**Molecular study on Methicillin-Resistant *Staphylococcus aureus* isolated from sheep**

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**Abstract:**

This study investigated the presence of MRSA in sheep. The study was conducted in 2022 using 100 samples, including 50 raw milk samples and 50 nasal swabs obtained from 10 flocks of sheep that were housed in various places along the North Coast area of Egypt to identify and characterize MRSA. *Staphylococcus aureus* was isolated and identified from the samples using conventional bacteriological techniques, while MRSA was found using a culture on oxacillin resistance screening agar basal medium (ORSAB). Therecovery rate of *S. aureus* in the examined samples of sheep was 54 and 18 %, in raw milk samples and nasal swabs of sheep, respectively. In addition, the prevalence of confirmed MRSA strains isolated from sheep samples was 51.6 and 66.7% in milk samples and nasal swabs, respectively with total prevalence of 55.6% (20 out of 36 isolates). The antimicrobial susceptibility testing of the MRSA isolates in this study revealed varying resistance patterns. The isolates had 100% resistance to Penicillin-G and Clindamycin, 90% resistance to Linezolid,80% resistance to Cefoxitin and 70% resistance to Erythromycin. In short, strict hygiene and biosecurity protocols should be implemented on sheep farms to avoid this from turning into a serious illness that may be deadly. According to the present inquiry, MRSA was discovered in the milk and nasal samples of sheep grown on Alexandria's North Coast that appeared to be in good health.

**Keywords**: MRSA, Antibiotic Resistance, Milk, Nasal swabs, sheep

**1. Introduction:**

*Staphylococcus aureus* is the most prevalent skin-colonizing bacterium and the main factor in nosocomial and community-associated skin infections is Staphylococcus aureus. **(Turner et al., 2019).** It is also an important opportunistic pathogen whose pathogenicity depends mostly on extracellular proteins, such as enzymes and exotoxins, which have a role in the emergence of a number of diseases in both human and animal species **(Cheung et al., 2021).**

*Staphylococci* are common bacteria that may infect the skin and soft tissues, especially when the host's immune system is weak. Cleaning pens, milking (either from the hands of the milkers’ to the animals or from the hands of the animals to the milkers’), feeding, or stroking the animals all involve close human-animal interaction. Some farms raise their own sheep, using the animals' meat or milk for either human consumption or the production of cheese**(Daaloul-Jedidi et al., 2016).**

Animals' skin and nasal mucosa are colonized by *S. aureus*, which lives there as a commensal. Due to the large number of animals that are in close proximity to one another in a confined space during lambing and kidding, infection is frequently a possibility. Small ruminants are therefore thought to be a possible source of staphylococcal infections and environmental pollution through their milk, skin, faeces, or nasal cavities**(Ali et al., 2017).**

*S. aureus,* which is the most common cause of mastitis in ruminants worldwide, has also been linked to cases of intra-mammary infections in cattle, sheep, and goats. The bacteria that is causing the infection in subclinical mastitis may be transmitted to milk without significantly altering the product's flavour, aroma, texture, or appearance, infecting consumers through the dairy business **(Schmidt et al., 2017)**.

This pathogen's high diversity, which allows it to appear at various times and locations with various clonal types and antibiotic resistance patterns across regions and nations, is the main factor contributing to its success **(Dai et al., 2019).**

By generating a particular penicillin-binding protein, PBP2 (or PBP2a), MRSA has decreased affinity for binding to -lactam antibiotics, leading to -lactam antibiotic resistance **(Lade and Kim, 2021).** The majority of the regularly used antimicrobial drugs, such as aminoglycosides, macrolides, chloramphenicol, tetracycline, and fluoroquinolones, are also resistant to Methicillin (oxacillin) -resistant *S. aureus* **(Kavya et al., 2018).** In addition, they have been reported to be resistant to all cephems, cephalosporins, and other -lactams, including ampicillin-sulbactam, carbapenems, and piperacillin-tazobactam, regardless of the results of in vitro tests conducted with those drugs **(Bitrus et al., 2016).**

Bovine (milk, milk products, and nasal tissue) had several MRSA strains identified; however there is limited data on the frequency and distribution of MRSA in sheep **(Gulani et al. 2016).** So, this study investigated the occurrence of MRSA in sheep reared in North coast and El America, Alexandria and determined the antimicrobial susceptibility and molecular characterization of the MRSA isolates.

**2. Materials and methods:**

**2.1. Sample collection and transportation:**

Samples were gathered from June through September over a 4-month period. In the North Coast and Al America District area of Egypt in 2022, 50 ewes from 10 sheep flocks living in various places provided milk and nasal swab samples. The animals were multiparous and ranged in age from 2 to 7 years. Prior to sampling, information was gathered from the farmers on the animals and management procedures, including the age, breed, housing arrangement, use of antibiotics, and mastitis issues. According to physical and clinical evaluations, none of the sheep in this research that had their milk tested had mastitis, and the milk samples themselves were normal with no obvious clots. Farmers confirmed the routine use of antibiotics when animals were sickly and lacked appetite.

After disinfection with 70% alcohol while wearing gloves, raw milk samples were taken from nursing ewes' udders in sterile universal bottles. Using sterile swab sticks placed into the left and right nostrils, nasal swab samples were obtained by gently rolling the swab on the nasal epithelial walls. The correctly labeled milk and nose swab samples were taken to the lab for quick examination, where they were kept in insulated boxes with ice packs.

**2.2. Isolation and identification of *S. aureus* from milk and nasal swabs:**

Each nasal swab was pre-enriched with 5 mL of TSB supplemented with 6.5% sodium chloride, and two microliters of each milk sample were pre-enriched with 18 mL of TSB supplied with 6.5% sodium chloride. These samples were then incubated at 37 °C for 24 hours. A loopful of the overnight broth culture was then streaked onto plates of Mannitol salt agar. Suspected yellow colonies were subcultured on sterile nutrient agar plates after 24 hours of incubation at 37 °C. For further biochemical studies, the purified S. aureus isolates were stored on nutrient agar slopes at 4 °C. Gram staining, cultural morphology and biochemical traits such as the catalase test, oxidase test, and coagulase test were used to identify *S. aureus* **(Cheesbrough, 2006).**

**2.3. Phenotypic identification of methicillin-resistant *S. aureus* (MRSA):**

Coagulase-positive *S. aureus* isolates were determined phenotypically by placing them on selective media oxacillin resistance screening agar basal medium (ORSAB). All plates were incubated at 35 °C for 18 h. Isolates that grew on the medium were categorized as methicillin resistant *S. aureus* **(Arunava et al., 2014).**

**2.4. Antimicrobial susceptibility testing:**

To assess the antimicrobial susceptibility pattern using the Bauer-Kirby approach, antimicrobial susceptibility testing was done using the disc diffusion method **(CLSI, 2018).** Ten antimicrobial agents (Oxoid, Hampshire, UK) were used including Amikacin, Cefoxitin, Clindamycin, Erythromycin, Gentamicin, Levofloxacin, Linezolid, Penicillin-G, Sulphamethoxazol and Vancomycin for the antimicrobial susceptibility testing.

**2.5. DNA extraction and molecular confirmation of MRSA by PCR assay**

DNA extraction was performed by boiling method **(Sambrook et al., 1989)** after subculturing the presumptive MRSA isolates on nutrient agar plates. The extracted DNA was amplified using species-specific primer targeting *nuc* gene of *S. aureus*. The forward primer Sa-*nuc*-F 5′- GTGCTGGCATATGTATGGCAATTG -3′ and reverse primer Sa-*nuc*-R 5′- CTGAATCAGCGTTGTCTTCGCTCCAA -3′ encoded an amplicon size of 660 bp. Furthermore, molecular identification of MRSA strains was done by using the primers (*mec*A-F: 5′ - GATTGGGATCATAGCGTCA-′3 and *mec*A-R: 5′ - CAGTATTTCACCTTGTCCG-′3) and the product size was 1200 bp **(Sallam et al., 2015).**

The uniplex PCR reaction mixture, with a total of 25µl, consisted of 12.5 µl of 2X TOP simple TM DyeMIX-nTaq (enzynomics, Cat. #P501T), 1 µl of each primer of 10 µM working concentration (Europhins Scientific laboratories, Japan), 5.5 µl of nuclease-free water and finally 5 µl of DNA template. The reaction mixture was moved to the thermal cycler (Applied Biosystems, Foster City, CA, USA) and cycled once at 94°C for 2 min, followed by 35 cycles at 98°C for 10 s, 58°C for 30 s, and 68°C for 1 min, and then a final extension once at 68°C for 7 min. PCR products (Five µL) were analyzed by electrophoresis in 1x TBE electrophoresis buffer and on 1.5% (w/v) agarose gel at 100 V for 60 min.

Ladder (100 bp) (enzynomics, Cat. #DM001) was used to conclude the sizes of DNA products. The gel documentation system took pictures of agarose gels after they had been stained with ethidium bromide (Alpha Innotech, Biometra).

**2.6. Statistical analysis:**

ANOVA statistical analysis was carried out using SAS software **(SAS, 2014).**

**3. Results:**

**Table (1):** Recovery rate of *S. aureus* in the examined samples of sheep

|  |  |  |  |
| --- | --- | --- | --- |
| **Source of samples** | **No. of samples** | **Positive** | |
| **No.** | **%** |
| **Raw milk** | 50 | 27 | 54.0 |
| **Nasal swabs** | 50 | 9 | 18.0 |
| **Total** | **100** | **36** | **36.0** |
| Recovery rate of *S. aureus* was significantly higher (p < 0.05) in raw milk than in nasal swabs. | | | |

**Table (2):** Prevalence of confirmed MRSA strains isolated from sheep samples

|  |  |  |  |
| --- | --- | --- | --- |
| **Source of samples** | **No. of samples** | **Positive isolates on ORSAB** | |
| **No.** | **%** |
| **Raw milk** | 27 | 14 | 51.6 |
| **Nasal swabs** | 9 | 6 | 66.7 |
| **Total** | **36** | **20** | **55.6** |
| Prevalence of confirmed MRSA strains was not significantly higher(p > 0.05) in milk samples than in nasal swabs. | | | |

**Table (3):** Antimicrobial sensitivity testing of MRSA isolates (n= 20) obtained from milk samples of sheep

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Antimicrobial agents** | **Sensitive** | | **Resistant** | |
| **No.** | **%** | **No.** | **%** |
| **Amoxicillin-clavulanate** | 4 | 20.0 | 16 | 80.0 |
| **Cefoxitin** | 4 | 20.0 | 16 | 80.0 |
| **Clindamycin** | 0 | 00.0 | 20 | 100.0 |
| **Erythromycin** | 6 | 30.0 | 14 | 70.0 |
| **Gentamicin** | 17 | 85.0 | 3 | 15.0 |
| **Levofloxacin** | 9 | 45.0 | 11 | 55.0 |
| **Linezolid** | 2 | 10.0 | 18 | 90.0 |
| **Penicillin-G** | 0 | 00.0 | 20 | 100.0 |
| **Sulphamethoxazol** | 11 | 55.0 | 9 | 45.0 |
| **Vancomycin** | 12 | 60.0 | 8 | 40.0 |

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| **Fig. (1):** Agarose gel electrophoresis for PCR product of *nuc* and *mec*A genes for characterization of MRSA strains obtained from milk samples of sheep. The amplified amplicon size was of 660 bp and 1200 bp for *nuc* and *mec*A genes,respectively.  Lane M: 100 bp DNA marker,  Lanes from 1 to 14 are representative of positive MRSA strains. |

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| **Fig. (2):** Agarose gel electrophoresis for PCR product of *nuc* and *mec*A genes for characterization of MRSA strains obtained from nasal swabs of sheep. The amplified amplicon size was of 660 bp and 1200 bp for *nuc* and *mec*A genes,respectively.  Lane M: 100 bp DNA marker,  Lanes from 1 to 6 are representative of positive MRSA strains. |

**Table (4):** Prevalence of confirmed MRSA strains in milk samples of sheep in relation to age groups

|  |  |  |  |
| --- | --- | --- | --- |
| **Age groups** | **No. of samples** | **MRSA** | |
| **No.** | **%** |
| **< 2 years** | 11 | 2 | 18.2 |
| **2 - ˂ 5 years** | 27 | 8 | 29.6 |
| **≥ 5 years** | 12 | 4 | 33.3 |
| **Total** | **50** | **14** | **28.0** |
| Prevalence of confirmed MRSA strains was significantly associated with age groups of sheep (p < 0.05). | | | |

**Table (5):** Prevalence of confirmed MRSA strains in milk samples of sheep relation to breeds

|  |  |  |  |
| --- | --- | --- | --- |
| **Breeds** | **No. of samples** | **MRSA** | |
| **No.** | **%** |
| **Baladi** | 10 | 3 | 30.0 |
| **Barki** | 20 | 5 | 25.0 |
| **Rahmani** | 20 | 6 | 30.0 |
| **Total** | **50** | **14** | **34.0** |
| Prevalence of confirmed MRSA strains was not significantly associated with breeds of sheep (p > 0.05). | | | |

**Table (6):** Prevalence of confirmed MRSA strains in milk samples of sheep in relation to housing systems

|  |  |  |  |
| --- | --- | --- | --- |
| **Housing system of sheep** | **No. of samples** | **MRSA** | |
| **No.** | **%** |
| **Closed** | 15 | 2 | 13.3 |
| **Opened** | 35 | 12 | 34.3 |
| **Total** | **50** | **14** | **28.0** |
| The rate of MRSA detection was not significantly associated with housing of sheep (p > 0.05). | | | |

**4. Discussion:**

The most common and commercially significant bacterium causing intramammary infections in sheep is *Staphylococcus aureus*. About 30% to 40% of all instances of mastitis are brought on by the organism. When handling and processing raw milk, *S. aureus* can enter the milk either directly from udders with clinical or subclinical staphylococcal mastitis or indirectly from the surroundings**(Olechnowicz and Jaskowski 2014).**

The recorded result in **Table (1)** showed that therecovery rate of *S. aureus* in the examined samples of sheep was 54 and 18 %, in raw milk samples and nasal swabs of sheep, respectively. The prevalence of *S. aureus* in milk was nearly similar to that recorded by **Chu et al., (2012)** (50%) while it was lower than that recorded by **Mork et al., (2007)** (65.3%). On contrary, it was higher than that recorded by **Mai- Siyama et al., (2014)** (2.3%) and **Caruso et al., (2015)** (1.23%) in Southern Italy. Differences in the farms' management systems, medication use, isolation techniques, and the impact of the local climate on the research sites might all contribute to the inconsistent outcomes **(Graveland et al. 2011).**

In addition, the overall prevalence of MRSA in nasal samples from sheep in this study (18%) was higher than **Rahimi et al., (2015) (9.1%)** in Iran and **Mai- Siyama et al., (2014)** (8%) in Maiduguri while it disagreed with **Chu et al., (2012)** in Taiwan who failed to isolate *S. aureus* from nasal swabs.

Self-care habits like nose-licking in the herd and the farm management system may both have some bearing on the occurrence of MRSA in the nasal passage of sheep **(Daaloul-Jedidi et al. 2016).**

The fact that sheep aid in the spread of dangerous pathogens like MRSA and other zoonotic infections is widely accepted. It may be difficult to prevent the transmission of infection during the critical lambing seasons, when several animals are gathered in a small area. This is because many animals are healthy carriers of coagulase-positive Staphylococcus strains. Therefore, staphylococcal infections may be disseminated by using ruminant nasal cavities, milk, or faeces to contaminate the surroundings **(Daaloul-Jedidi et al., 2016).**

As recorded in **Table (2),** the prevalence of confirmed MRSA strains isolated from sheep samples was 51.6 and 66.7% in milk samples and nasal swabs, respectively with total prevalence of 55.6% (20 out of 36 isolates).

In this study, antibiotic susceptibility tests of MRSA isolates showed various patterns of resistance. The isolates were completely resistant to Clindamycin and Penicillin-G, 90% resistant to Linezolid, 80% resistant to Cefoxitin, and 70% resistant to Erythromycin. This discovery matched those made by **Stastkova et al. (2009)** in regard to MRSA isolates from milk in the Czech Republic and by **Caruso et al. (2015)** in regard to MRSA isolates from bulk tank milk in Southern Italy.

The public health particularly that of milk consumers and those who come into touch with ruminants often, such as farm workers, is at risk due to the high cefoxitin resistance **(Lee, 2003).** The penicillin-binding protein PBP2a' (or PBP2a), which has a very low affinity for β-lactam antibiotics and is thought to help cell wall assembly when normal penicillin-binding proteins are inactivated, confers resistance to all β-lactam antibiotics, including the semi-synthetic penicillin, in MRSA **(Sharon and Gavin, 2015).**

The *mec*A gene, which is found on the staphylococcal cassette chromosome encodes the protein. In contrast to 12.1% of MRSA isolates resistant to oxacillin reported in South Africa that displayed the PBP2a', all MRSA isolates resistant to cefoxitin in the current investigation expressed the penicillin binding protein 2a', indicating the existence of the *mec*A gene **(Ateba et al., 2015).**

The use of cefoxitin for further testing of oxacillin-resistant isolates as confirmation for methicillin resistance may be the cause of this discrepancy. Contrary to oxacillin, cefoxitin has been demonstrated in several studies to be a powerful inducer of the mecA gene **(CDC, 2018).** The Clinical and Laboratory Standards Institute (CLSI) has recommended cefoxitin as an alternative to polymerase chain reaction (PCR) where it is not available because it is known to be more sensitive than oxacillin in screening for MRSA in coagulase-positive S. aureus (COPS) isolates **(Ibrahim et al., 2017).**

The 70% resistance of the MRSA isolates to erythromycin in this study was higher than the 58.4% in Maiduguri and 11.7% in Brazil but lower than 97% in Egypt **(Gulani et al., 2016; Sohad et al., 2013; Franca et al., 2012).**

Due to their accessibility, macrolides are among the most popular antibiotics used to treat infections in both humans and animals. Regular use of these antibiotics causes the development of resistant bacteria, and meat, milk, and milk products can transmit resistance genes from animals to people **(Schlegelová et al. 2004).** 15% of MRSA in this research exhibited gentamicin resistance, which was greater than the 0% recorded in Brazil but lower than the 57% in India and the 97% in Egypt **(Franca et al. 2012; Gade and Qazi 2014).**

This investigation found that not all MRSA isolates were amoxicillin-clavulanate (β-lactamase inhibitor) combination resistant. This could be because the extra beta-lactamase that the oxacillin-resistant isolate generated was inactivated. According to reports, oxacillin-resistant *S. aureus* becomes sensitive when tested with beta-lactam antibiotics coupled with a -lactamase inhibitor (clavulanic acid). Contrary to non-beta-lactamase-producing MRSA, which exerts its resistance through a chromosomally (altered PBP2a') based method, beta-lactamase-producing MRSA are able to achieve this through a plasmid-mediated (hyper-production of beta-lactamase) mechanism. All beta-lactam antibiotics are known to be ineffective against MRSA, although certain beta-lactam medicines have been proven to reverse this process when -lactamase inhibitor is added **(Jamil et al., 2017).**

When compared to phenotypic approaches, PCR method for the identification of the *mec*A gene has better sensitivity and specificity **(Datta et al., 2011).** The use of primers that target the particular gene is one of these advantages. In order to better identify MRSA in humans and animals, nucleic acid amplification techniques like *spa* typing and *nuc* detection have recently been developed **(René et al. 2010).**

Polymerase chain reaction continues to be the gold standard for MRSA detection despite the fact that it has certain drawbacks of its own, including the requirement for enrichment medium, high cost, and difficulty to discriminate between coagulase-positive and coagulase-negative MRSA **(Datta et al., 2011).**

Because of their minimal inconsistencies, it is advised to employ these two distinct phenotypic approaches to identify MRSA in clinical samples in laboratories where PCR are not easily accessible for detection of *mec*A gene. However, due of its high degree of sensitivity, specificity, and accuracy, the use of PCR for the identification of the *mec*A gene continues to be the gold standard.

Multidrug-resistant bacteria are becoming more prevalent in food animals, people, and their environment as a result of the study area's subtherapeutic doses of antibiotics being given to food animals and indiscriminate antimicrobial usage **(Alhaji and Isola, 2018).**

The effect of age groups on the prevalence of MRSA in milk samples of sheep was tabulated in **Table, (4),** it was found that the highest prevalence was recorded in the age group ≥ 3 years (33.3%) followed by age group 2 - ˂ 5 years (29.6%) and the age group < 2 years (18.2%). Prevalence of confirmed MRSA strains was significantly associated with age groups of sheep (p < 0.05).

The effect of breed difference on the prevalence of confirmed MRSA strains in milk samples of sheep was illustrated in **Table (5).** It was found that the highest prevalence was recorded in Rahmani sheep and Baladi sheep (30 % for each) followed by Barki sheep (25%). Prevalence of confirmed MRSA strains was not significantly associated with breeds of sheep (p > 0.05).

The vulnerability of various sheep breeds varies greatly; Malta sheep are particularly resistant, whilst fat-tailed sheep are very vulnerable **(WHO, 2006).**

The effect of type of housingon the prevalence of confirmed MRSA strains in milk samples of sheep was illustrated in **Table (6).** The rate of MRSA detection was not significantly associated with housing of sheep (p > 0.05) although it was higher in sheep housed in opened system (34.3%) compared to those housed in closed system (13.3%). The environment's pH and humidity have an impact on *S. aureus* ability to survive. The organism is susceptible to pasteurization, disinfectants, and bright sunshine **(WHO, 2006).**

5. **Conclusion:**

In brief, the current investigation found MRSA in the milk and nasal samples of seemingly healthy sheep raised in Alexandria's North Coast. MRSA may spread among owners' family members and their animals because to the intimate contact owners have with their pets and the drinking of unpasteurized milk. Strict hygienic and biosecurity procedures should be used on sheep farms to prevent this from developing into a major infection that might be fatal. Additionally, the development of MDR MRSA strains poses a serious risk to consumer health and under certain conditions may cause staphylococcal food poisoning in addition to the spread of MRSA in the neighborhood. Finally, we ask the relevant authorities to implement laws that prohibit the inappropriate and indiscriminate use of antimicrobials in human and animal treatment.

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