 48 hours, resulting in a typical greyish-black zone surrounded by a halo zone, later was subjected to molecular analysis. The biochemical identification of the bacterial isolates following isolation was carried out in accordance with the method given by Agarwal *et al*. (2003). Primary test reactions included grams staining, motility test, catalase test, and oxidase test. Voges-Proskauer was performed as a secondary biochemical test reaction. The *S. aureus* isolates tested positive in all the above said biochemical test reactions except the oxidase test.

*D. Detection of S. aureus by molecular method*

DNA was isolated from the *S. aureus* isolates using Genomic DNA Extraction kit (Origin™). The isolated DNA of standard cultures was quantified by a NanoDropTM 2000c spectrophotometer (Thermo Scientific, USA). PCR targeting the *nuc* gene was used to confirm isolated colonies of *S. aureus*. The forward primer (5′- GCGATTGATGGGTGATACGGTT -3′) and reverse primer (R-5′-AGCCAAGCCTTAGACGAACTAAAGC -3′) were used to amplify the *nuc* gene, as described by Brakstad *et al*. (1992). The PCR mix was prepared using ORIonX Taq PCR Smart mix (2X) kit. PCR was performed in an automated thermal cycler (Bio-Rad, USA). The cycling conditions were standardised to arrive at an optimum PCR condition for the specific genes. The PCR cycling conditions consisted of initial denaturation for 5 minutes at 94°C, followed by 35 cycles of amplification (denaturation at 94°C for 50 s, primer annealing at 57°C for 60 s and primer extension at 72°C for 60 s), and final extension at 72°C for 5 minutes.

*E. Phenotypic characterisation of MRSA in the isolates*

By using the disc diffusion method, antibiotic resistance to oxacillin and cefoxitin was examined. A loopful of culture of isolates was streaked onto BHI agar and left to incubate at 37º C for 24h. To obtain an optical density equivalent of 0.5 McFarland Units or 1.5108 CFU/mL for each test isolate, a single colony from the nutrient agar plate was transferred to 1 mL of phosphate-buffered saline (PBS) in a 1.5 mL microcentrifuge tube and vortexed vigorously. On a Mueller-Hinton (MH) agar plate, inoculums with an optical density set to 0.5 McFarland Units were equally distributed using a sterile cotton swab. Antibiotic discs, oxacillin (5µg) and cefoxitin (30µg) were gently pressed onto the infected agar surface at a distance of two to three centimeters, and then incubated at 37 °C for 16 to 18 hours.

The zone of inhibition for each antibiotic was evaluated for the quality control strain first, and subsequently for all test strains. In order to categorise the test isolates for MRSA as either susceptible (S) or resistant (R) to specific antibiotics, the data were compared to an interpretive manufacturer's chart. By measuring the zone of inhibitions with cefoxitin and oxacillin at 21 mm and below, MRSA can be determined. Due to its simplicity of use and greater sensitivity, the cefoxitin disc diffusion test is thought to be superior to the oxacillin disc diffusion test (Joshi and Devkota 2014).

*F. Genotypic identification of the MRSA isolates*

The *S. aureus* isolates confirmed by *nuc* gene expression and biochemical tests was screened for the

presence of *mecA* gene by PCR. To amplify the *mecA* gene, the forward primer (5'-AAAATCGATGGTAAAGGTTGGC-3') and the reverse primer (5'-GTTCTGCAGTACCGGAATTTGC -3') were used as described earlier by Vannuffel *et al.* (1995). The PCR cycling conditions consisted of initial denaturation for 5 minutes at 94°C, followed by 35 cycles of amplification (denaturation at 94°C for 30 s, primer annealing at 58°C for 30 s and primer extension at 72°C for 30 s), and final extension at 72°C for 5 minutes. The PCR product for *nuc* gene and *mec*A gene were confirmed by performing 1.5% agar gel electrophoresis stained with ethidium bromide and then visualised under gel documentation system.

## RESULTS AND DISCUSSION

*A. Isolation and confirmation of S. aureus*

*A* total of 60 samples, milk samples (20), udder washings (20), and hand washings (20), were collected from 20 different dairy farms of Wayanad. All the samples were screened for the presence of *S. aureus*. The isolates found positive was confirmed using the molecular method. The results obtained are outlined in the Table 1.

*Culture based identification of S. aureus*: The sample displayed typical greyish-black colonies with a halo around them after being streaked in BP agar (37°C for 48 hours) after inoculation in Tryptone soya broth at 37°C for 24 hours (Fig. 1). The *S. aureus* isolates was culture positive in 20% (4 samples), 80% (16 samples), and 90% (18 samples) of the milk, udder washings, and hand washings, respectively.

*Confirmation of S. aureus by biochemical tests:* The biochemical tests mentioned in the table 2 were performed to confirm *S. Aureus* isolates. The presence of *S. aureus* was biochemically confirmed in all the samples that was detected culture positive.

*Molecular confirmation of S. aureus*: By detecting the *nuc* gene in the PCR, the recovered *S. aureus* isolates were validated (Fig. 2). Out of 20 each sample collected, 4 milk samples, 16 samples of udder washes, and 18 hand washes were confirmed as *nuc* gene positive.

*B. Antibiotic susceptibility to methicillin resistance*

*Phenotypic characterisation of MRSA*: The phenotypic assay was done using a double disc diffusion assay (CLSI, 2019). Among the 38 *S. aureus* isolates obtained from various samples, resistance to oxacillin (42.11%) was commonly discovered followed by resistance to cefoxitin (7.89%). The phenotypic assay for MRSA is depicted in Fig. 3 and Table 3.

*Genotypic detection of MRSA*: Base on genotypic assay, MRSA was detected in 11.11% (2) and 6.25% (1) in the recovered isolates from hand washings and udder washings. From the total samples collected (n=60), 7.89% of the recovered isolates harboured *mecA* gene. The genotypic assay is shown in Table 3 and Fig. 4.

*S. aureus* is an opportunistic bacterial pathogen and harbours in human skin, hands, noses and throats. This bacterium produces a variety of toxins and may lead to toxin-mediated illness in animals and humans. The overall occurrence rate (63.33%) of *S. aureus* observed in this study was a little higher when compared to the studies conducted by Bhati *et al*., 2018 (54.3%), Kutar *et al.,* 2015 (56%) and Parth *et al.* (2016) (54.29%). The present study reported a higher occurence rate of *S. aureus* in hand washings of milkers when compared to the udder washings and milk samples. This could be because 10 to 35% and 20 to 75% of individuals are chronic and intermittent carriers of *S. aureus*, respectively (Tarekgne *et al*., 2016; Sakr *et al*., 2018). These findings unequivocally show that milk contamination may come from the hands of the milkers. But the *S. aureus* isolated from the milk samples were less when compared to udder washings and the hand washings. This may be due to the better hygienic measures followed by the animal handlers during milking.

Antibiotic susceptibility test conducted under the present study revealed that the *S. aureus* isolates were more sensitive to cefoxitin when compared to oxacillin. Antibiotic susceptibility data obtained in this study are consistent with the experiments conducted by Myllys *et al.* (1998); Gooraninejad *et al.* (2007). A higher percentage of resistance (42.11%) towards oxacillin indicates the presence of MRSA in the environment which gives an alarming signal regarding the indiscriminate use of β- lactam antibiotics in the human and animal health sectors. The concerned class of antibiotics may be used frequently earlier in the area of study have resulted in the development of these antibiotic resistant strains.

The occurrence of MRSA from the recovered *S. aureus* isolates was 7.89% confirmed by detection of *mecA* gene and these isolates were resistant to cefoxitin. High levels of antimicrobial resistance to these therapeutic medications may also be caused by a lack of strict regulation and monitoring in the country regarding the selling and use of antibiotics along with the indiscriminate use. There may be a cross contamination of these resistant organisms between animals and humans while milking and consuming the animal derived products. The AMR residues in the milk can be delivered to humans by consuming milk while the unhygienic practices during milking can transmit these organisms to animals too.

**Table 1: Isolation rate of *S. aureus* in different samples.**

| Sr. No. | Sample Type | Total no. of samples | *S. aureus* | |
|---|---|---|---|---|
| | | | Positive | Percentage (%) |
| 1. | Milk | 20 | 4 | 20 |
| 2. | Udder washings | 20 | 16 | 80 |
| 3. | Hand washings | 20 | 18 | 90 |
| Total | | 60 | 38 | 63.33 |

**Table 2: Biochemical identification of *S. aureus.***

| Tests | Test reaction of *S. aureus* |
|---|---|
| (a) Primary test reactions | |
| *Motility* | + |
| *Catalase test* | + |
| *Oxidase test* | - |
| (b) Secondary test reactions | |
| *Voges Proskauer* | + |

(-) - Negative, (+) – Positive

**Table 3: Antibiotic Susceptibility of *S. aureus* isolates.**

| Sr. No. | Sample Type | *S. aureus* isolates | MRSA genotypic assay | | MRSA phenotypic assay | | | |
|---|---|---|---|---|---|---|---|---|
| | | | *mecA* | | OX | | CX | |
| | | | Positive | Percentage (%) | R | S | R | S |
| 1. | Milk | 4 | 0 | 0 | 3 | 1 | 0 | 4 |
| 2. | Udder washes | 16 | 1 | 6.25 | 6 | 10 | 1 | 15 |
| 3. | Hand washings | 18 | 2 | 11.11 | 7 | 11 | 2 | 16 |
| Total | | 38 | 3 | 7.89 | 16 | 22 | 3 | 35 |

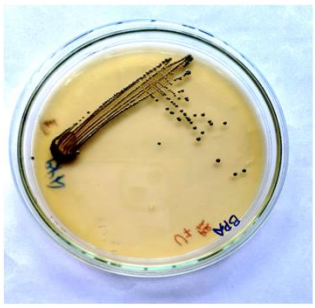

**Fig. 1.** Greyish black colonies in BP agar.

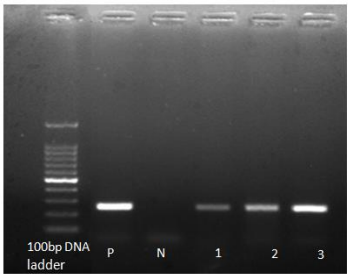


**Fig. 2.** Agarose gel of PCR products of *S. aureus* in lanes 1-3. P: Positive control, N: Negative control.

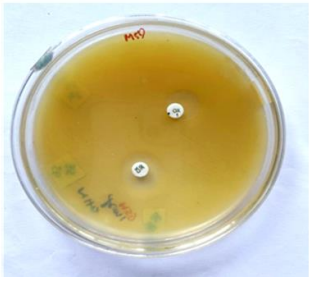

**Fig. 3**. Phenotypic assay for *MRSA.*

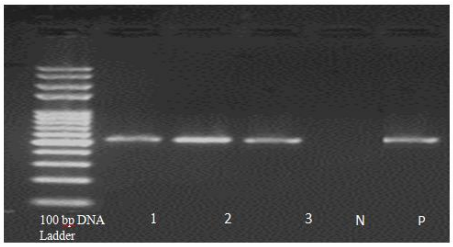


**Fig 4.** Agarose gel of PCR products of *mecA* gene (533bp) of *S. aureus* in lane 1-3; N: Negative control, P: Positive control.

## CONCLUSIONS

The present study confirmed a high occurrence of *S. aureus* in the milker's hand followed by udder washings and milk samples. The recovered *S. aureus* isolates had a high rate of antibiotic resistance, notably to oxacillin. The detection of MRSA in the current study highlights how crucial it is to stop the emergence of resistance genes in the environment. To minimise the growth and spread of drug-resistant bacteria, stringent adherence to antibiotic regime, hygienic practises of milking, along with strict monitoring in the distribution of antibiotics, should be mandatory. Promoting mass awareness campaigns and bolstering the public health education system can both serve as useful means of achieving the objective. In this case, AMR surveillance and monitoring initiatives must be implemented immediately.

## FUTURE SCOPE

Antibiotic resistance levels remain a major concern despite of several strategies followed. A nationwide plan for the containment of AMR, standard treatment recommendations, and antimicrobial policy must be developed and strengthened immediately. To overcome resistance and lessen the selection pressure associated with non-targeting broad-spectrum antibiotics, more researches can be focussed on the use of biologics in targeted delivery using nanocarriers. With the support of continuing development of novel technologies like SELEX, identification of newer therapeutic targets, improved stewardship, and better-informed judgements regarding combination therapy, we anticipate being able to treat MRSA infections for the foreseeable future.

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**Conflict of Interest.** None.

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