Penicillin resistance against Staphylococcal isolates recovered from subclinical mastitis in Sohag City, Egypt

Mohamed Wael Abd Al-Azeem[1], Hazem Mahmoud Shaheen[2], Karima Galal Abdel Hameed[3*], Manar Mahmoud Helmy[1]

[1]Department of Microbiology, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt
[2]Department of Pharmacology and Therapeutic, Faculty of Veterinary Medicine, Damanhour University, Damanhour, Egypt
[3]Department of Food Hygiene, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt
***Corresponding author:** Karima Galal Abdel Hameed, B.V.Sc., M.V.Sc., Ph.D.
Associate Professor of Milk Hygiene,
Department of Food Hygiene, Faculty of Veterinary Medicine,
South Valley University, 83523 Qena, Egypt
E- mail: karima_galal2004@yahoo.com
Mobile No.: (002) 01063539006

Abstract

Staphylococcus aureus (S. aureus) is considered a major pathogen associated with mastitis infections. Resistance of *S. aureus* to Penicillin has a wide distribution. The aim of this study was to investigate the prevalence of MRSA with special reference to Penicillin in Sohage City, Egypt. Therefore 85 milk samples were collected from subclinical mastitis cows, and examined for the prevalence of MRSA using conventional methods and by PCR. Out of 85 milk samples studied, 70 samples (97.2%) were positive for *S. aureus*. All of these were confirmed on Mannitol and Baired Parker agar and only 15 of them were confirmed on Oxacillin Resistance *S. aureus* Base (ORSAB) as MRSA. Polymerase Chain Reaction (PCR) method confirmed only 3

samples out of the 15 MRSA positive. The highest resistance of the isolated strains was recorded for Penicillin and the lowest was recorded for Amoxicillin/Clavulanate. Therefore, the appropriate therapy of ß-lactame resistance *S. aureus* infection requires more about the knowledge of antimicrobial resistance profile.

Keywords: mastitis, *S. aurues*, Penicillin, Resistance, PCR

{**Citation**: Mohamed Wael Abd Al-Azeem, Hazem Mahmoud Shaheen, Karima Galal Abdel Hameed, Manar Mahmoud Helmy. Penicillin resistance against Staphylococcal isolates recovered from subclinical mastitis in Sohag City, Egypt. American Journal of Research Communication, 2013, 1(7): 116-130} www.usa-journals.com, ISSN: 2325-4076.

1. Introduction

S. aureus is the most important pathogen among *Staphylococci* species related to sub-clinical intramammary infections in dairy cows leading to severe economic losses in industry worldwide (1). Although a variety of antibiotics can be used against this organism, *S. aureus* mastitis has been found to respond poorly to antibiotic treatment (2). The increased resistance of *S. aureus* isolates to several antimicrobial agents has been reported (3). The determination of antimicrobial susceptibility of clinical isolates is required not only for therapy but also for monitoring the spread of resistant strains throughout the populations. ß-lactam antibiotics are the most frequently used in intramammary infusion therapy. Bacterial resistance mechanisms to this class of antibiotics include production of ß-lactamase and low-affinity penicillin-binding protein 2a (PBP2a) determined by the presence of the chromosomal gene *mec*A. The latter, designated

for methicillin resistance, precludes therapy with any of the currently available ß-lactam antibiotics, and may predict resistance to several classes of antibiotics (4).

The isolation of *S. aureus* methicillin resistant (MRSA) from animals was first reported in 1972 following its detection in milk from mastitic cows. The potential for animals to act as a source of *S. aureus* zoonotic infections of humans is exemplified by recent descriptions of human infections caused by pig-associated strains of MRSA (5). In addition, recent evidence implies that some *S. aureus* strains may have the capacity to colonize humans (6) and that humans represent an important source of new pathogenic strains affecting livestock (7). Methicillin-resistant *S. aureus* (MRSA) is among the most important pathogens in terms of increasing prevalence and impact. Therefore, the purpose of this study was to investigate the prevalence of MRSA with special reference to Penicillin in Sohage City. The phenotypic and genotypic characteristics of MRSA isolates were analyzed to gain further insight into short and long term evolution of MRSA.

2. Materials and methods

A total of 85 milk samples from sub clinical mastitic cows were collected from different localities in Sohag City in clean, dry and sterile containers and transferred immediately to the laboratory for examination. The samples were prepared according to A.P.H.A. (8).

2.1. Enrichment procedures:

One milliliter of homogenized milk sample was aseptically inoculated into 5 ml sodium chloride 10% broth. The inoculated tubes were incubated at 37°C for 18-24 hrs.

2.2. Isolation and identification of *S. aureus*:

Loopful of the incubated broth was streaked into plates of selective media Baird- Parker agar (Oxoid) (9) and Mannitol Salt agar media (Oxoid) (10) in a manner to obtain separated colonies. Inoculated plates were incubated at 37°C for 2 days. The suspected colonies were inoculated into slope of nutrient agar for morphological and biochemical tests. The identification was carried out using the following tests: Gram staining, production of coagulase, catalase and fermentation of mannitol (11).

2.3. Antimicrobial susceptibility of *S. aureus* isolates

S. aureus isolates were evaluated for antibiotic susceptibility with the disc diffusion method on Mucller- Hinton agar plates that were overlaid with the inoculum (turbidity equivalent to that of a 0.5 McFarland standard) of the *S. aureus* clinical strains. Zone diameters were measured at 24 and 48 h as recommended by the National Committee for Clinical Laboratory Standards (12). The following discs (Oxoid) were used: Penicillin 10 units, ampicillin (AMP), 10 µg; Amoxicillin 25 µg, Amoxicillin with Clavulanic Acid 30 µg, Tetracycline 10 µg, and Erythromycin 15 µg.

2.4. Detection of MRSA: was done according to Merlino et al. (13) using Oxacillin Resistance *S. aureus* Base (ORSAB) supplemented by (oxacillin, 2.0 µg/ml and polymixin B, 50,000 IU/l).

2.5. Confirmation of the isolates and PCR amplification of the *mecA* gene

Specific primer of *mec A* gene was used in the molecular detection of methicillin-resistant *S. aureus* according to the protocol illustrated by Strommenger et al. (14) with some

modification as follow: Bacterial DNA was extracted following grown up in the phosphate buffered saline (PBS), one millilitre of inoculated PBS was centrifuged at 8000 rpm g for 5 minutes. The QIAamp®DNA (51304 Qiagen, USA) was used to obtain bacterial DNA, according to the manufacturer recommendations and stored at -20°C until use.

2.5.1. DNA amplification: PCR were carried out in 50 µl reaction volumes containing 5 µl template DNA, 1 mM $MgCl_2$, 50 Mm KCl 1 mM of dNTP, 5 µl of x 10 PCR buffer (Qiagen), 1.25 unit of *Taq* DNA polymerase *(Ampli Taq Gold* Qiagen) and 20 pM of each primer. The sequences of the primers are given in following Table.

Primer name	Nucleotide sequence (5 -3)	Accession No.	Size of PCR product (bp)
mec A, forward	AAAATCGATGGTAAAGGTTGGC	1282/1303 (bp)	533
mecA, reverse	AGTTCTGCAGTACCGGATTTGC	1814/1793 (bp)	533

The PCR cycles consisted of pre-heating at 94°C for 5 min, denaturation at 94°C for 40 sec. min, annealing at 50°C for 40 sec and extension at 72°C for 1 min. the amplifications were performed for 40 cycles in a model T professional basic 070-701 thermocycler, in the Molecular Biology Research and Genetic Engineering Center, Assiut University, Egypt, with a final extension step at 72°C for 10 min. The PCR products were visualized using a 1.5% agarose gel containing 0.5 µg of ethidium bromide/ml in relation to DNA mass ladder standard (100-bp DNA ladder Promega Corp., Madison, Wis).

2.6. Statistical analysis:

Using Pearson's correlation coefficient (SAS program) to compare rapid PCR method and conventional methods did the statistical evaluation.

3. Results

According to the results in Table (1) it was found that 72 isolates out of the total examined samples were recorded as staphylococci (84.7%) and 70 isolates of them were confirmed as *S. aureus* (97.2%) by conventional methods, and 18.5% were belonging to other pathogens. Growth of *S. aureus* isolated from mastitis milk samples on different media was illustrated in (Table 2). *S. aureus* showed identical growth rate on both Mannitol salt agar and Baird Parker (100%), however, it was only 21.4% on ORSAB. The Antibiotic resistance of *S. aureus* against 6 antibiotics with different disc potency was determined by disc diffusion method and recorded in (Table 3). Highest resistance was detected for Penicillin (45.7%), Ampicillin (42.8%), Amoxicillin (34%) respectively. On the other hand, lowest resistance was seen for Amoxicillin / Clavulanate (4%). Concerning the PCR method for confirmation of *S. aureus* strains as diagnosed by ORSAB, only 3 out of the 15 ORSAB positive isolates were confirmed by PCR (Table 4) through amplification of *mecA* gene that was amplified at 533pb (Figure 1).

Table (1): Incidence of *S. aureus* in the examined mastitic milk samples

No. of the milk samples	Positive samples				Negative samples	
	Staphylococci		*S. aureus*		Other pathogens	
	No.	%	No.	%	No.	%
85	72	84.7	70	97.2	13	18.57

Table (2): Growth of *S. aureus* isolated from mastitis milk samples in the examined milk samples on different media

No. of isolated sample	Mannitol salt agar		Baird Parker		ORSAB	
	No.	%	No.	%	No.	%
70	70	100	70	100	15	21.4

Table (3): Antibiotic resistance of *S. aureus* by disc diffusion method

Antibiotics	**Disc potency**	**No. of resistant**	**% of resistant**
Penicillin	10 I.U	32	45.7
Ampicillin	25 µg	30	42.8
Amoxicillin	10 µg	24	34
Amoxicillin / Clavulanate	30 µg	4	5.7
Tetracyclin (doxycycline)	10 µg	9	12.8
Erythromycin	15 µg	11	15.7

Table (4): *S. aureus* as diagnosed by ORSAB and by PCR methods

Diagnosis by conventional methods		Diagnosis by PCR method			
Bacterial strain	No. on ORSAB	Confirmed		Not confirmed	
		No.	%	No.	%
S. aureus	15	3	20	12	80

Figure 1: PCR products for *S. aureus mecA* resistance gene

Line M - molecular size marker 1000 bp. Line 1: negative control
Line 4, 5, 6, 7, 8, and 10 negative samples
Line 2, 3 and 9 positive samples 533 bp

4. Discussion

Because of the importance of Staphylococci specifically *S. aureus* as major mastitis pathogen which is very difficult to be treated, this work focused on studying Staphylococci causing sub clinical mastitis in bovine and their antibiotic resistance. According to the results in

Table (1) it was found that high prevalence of *S. aureus* (97.2%) by conventional methods. This was in accordance with Jorgensen et al. (15). Higher results recorded by Ozkan et al. (16).

The most common strategy used by *S. aureus* to circumvent the action of the penicillins is by the production of the enzyme ß-lactamase, which hydrolyses the ß-lactam ring, rendering the entire compound inactive (17). The use of methicillin and ß-lactamase resistant penicillin, initially overcame the problem experienced with beta-lactamase producing bacteria. Unfortunately certain groups of bacteria, including the staphylococci, have evolved new strategies that led to the emergence of methicillin-resistant strains. This has had the greatest impact in human medicine, where methicillin resistant *S. aureus* (MRSA) has emerged as a major nosocomial pathogen. Until recently the problem was limited to hospitals. Bacterial infection of human beings through consumption of contaminated food is a common finding worldwide, if care is not taken during milk processing (18).

In present study (ORSAB) were used for screening of MRSA and the results in Table (2) showed that (21.4%) out of the *S. aureus* isolates were MRSA. These results correlates with the findings by Kumar et al. (19), but higher than those obtained by Moon et al. (4).

The primary tool for controlling *S. aureus* mastitis is antimicrobial therapy. The penicillins and synthetic penicillins those are resistant to ß-lactamase account for the most of the treatments (20). The present study aimed to detect the prevalence of ß- lactam resistance among *S. aureus* isolates. Regarding antibiotic susceptibility testing of *S. aureus* to various antibiotics, we demonstrated existence of alarming level of resistance of frequently isolated mastitis bacteria to commonly used antimicrobial agents in the study farms. Therefore, it is very important to

implement a systematic application in vitro antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of intra mammary infections.

The results of penicillin resistance were similar to those reported in Argentina (21), but lower than that recorded in Iran (22). Regarding ampicillin 42.8% of *S. aureus* isolates showed resistance. Higher resistant reported by Moronie et al. (23). The result of amoxicillin resistance represent by this study were (34.2%), are less than that illustrated by Moronie et al. (23). Resistant profile of amoxicillin similar to penicillin G could be associated with the same biochemical mechanism of the bacterial for resistance these class of agents. It was not possible to get more than one report to compare this result with other reports. Lowest resistance against *S. aureus* in this study was recorded for Amoxicillin/ clavulanate (5.7%). Moronie et al. (23) reported lower percent. Erythromycin resistant against *S. aureus* was (15.7%), Saad et al. (22) reported similar results in Iran. Present results regarding doxycycline resistance for *S. aureus* (12.8%) were similar to those recorded in Iran by Saad et al. (22). The high number of ß-lactamase producing isolates found in present study suggests that the administration of ß-lactamse, especially penicillin and related drugs, should be carefully considered for mastitis control.

The Phenotypic base of identification and susceptibility testing methods are time consuming and have inherent limitations (24), In addition to the low specificity of the ORSAB medium that prevents its use at least alone in predicting methicillin resistance *S. aureus*. Therefore, PCR based methods have shown to be a rapid and reliable approach for the identification and genotypic characterization of these organisms. *mecA*-based PCR method has

accepted as gold standard (25). Because occasional susceptible strains carrying a non functional or non-expressed *mecA* will also be detected, but the presence of *mecA* is generally considered to indicate a potential for resistance and is used as a marker to identify MRSA.

In the present study *mecA*-based PCR methods was carried out using all positive isolates by ORSAB, 3 out of 15 were positive to *mecA* Table (4) and Figure (1). This indicated the absence of correlation between the phenotypic and genotypic tests was, the positive samples to ORSAB did not carry *mecA* gene but phenotypically resisted oxacillin. Non *mecA* methicillin resistance or MRSA lacking *mecA* gene are classified as false resistant by agar screening method. This could be due to another resistance mechanism such as inactivation of oxacillin or methicillin by hyper production of ß-lactamase (26), or modification of normal penicillin-binding proteins (PBPs) and altered affinity for methicillin (27). This minor miscorrelation between the phenotypic and genotypic tests was also mentioned by Moon et al. (4) and Hososaka et al. (28). Therefore, the appropriate therapy of ß-lactame resistance *S. aureus* infection requires more about the knowledge of antimicrobial resistance profile.

References

1. Godden, S.M., Jansen, J.T., Leslie, K.E., Smart, N.L., Kelton, D.F.: The effect of sampling time and sample handling on the detection of *Staphylococcus aureus* in milk from quarters with subclinical mastitis. Canad. Vet. J., 2002; 43: 38-42.

2. Barkema, H.W., Schukken, Y.H., Zadoks, R.N.: Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. J. Dairy Sci., 2006; 89:1877-95.
3. Gentilini, E., Denamiel, G., Llorente, P., Godaly, S., Rebuelto, M., DeGregorio, O.: Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Argentina. J. Dairy Sci., 2000; 83:1224-7.
4. Moon, J.S., Lee, A.R., Kang, H.M., Lee, E.S., Kim, M.N., Paik, Y.H., Park, Y.H., Joo, Y.S., Koo, H.C.: Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. J. Dairy Sci., 2007; 90:1176-1185.
5. Van der Mee-Marquet, N., François, P., Domelier-Valentin, A.S., Coulomb, F., Decreux, C., Hombrock-Allet, C., Lehiani, O., Neveu, C., Ratovohery, D., Schrenzel, J., Quentin, R.: Emergence of unusual bloodstream infections associated with pig-borne-like *Staphylococcus aureus* ST398 in France. Clin. Infect. Dis., 2011; 52: 152-153
6. Garcia-Alvarez, L., Holden, M., T., Lindsay, H.: Meticillin-resistant *Staphylococcus aureus* with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect. Dis., 2011; 11: 595-603.
7. Price, L. B., Stegger, M., Hasman, H., Aziz, M., Larsen, J., Andersen, P. S., Pearson, T., Waters, A. E., Foster, J. T., Schupp, J., Gillece, J., Driebe, E., Liu, C. M., Springer, B., Zdovc, I., Battisti, A., Franco, A., Zmudzki, J., Schwarz, S., Butaye, P., Jouy, E., Pomba, C., Porrero, M. C., Ruimy, R., Smith, T. C., Robinson, D. A., Weese, J. S., Arriola, C. S., Yu, F., Laurent, F., Keim, P., Skov, R., Aarestrup, F. M.*: Staphylococcus aureus* CC398:

host adaptation and emergence of methicillin resistance in livestock. mBio 3(1):e00305-11. doi:1s0.1128/mBio.00305-11.

8. American Public Health Association (A.P.H.A.) Standard Methods for Examination of Dairy Products. 17th ed. Washington D.C., USA, 2004.
9. Finegold, S. H., Martin, W. J.: Diagnostic Microbiology. Bailly and Scott. 6th ed. Mosby Co. St., Louis, Toronto, London, 1982.
10. Murray PR, Baron EJ, Jorgensen JH**,** Pfaller MA, Yolken RH **(**editors): Manual of clinical microbiology**.** 9th ed. American Society for Microbiology**,** Washington**,** D.C.**,** 2007. pp. 477.
11. Bennett RW, Lancette GA. *Staphyloccus aureus*. In: Food and Drug Administration. Chapter 12, Bacteriological Analytical Manual. 8th ed., AOAC International, Gaithersburg, MD, 1995. pp. 12.01-12.05.
12. National Committee for Clinical Laboratory Standards: Performance Standards for Antimicrobial Disc Susceptibility Testing: Fourteenth Informational Supplement M100-S14. NCCLS 2004, Wayne, PA, USA.
13. Merlino, J., Leroi, M., Bradbury, R., Veal, D., Harbour, C.: New Chromogenic identification and detection of *S. aureus* and methicillin-resistant *S. aureus*. J. Clin. Microbiol., 2000; 38: 2378-2380.
14. Strommenger, B., Kettlitz, C., Werner, G., Witte, W.: Multiplex PCR Assay for Simultaneous Detection of Nine Clinically Relevant Antibiotic Resistance Genes in *Staphylococcus aureus*. J. Clin. Microbiol., 2003; 41: 4089-4094.

15. Jorgensen, H., Mork, H., Hogase, R., Rovik, L.: Enterotoxigenic *Staphylococcus aureus* in bulk milk in Norway. J. Applied Microbiol., 2005; 99: 158-166.
16. Ozkan, A., Cemil, D., Hulya, T., Zafer C., Yaflar, E., Gokhan, D.: Coagulase gene polymorphism of *Staph. aureus* isolated from subclinical bovine mastitis. Turk. J. Vet. Anim. Sci., 2007; 31: 253-257.
17. Kernodle DS.: Mechanisms of resistance to β-lactam antibiotics. In: Gram-positive pathogens. Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI, editors. American Society for Microbiology. Washington, DC, USA. 2000. Pp. 609-620.
18. Spohr, M., Rau, J., Friedrich, A., Klittich, .G, Fetsch, A., Guerra, B., Hammerl, J. A., Tenhagen, B. A.: Methicillin-resistant *Staphylococcus aureus* (MRSA) in three dairy herds in southwest Germany. Zoono. Publi. Health. 2011; 58: 252-261.
19. Kumar, R., Yadav, B. R., Singh, R. S.: Antibiotic resistance and pathogenicity factors in *S. aureus* isolated from mastitic Sahiwal cattle. J. Biosci., 2011; 36:175-188.
20. Tenhagen, B., Köster, G., Wallmann, J., Heuwieser, W.: Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. J. Dairy Sci., 2006; 89: 2542-2551.
21. Nunes, S., Bexiga, R., Cavaco, L., Vilela, C.: Antimicrobial susceptibility of Portuguese isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* in subclinical bovine mastitis. J. Dairy Sci., 2007; 90: 3242-3246.
22. Saad, G., Masoud, G., Amir, P.: Antibiotic Susceptibility of Staphylococci Isolated from Bovine Subclinical milk Matitis. Pakis. J. Biol. Sci., 2007; 10: 2781-2783.

23. Moroni, P., Pisoni, G., Antonini, M., Villa, R., Boettcher, P., Carli, S.: Antimicrobial drug susceptibility of *Staphylococcus aureus* from sub-clinical bovine mastitis in Italy. J. Dairy Sci., 2006; 89: 2973-2976.
24. Van Griethuysen, A., Pouw, M., Van Leeuwen, N., Heck, M., Willemse, P., Buiting, A., Kluytman, J.: Rapid slid latex agglutination test for detection of methicillin resistance in *Staphylococcus aureus*. J. Clin. Microbiol., 1999; 37: 2789-2792.
25. Wannet, W.: Spread of an MRSA clone with heteroresistance to oxacillin in the Netherlands. Euro. Surveill., 2002; 7: 73-4.
26. Keserû, J., Szabó, I., Gál, Z., Massidda, O., Mingoia, M., Kaszanyitzky, É.: Identification of â-lactamases in human and bovine isolates of S. aureus strains having borderline resistance to penicillinase-resistant penicillins (PRPs) with proteomic methods. Vet. Microbiol., 2011; 147: 96-102.
27. Frey, K., Lombardo, M., Wright, D., Anderson, A.: Towards the understanding of resistance mechanisms in clinically isolated trimethoprim-resistant, methicillin-resistant *Staphylococcus aureus* dihydrofolate reductase. J. Struct. Biol., 2010; 170: 93-97.
28. Hososaka, Y., Hanaki, H., Endo, H., Suzuki, Y., Nagasawa, Z., Otuska, Y., Nakae, T., Sunakawa, K.: Characteriz-ation of oxcillin- susceptible mecA- positive *S. aureus*: a new type of MRSA. J. Infect. Chaemother., 2007; 13: 79-86.