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Isolation of Multi Drug Resistant MRSA from Cattle in Mizoram

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ABSTRACT

The experiment was conducted during January to March, 2023 at College of Veterinary Sciences and Animal Husbandry, Selesih, Aizawl, Mizoram to investigate and isolate MDR *S. aureus* from cattle in Mizoram. Nasal swab samples collected from cattle and inoculated in nutritional broth. Then samples were streaked on Baird Parker Agar (BPA) media plates and positive isolates were streaked on the Mannitol Salt Agar (MSA). The yellow colonies were coagulase-positive cocci and *Staphylococci* were recognized as gram-positive cocci by the Gram's staining technique. Suspicious colonies were placed into the nutrient broth tubes and cultured. Catalase test was performed for the confirmation of *Staphylococci* along with standard biochemical verifications. All the phenotypically validated *S. aureus* isolates were kept in nutritional agar, as well as in Luria Bertani (LB) broth for genotypic confirmation. MHA agar was used for the antimicrobial sensitivity test (Methicillin, Sulfafurazole, Cefotaxime, Clindamycin, and Doxycycline) . Isolates were subjected to PCR to detect the presence of the *mecA*, and *femA* genes responsible for MRSA. In this study, 19.05% (8/42) of nasal swab samples were showing the presence of *S. aureus*. The overall percentage of MRSA was found to be 7.14% (3/42). Anti-microbial sensitivity test using different antibiotics revealed MDR *S. aureus* in samples collected from cattle. The genes specific for the MRSA *i.e.*, *mecA* and *femA* could be detected in PCR that is responsible for the virulence of MDR *S. aureus*. This report dealt with the development and possible transmission of MDR *S. aureus* in cattle population of the region.

KEYWORDS: Cattle, antibiotic, MRSA, MDR, *Staphylococcus aureus,* zoonoses

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Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

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1. INTRODUCTION

Staphylococcus aureus is a widespread bacterium that causes a variety of illnesses in people and animals, ranging from minor infections to potentially fatal bacteraemia (Yadav et al., 2018). The bacterium is present in people, domestic animals, and wild animals' anterior nares on a regular basis (Sakr et al., 2018). These resistant have been reported from almost all animals, namely cows, pigs, sheep, dogs, cats and horses (Pexara et al., 2013). The most perplexing issue facing public health in the first ten years of the 21st century is antibiotic resistance (Coque et al., 2023). Bacteria have quick generation times and effective gene recombination systems; this question appears to be more concerning. The best illustration of how antibiotic resistance poses a major global issue is *Staphylococcus aureus* (Urban-Chmiel et al., 2022). Because of its capacity to withstand the effects of several antimicrobial drugs and develop drug resistance, *Staphylococcus aureus* is a matter for concern (De Souza et al., 2012). The two families of antibiotics approved by the Food and Drug Administration to treat *S. aureus* infections in humans and animals are lincosamides (like pirlimycin) and β-lactams (like amoxicillin, penicillin, oxacillin, ceftiofur, hetacillin, and cephapirin) (Kerro Dego and Vidlund, 2024). MDR bacteria, such as MRSA, have emerged as a result of the widespread and indiscriminate use of antibiotics in both human and animal medicine. The development of antibiotic resistance exacerbates *S. aureus* pathogenesis, making treatment of *S. aureus* infections more difficult (Eid et al., 2022).

As a result, *S. aureus* infections in domestic animals have been reported more often recently, seemingly on the rise. According to the pattern of drug resistance, antimicrobial-resistant *S. aureus* is also known as oxacillin-resistant *S. aureus* (ORSA), methicillin-resistant *S. aureus* (MRSA), glycopeptide-resistant *S. aureus* (GRSA), vancomycin-intermediate *S. aureus* (VISA), and vancomycin-resistant *S. aureus* (VRSA) (IB et al., 2014).

Among these resistant bacteria, MRSA has developed serious and terrifying condition for the treatment as it results in medications, such as cephalosporin, and betalactams, being resistant to them and has grown to be a serious concern to the population, responsible for nosocomial and community-acquired illnesses (Pesavento et al., 2007). The World Health Organization has identified MRSA, commonly referred to as "superbug" or "resistant staph," as an organism that requires urgent research and treatment efforts. The rise of livestock-associated MRSA, originating from animals and their products, is concerning due to its growing prevalence and the significant risk of zoonotic transmission across the globe (Anjum et al., 2021, Tamendjari et al., 2021). A major worldwide health concern, the emergence of multidrug-resistant organisms (MDROs) has increased the urgency of managing MDR sepsis (Kumar et al., 2024). The researchers and different pharmaceutical companies are in search of novel antibacterial agents that can resolve the problems of development of resistance (Chandnani et al., 2023). Many different virulence factors are present in *S. aureus*, which gives it considerable pathogenic capabilities. The propensity of *S. aureus* to develop resistance to nearly all kinds of antibiotics is another significant barrier to the management of *S. aureus* infections (Cheung et al., 2021). Livestock have a close relationship with people (farmers, farm workers, and veterinarians), and once they are in the food chain, they may act as easy ways for bacteria to spread, endangering food handlers and consumers (Vanderhaeghen et al., 2010).

Therefore, in order to prevent human infections, MRSA and other antibiotic-resistant *S. aureus* must be continuously monitored by continuing surveillance and management in domestic animals. Hence, it was intended for the current study to isolate and characterise MRSA prevalence and antibiotypes to determine its presence in cattle from the farms in Mizoram, India.

2. MATERIALS AND METHODS

The experiment was conducted during January to March, 2023 at College of Veterinary Sciences and Animal Husbandry, Selesih, Aizawl, Mizoram (Latitude is 23.24733' N and Longitude is 92.83965' E). Nasal swabs were collected from cattle at different parts of Mizoram using separate sterile cotton tipped swabs (HiMedia, Mumbai, India). A total of 42 nasal swabs were taken from different animals and 5 ml of nutritional broth was used to inoculate them for 24 hours at 37°C. The swab sample was streaked on Baird Parker Agar (BPA) media plates and stored for 24 hours at 37°C in the incubator to observe the colony development. Black colour colonies started to develop on the BPA plates after they had been incubated.

The positive isolates were streaked on the selective Mannitol Salt Agar (MSA) for 24 to 48 hours at 37°C after being inoculated in sterile peptone water for 24 hours. This method yields yellow colonies with yellow zones, whereas other coagulase-negative *Staphylococci* produce small pink or red colonies with no colour change to the medium. *Staphylococci* were recognized as gram-positive cocci by the Gram's staining technique and coagulase-positive cocci. From each MSA plate, one or two typical suspicious colonies were placed into nutrient broth tubes and cultured at 37°C for 24 to 48 hours. After the incubation time, a loop full of nutrient broth was spread over the nutrient agar plates and incubated at 37°C for 24–48 hours. For the catalase test, a tiny portion of the colony was then exposed to a clean, dry

glass slide by using a loop, and a few drops of 10% hydrogen peroxide were then applied to the culture. The presence of oxygen bubbles served as *Staphylococcus*' confirmation. The pure isolate colonies underwent further biochemical verification. Standard biochemical assays, such as the citrate utilization, Indole, Methyl red, and Voges-Proskauer, were performed on all of the isolates. All of the *S. aureus* isolates that had been phenotypically validated were kept as pure cultures in semi-solid nutritional agar at 4°C after being properly sealed with paraffin wax, as well as in Luria Bertani (LB) broth containing 25% glycerol (v/v) at -20°C for future genotypic confirmation.

2.1. Antimicrobial susceptibility test

Muller-Hinton agar (MHA) was used to conduct antimicrobial susceptibility tests using the disc diffusion technique. The following antibiotic disks were used: Methicillin, Sulfafurazole, Cefotaxime, Clindamycin, and Doxycycline respectively. Under steady shaking at 37°C for an overnight period, the pure positive isolates were added to 5 mL of sterile Luria Bertani (LB) broth. With the use of sterile spreaders, the overnight broth cultures were distributed equally across MHA plates. Using sterile forceps, antibiotic discs were positioned on the inoculated MHA surface at a distance of roughly two cm. and kept it for overnight in the incubator at 37°C. Antibiotic sensitivity testing with methicillin disc allowed for the diagnosis of MRSA.

2.2. Molecular characterization of S. aureus

To detect the presence of the *mecA,* and *femA* genes responsible for MRSA isolates were subjected to PCR. Snap chilling method was used to recover DNA from the isolates (Franco et al., 2008). The PCR amplification of *mecA* gene, *femA* and *femB* gene was done (Zuniga et al., 2020). Details of primers are given below (Table 1).

The PCR was carried out in a final volume of 25 µl, which contained 12.5 µl of 2 x master mixes, 1 µl of forward and reverse primer, 4 µl of DNA template, and 6.5 µl of nuclease-free water. The *mecA* gene's PCR cycling conditions were initial denaturation at 94°C for 30 seconds, followed by 40 cycles at 94°C for 30 seconds and, annealing at 55°C for 30 seconds. Then elongation had done at 72°C (30 seconds) and final extension at 72°C (5 minutes). While the *femA* gene was amplified, the temperature was maintained at 94°C for 30 seconds for initial denaturation, 30 cycles at 94°C for 30 seconds, 50°C for 30 seconds for annealing, 72°C for 30 seconds for elongation, and 72°C for 5 minutes for final extension. According to the procedure Heng (2022) the amplified products were visualized by passing them across a 1.5% agarose gel that contained 0.5 ug ml^{-1} ethidium bromide and validated by seeing a certain band size (bp) on an Agarose gel and interpreting the results using a gel documentation system.

Table 1: Oligonucleotide primers used for detection of *mecA* gene, and *femA* gene

Target gene	Primer sequence (5'-3')	Base pair	Reference
mecA	F: AAAATCGATGGTA-AAGGTTGGC R: AGTTCTGCAGTAC-CGGATTTGC	533	Idrees et al. (2023)
femA	F: AGACAAATAGGAG-TAATGAT R: AAATCTAACACT-GAGTGATA	509	Zuniga et al. (2020)

3. RESULTS AND DISCUSSION

Among the 42 samples subjected for culturing in BPA media, 36 samples were showing the growth pattern specific for *Staphylococcus*. These 36 samples were cultures in MSA and found 8 samples as positive for *S. aureus*. All these 8 samples were subjected for Grams' staining, Catalase test, MR test and VP test. The results are depicted in Table 2.

Table 2: Isolation and identification of MRSA isolates in different media

Laboratory Test	Total No.	Positive No.	Percentage (%)
Culture on BPA media	42	36	85.71
Culture on manitol salt agar	36	8	22.22
Grams staining	8	7	87.5
Catalase test	8	8	100
MR (Methyl Red) test	8	7	87.5
VP (Voges Proskauer) test	8	6	75
Indole test	8	0	0
Citrate utilization test	8	6	75
MRSA in antibiotic sensitivity test	8	3	37.5

Antibiotic Sensitivity Test (ABST) was done for all these 8 samples after the isolation and identification. Methicillin resistance was observed in 3 samples among the 8 samples. Cefotaxime, Sulfisoxazole, Clindamycin and Doxycycline resistance was observed in 5 samples. The results are given in Table 3.

In PCR, *mecA* and *femA* could be identified in samples. The PCR results are depicted in Figure 1.

In this study, it was observed that 19.05% (8/42) of nasal swab samples were showing the presence of *S. aureus*. Nasal carriage of *S. aureus* was 9.97% in 421 cattle at Morocco (Mourabit et al., 2020). But it is recorded that 12% of *S.*

Table 3: Antibiotic sensitivity of *S. aureus* isolates from nasal swabs of healthy dairy cattle

Antibiotics	Conc. (µg/disc)	No. of resistant isolates	No. of susceptible isolates
Methicillin (MET)	5 mcg	3	5
Cefotaxime (CTX)	30 mcg	5	3
Sulfisoxazole (SF)	300mcg	5	3
Clindamycin (CD)	2 mcg	5	3
Doxycycline (DO)	30 mcg	5	3

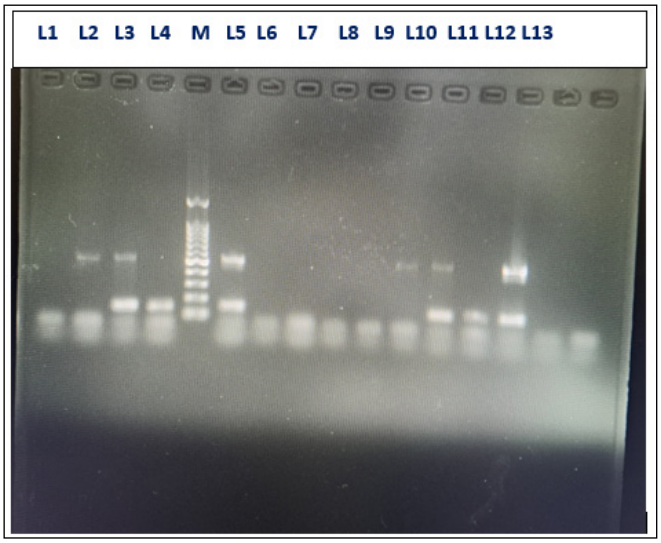

Figure 1: Lane 2, 3: *mecA*, M: 100 BP Ladder, Lane 5: *femA*, Lane 9: Negative control and Lane 10, 11 and 12: Positive Control

aureus prevalence in nasal samples from 249 cows (Titouche et al., 2019). Nasal carriage of *S. aureus* in healthy cattle is a potential source of antimicrobial resistance (Rahimi et al., 2015).

In this work, 42 samples collected from cattle were studied for the presence of MRSA using isolation, identification, ABST and PCR. The overall percentage of MRSA was found to be 7.14% (3/42). Khanal et al. (2022) observed that MRSA is prevalent in several dairy cattle farms with 3.91% prevalence in cattle milk and there is a possibility of transmission to general public. In general the presence of *Staphylococcus aureus* is an indication of unhygienic condition of premises (Roy et al., 2023). Subclinical mastitis cases in Hungary were caused by MRSA and these strains were indistinguishable from the isolate of a worker in the farm pointing to the possibility of animal-human transmission or *vice versa* (Krukowski et al., 2020).

The *S. aureus* isolated in this work were resistant to multiple antibiotics. Presence of multi drug resistant (MDR) *S. aureus* in their research work in cattle. This is an alarming situation where the isolates were becoming MDR and they are becoming a potential source of transmission to human (Eid et al., 2022, Khanal et al., 2022).

In our study, *mecA* and *femA* genes were used for the detection of MRSA. This way of MRSA detected where they concluded that healthy bovines can be reservoirs of this zoonotic pathogen (Van Duijkeren et al., 2014). The presence of *mecA* genes were reported by several authors (Eid et al., 2022). The methicillin resistance could be attributed to this gene as it encodes an additional Penicillin Binding Protein (PBP) termed PBP2' and PBP 2a that can reduce the affinities for binding to beta lactum antibiotics (Havaei et al., 2015, Rao et al., 2017). Similarly, *femA* genes were reported by other researchers like Mekonnen et al., 2018.

4. CONCLUSION

The isolation of multidrug-resistant MRSA from cattle in Mizoram was documented in this study. The possibility of zoonotic transmission and the emergence of antibiotic resistance made the detection of MRSA in cattle a serious public health concern. This has to increased livestock MRSA surveillance and monitoring, better farm biosecurity protocols, and appropriate antibiotic use.

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