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Molecular identification of Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin- resistant *Staphylococcus aureus* (VRSA) strains isolated from milk and milk products.

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ABSTRACT

In developing countries, resistance to antimicrobial agents is a serious public health problem, and the indiscriminate use of antimicrobials is a key contributor to the development of resistant bacterial diseases. The following research was aimed to determine the in-vitro antimicrobial activity of (23 strains) of *Staphylococcus aureus* isolated from 80 random samples of milk and dairy products (soft cheese "Kareish and white" and Roomy cheese) 20 samples from each product, with molecular identification of their resistant genes. The results declared that all isolated strains were totally resistant to Ampicillin (100%), Amoxicillin, and Erythromycin (86.9%), then Oxacillin (65.2%), while all other strains showed intermediate susceptibility to Vancomycin, Cefoxitin, Cefotaxime, and Norfloxacin. While Gentamicin had the highest level of susceptibility (78.3%). Moreover, by the cultivation of the isolated strains on oxacillin resistance screening agar base plates "ORSAB," there were 15/23 MRSA strains; also, 5/23 strains showed resistance to Vancomycin and grow well in vancomycin screening agar. The molecular identification for the *mecA* gene and *vanA* gene was made on seven strains (3 strains resistant to both Oxacillin and Vancomycin, two strains with intermediate resistance, and two strains sensitive to them) isolated from different products (milk, Kariesh cheese, soft white cheese, and Roomy cheese). The results showed that all the samples carried the *mecA* gene, while only two samples had the *vanA* gene (isolated from milk).

1. INTRODUCTION

Staphylococcus genus, a heterogenous group, comprises 51 species and 27 subspecies (Swetha et al., 2017). They are related to Gram-positive bacteria that appeared to be grape-like clusters under the microscope. Staphylococci strains are divided into two groups based on the ability to coagulate plasma, i.e., coagulase-positive Staphylococci and coagulase-negative Staphylococci (Otto, 2013). Staphylococcus aureus (S. aureus) is the most significant pathogen that coagulates plasma, causing severe public health hazards for animals and humans (Shrivastava et al., 2017). Its importance is not only because of its widespread diffusion and pathogenicity but also due to its resistance to many antimicrobial agents. For instance, treating human infections induced by strains of Methicillin-resistant S. aureus requires much attention from the researcher. The acquisition of a gene (mecA) expressing a penicillin-binding protein (PBP2a) with a decreased affinity for beta-lactams is commonly used to establish resistance (Peacock and Paterson, 2015). More than twenty years after the first MRSA cases were reported, the mecA gene was discovered (Katayama et al., 2000; Rolo et al., 2017). It is encoded on a portable genetic element called staphylococcal cassette chromosome (SCC) (Ubukata et al., 1989; Yang et al., 2016). Following a crisis of Methicillin resistance, MRSA bacteria were treated with the glycopeptide medication Vancomycin (Sorrell et al., 1982; Murray, 2000). In 1996, the resistance developed against Vancomycin and VRSA isolated from a Japanese patient; after that, it was isolated from the USA, France, Korea, South Africa, and Brazil (Hiramatsu, 2001; Weigel et al., 2007). Vancomycin resistance is acquired either by mutation or thickening of the cell wall due to the buildup of too much peptidoglycan. (Umaru et al., 2014). S. aureus vancomycin resistance is given by the vanA operon on transposon Tn1546, which was initially part of a conjugative plasmid for vancomycin-resistant enterococci (VRE). (Arthur et al., 1993; McGuinness et al., 2017). The present work was carried out to examine the antibiotic resistance of isolated S. aureus strains isolated from raw milk and different varieties of cheeses "Kariesh, Soft cheese, and Hard cheese" collected from October 2022 to December 2022 by using disc diffusion assay and then confirmed by presence the genes coding for MRSA and VRSA.

2. MATERIAL AND METHODS

2.1. Bacterial strains:

Twenty-three *S. aureus* isolates from raw milk and different varieties of cheeses, "Kariesh, Soft cheese, and Hard cheese," collected from October 2022 to December 2022.

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2.2. Antimicrobial sensitivity test:

The strain of *S. aureus* was tested for antibiotic sensitivity using the disc diffusion technique with antibiotic discs (Oxoid Ltd., England) on Mullar-Hinton agar (Koneman *et al.*, 1997). The clarification of results was done according to CLSI, (2017) as reported in Table (1).

Table 1 Antimicrobial standardized discs, concentrations, and interpretation of their effect (Oxoid).

Antimicrobial disks		Disk concentrations	Zone of inhibition (mm)		
			Resistant ≥ mm (R)	Intermediate mm range (IS)	Sensitive ≤ mm (S)
Oxacillin	OX1	1µg	10	11-12	13
Vancomycin	VA30	30µg	14	15-16	17
Ampicillin	AM	20µg	28	-	29
Amoxicillin	AMX/25	25 µg	28	-	29
Erythromycin	E15	15 µg	13	14-22	23
Cefoxitin	FOX30	30 µg	14	15-22	23
Cefotaxime	CTX/30	30 µg	14	15-22	23
Gentamycin	CN/10	10 µg	12	13-14	15
Norfloxacin	NOR/10	10 µg	12	13-16	17

2.3. Phenotypic detection of MRSA according to Becker et al., (2002) and VRSA according to Tiwari and Sen (2006) with modification:

Colonies from each isolated strain of *S. aureus* were inoculated in Brain Heart Infusion (BHI) broth for 24 h before being streaked on the surface of oxacillin resistance screening agar base plates (ORSAB; Oxoid Limited, Basingstoke, England) with ORSAB Selective Supplement (SR 195 E) and incubated at 37 °C for 24 h for revealing of MRSA. The strains were streaked into mannitol salt agar containing vancomycin (vancomycin screening agar) prepared as modified to the method of Tiwari and Sen (2006) in which 1000 ml of MSA contains 6.0 gm of vancomycin.

2.4. Molecular detection of resistant genes (mec A gene) for methicillin-resistant S. aureus and (Van A gene) for Vancomycin- resistant S. aureus:

Seven isolates (3 resistant isolates for oxacillin and vancomycin, 2 strains intermediate resistant, and 2 sensitive strains) from (3 isolates from milk samples, 2 isolates from Kariesh cheese, one from soft cheese and one from roomy cheese) were used to identify resistance genes. It was operated in the molecular diagnosis unit of, Animal Health Research Institute (AHRI), Egypt.

2.4.1. Extraction of DNA: Based on the instructions of QIAamp DNA mini kit. Catalog no.51304. The following steps were followed to extract DNA, briefly; 200 µl of the sample were mixed with 20 µl of QIAGEN protease and 200 µl of buffer AL. the mixture was vortexed and incubated at 56˚C for 10 minutes to allow the cells to lyse. The mixture was centrifuged for 15 seconds to remove any debris, then the resulting liquid was transferred to a QIAamp mini spin column placed in a 2ml collecting tube. The column was centrifuged at 8000 rpm for 1 minute, discard the tube containing the filtrate and wash the column twice using AW1 and AW2 buffers, with centrifugation after each wash to remove debris. QIAamp mini spin column was placed in a clean 1.5 ml micro-centrifuge tube and then 100 µl buffer AE was added, then it was allowed it to stand for 1 minute at room temperature. Centrifuged at 8000 rpm for 1 minute and collect the PCR product.

2.4.2. Detection of resistant genes by using PCR:

To create the PCR mixture, 25 ml of PCR miliQ water, 12.5 ml of Emerald Amp GT PCR master mix (Takara, Japan), 1 ml of forward primer (20 pmol), 1 ml of reverse primer (20 pmol), and 5 ml of template DNA were added. (*mec*A and *van*A), the resistance genes under investigation, were amplified using certain primers, as shown in Table (2). A heat cycler with an applied bio system 2720 was used to finish the process.

Table 2 Oligonucleotide primers sequences.

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
mecA	GTA GAA ATG ACT GAA CGT CCG ATA A	310 bp	McClure *et al.*, 2006
	CCA ATT CCA CAT TGT TTC GGT CTA A		
vanA	CATGACGTATCGGTAAAATC	885 bp	Patel *et al.*, 1997
	ACCGGGCAGRGTATTGAC		

After the cycling condition, the ladder was directly loaded into Agarose gel electrophoreses according to the instruction of Sambrook *et al.,* (1989). The gel was then photographed using a gel documentation system, and the data was analyzed using software.

3. RESULTS

3.1. In-Vitro anti-microbial Sensitivity test

Table (3) indicated that, the isolated *S. aureus* from raw milk and different varieties of cheeses "Kariesh, Soft cheese and hard cheese" were resistant to oxacillin (65.2%), Ampicillin (100%), Amoxicillin (86.9%), and Erythromycin (86.9%). Meanwhile, they were highly susceptible to Gentamycin (78.3%). Moreover, they showed intermediate susceptibility to Vancomycin (43.5%), Cefoxitin (65.2%), Cefotaxime (65.2%) and Norfloxacin (69.6%).

Table 3 In-Vitro anti-microbial Sensitivity test.

Antimicrobial agents	Disk Concentration	Sensitive		Intermediate		Resistant		A A
		No.	%	No.	%	No.	%	
Oxacillin	1µg	3	13.1	5	21.7	15	65.2	R
Vancomycin	30µg	8	34.8	10	43.5	5	21.7	IS
Ampicillin	10µg	0	00	0	00	23	100	R
Amoxicillin	25µg	0	00	3	13.1	20	86.9	R
Cefoxitin	30µg	3	13.1	15	65.2	5	21.7	IS
Cefotaxime	30µg	3	13.1	15	65.2	5	21.7	IS
Norfloxacin	10µg	7	30.4	16	69.6	0	00	IS
Gentamycin	10µg	18	78.3	4	17.4	1	4.3	S
Erythromycin	15µg	0	00	3	13.1	20	86.9	R

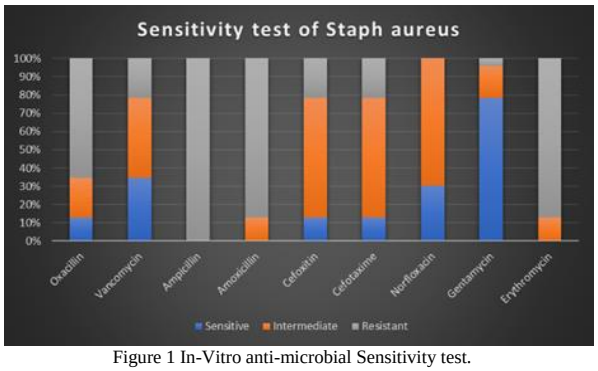

Figure 1 In-Vitro anti-microbial Sensitivity test.

3.2. Phenotypic detection of MRSA and VRSA.

Fifteen strains out of 23 grew extremely on ORSAB agar as blue-colored colonies, which indicated that they are MRSA strains. Table (4) determined that 15 strains out of 23 strains can be grown well on ORSAB agar confirming that it's MRSA. Only 5 strains can be grown on VSA.

Table 4 Incidence of S. aureus strains on ORSAB agar and VSA.

Samples	No.	Coagulase positive *S. aureus* strains		*S. aureus* on ORSAB media		*S. aureus* on VSA media	
		No.	%*	No.	%*	No.	%*
Raw milk	20	10	50	6	30	4	20
Soft (kariesh) cheese	20	5	25	4	20	1	5
Soft (white) cheese	20	4	20	1	5	0	0.0
Hard (Roomy) cheese	20	4	20	4	20	0	0.0
Total (80)	80	23	28.75	15	18.75	5	6.25

%*: Percentage concerning the total number of samples in each row.

3.3. Molecular detection of resistant genes.

The results of PCR revealed that the *mecA* gene was intensified in all examined *S. aureus* strains samples (100%) giving a product at 310 bp. as presented in Fig. (2). While the *vanA* gene was amplified in two samples only (4 and 6) giving a product at 885 bp. while failing to be amplified in other examined samples as presented in Fig (3).

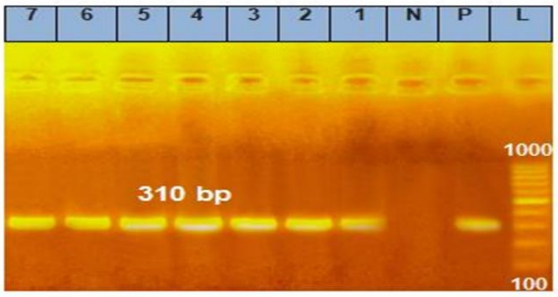

Figure 2 Gel electrophoresis of Methicillin-resistant (*mecA*) gene at 310 bp. Lane L: 100-1000 bp. DNA Ladder., N.: Negative control, P.: Positive control Lane 1-7:*S. aureus* (Positive at 310 bp).

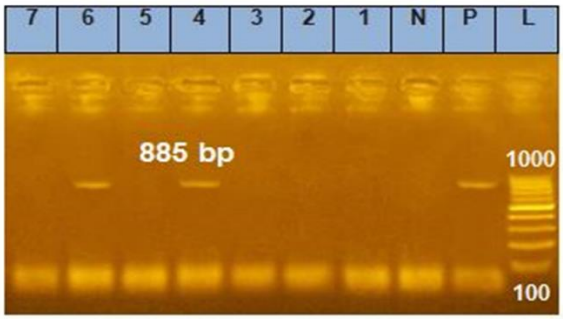

Figure 3 Gel electrophoresis of vancomycin-resistant (*vanA*) gene at 885 bp. Lane L: 100-1000 bp. DNA Ladder., N.: Negative control, P.: Positive control Lane 1-2-3-5-7: were negative, while Lane 4 &6: were positive for *vanA* gene.

4. DISCUSSION

Staphylococcus aureus is a pathogenic bacterium that causes various diseases, from skin infections to fatal systemic infections (Cheung *et al.*, 2021). *Staphylococcal* enterotoxins are a leading cause of gastroenteritis and vomiting from consuming contaminated food (Nedelkov et al., 2000). According to several earlier studies, both developed and developing nations experience an occurrence of milk contamination caused by antibiotic-resistant strains of *S. aureus* (Abdi *et al.*, 2018). The results in (Table 5 and Fig 1) demonstrated that the isolates were resistant to Oxacillin, Ampicillin, Amoxicillin, and Erythromycin while susceptible to Gentamycin. This is the same as that reported by Gonzalez *et al.* (2017), Omoshaba *et al.* (2017), Omoshaba *et al.* (2018), Ektik *et al.* (2018), and Abd El-Khalek *et al.* (2020). Methicillin susceptibility and resistance to another non-ß-lactam are significantly correlated (Otalu *et al.*, 2011). The spread of multi-antibiotic-resistant bacteria between people and food-producing animals via contaminated food consumption is blamed for the developed enormous resistance (Shalini and Rameshwar, 2005). For instance, poor and unsanitary milk handling during the milking process by contaminated farmer hands is a significant contributor to milk contamination. (Amenu *et al.*, 2019; Nyokabi *et al.*, 2021). Additionally, these isolated strains of *S. aureus* were extremely sensitive to Gentamycin. Similar results were also recorded by Abd El-Khalek *et al.* (2020).

The presence of *the mecA* gene in positive strains of methicillin-resistant *S. aureus* (MRSA) could be recorded as oxacillin-resistant. (CLSI, 2018). Table (6) determined that out of 23 strains of *S. aureus*, only 15 were suspected to be MRSA isolates as it has sufficiently grown on ORSAB media with developed colonies of blue color due to chromogenic aniline blue. These findings agree with those of Kapllan *et al.* (2013), Umaru *et al.* (2014), Swetha *et al.* (2017), Serrano *et al.* (2018), and Khairullah *et al.* (2022), who reported that the *mecA* gene was present in every isolate of *S. aureus* that determined as MRSA on CHROM agar (ORSAB). Only five strains of *S. aureus* showed resistance to Vancomycin (VRSA) and grew well in vancomycin-resistant screening agar, which agreed with Swetha *et al.* (2017) and contrary to Kapllan *et al.* (2013), who mentioned extraordinary rates of VRSA (47%) in the examined milk samples while Umaru *et al.*, (2014) from Nigeria, Ateba *et al.*, (2010) from S. Africa, Bandyopadhyay *et al.*, (2016) from West Bengal were reported with percentage of 4%, 3.4%, 0.95% in the examined raw milk samples respectively.

Regarding genotypic detection of MRSA strains using polymerase chain reaction (PCR), the *mecA* gene was intensified in all the examined isolates that were grown on ORSAB media and were resistant to Oxacillin by disc diffusion in-vitro sensitivity test, giving a product of 310 bp (Fig 2). This came in harmony with Gonzalez *et al.* (2017), Papadopoulos *et al.* (2018), Jang *et al.* (2018), and Ektik *et al.* (2018). The PCR results for the *vanA* gene were amplified in only two strains (4 and 6), giving a product of 885bp (Fig 3). This coordinated with Saha *et al.* (2008) and Okolie *et al.* (2015). The other strains that were not amplified agreed with Tiwari and Sen (2006), who reported that vancomycin resistance isolates could be detected phenotypically while not carrying *vanA* and *vanB* genes. Several reports showed various levels of correlation between gene detection and agar-based identification of staphylococcal vancomycin-resistant phenotype (Okolie *et al.*, 2015). Failure of PCR to detect the genes encoding vancomycin resistance could be multifactorial, ranging from poor primer to the absence of the target gene (or sequence) within the genome. Bandyopadhyay *et al.* (2016) reported that the *vanA* gene did not impart resistance in the VRSA isolates associated with livestock and that isolates may have

originated from humans or may have gained resistance because of exposure of animals to other antibiotics.

5. CONCLUSIONS

This study showed that several isolates of *S. aureus* isolated from milk and some varieties of cheese samples showed resistance against Methicillin and Vancomycin, and infection of humans by such strains may lead to severe biological hazards. This occurs mainly due to improper hygienic standards applied during milking, handling, or milk storage. Cheese contamination may arise either from the use of milk of poor quality or from the hands of workers. So, applying and keeping good hygienic practices in shops and markets is necessary to ensure public health safety.

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