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Molecular Screening of Staphylococcal Enterotoxin Type A Encoding Gene from MRS Clinical Isolates

H. A. Hussein[1,*], M. K. Ibrahim[2], H. A. Nour El-Din[3], E. H. El-Shatoury[2], M. E. El-Fouly[1], S. S. Abd-Eal[1], M. N. Abu El-Naga[1]

[1]National Center for Radiation Research and Technology, Atomic Energy Authority, Cairo 11787, Egypt
[2]Faculty of Science, Ain Shams University
[3]Agricultural Genetic Engineering Research Institute (AGERI)
*Corresponding author: hussienhalol@hotmail.com

Abstract Antibiotic resistance is a great problem in Egyptian hospitals and clinical centers. The aim of this study was the characterize of different clinical isolates of antibiotic resistant especially methicillin resistant staphylococci (MRS) on the basis of their production of enterotoxin A. Eighty four samples of bacteria were isolated from wound pus, throat, sputum, conjunctiva, urine and stool of patients in the Egyptian National Centre for Clinical and Environmental Toxicology. Sixty-two isolates had the typical biochemical characteristics of the *Staphylococcus aureus*, while two isolates were identified as *Staphylococcus epidermidis*. Forty two of the isolates were methicillin resistant, all were showed multidrug resistant to thirteen antibiotic. Twenty isolates encoded *ent* A gene (nineteen were *Staphylococcus aureus* and one was *Staphylococcus epidermidis*). Only twelve out of twenty *ent* A gene carriers can produce ent A protein which detected by enzyme linked immunosorbent assay (ELISA). Also, all carried *mec* A gene isolates were resistant to vancomycin. These findings prove that the staphylococcal clinical infections can cause food borne illness and vice versa.

Keywords: *MRSA, MRS, Vancomycin Resistance, Ent A gene, Staphylococcus aureus*

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1. Introduction

Staphylococcus aureus (*S. aureus*) represents a public health challenge worldwide. *S. aureus* is involved in a wide variety of diseases in humans and animals. Its pathogenicity is mainly related to a combination of toxin-mediated virulence, invasive capacity, and antibiotic resistance [1]. MRSA is any strain of *S. aureus* that has developed, through the process of natural selection, resistance to beta-lactam antibiotics. The multidrug resistant microorganisms (MDR) require higher costs, longer treatment times, and higher rates of hospitalization [2]. Enterotoxins are well-characterized virulence factors in *S. aureus*, and their genes and synthesis have been described in *S. epidermidis* [3,4], including *S. epidermidis* causing infections [5]. Staphylococcal food poisoning is a gastrointestinal illness. *S. aureus* enterotoxin A (SEA) is the most common cause of staphylococcal food poisoning worldwide [1]. Food handlers carrying enterotoxin producing *S. aureus* in their noses or on their hands are regarded as the main source of food contamination, *via* manual contact or through respiratory secretions [6]. Wounds, blisters and other bacterial infections sometimes arise in food handlers and kitchen staff when they come in contact with contaminated food. In Netherlands people working in animal food production were infected by a multidrug-resistant *S. aureus* strain, ST398 [7]. Retail meat and poultry are frequently contaminated with multidrug-resistant *S. aureus* [8]. In this study *Staphylococcus* isolates were studied in terms of: (i) Screening for Multi-drug resistant staphylococci specially MRSA using antibiotic sensitivity test; (ii) Detection of *ent* A and *mec* A coding gene using PCR; (iii) Detection of ent A protein productivity using ELISA.

2. Material and Methods

2.1. Bacterial Sources

Eighty four isolates were recovered from patients suffering from multidrug resistance infectious bacteria. Isolates were identified biochemically according to **Barrow and Feltham** [9]. *S. aureus* (ATCC 13565) strain was used as positive control for *ent* A gene detection.

2.2. Antibiotics Susceptibility Test

Antibiotic resistance disc diffusion method was used for determination the size of inhibition zone on Mueller Hinton Agar according to Clinical and Laboratory Standards Institute [10]. Thirteen antibiotics commonly used in the treatment of clinical infection were tested (concentrations are expressed in μg ml^{-1}): cefoxitin (FOX;

30 ug), oxacillin (OX; 1 ug), Amoxicillin/Clavulanic acid (AMC; 30 ug) Vancomycin (VA; 30 ug), Amikacin (AN; 30 ug), Ampcillin (AP; 10 ug), Erythromycin (E; 15 ug), Gentamicin (GM; 10 ug), Rifampin (RA; 5 ug), Teicoplanin (TEC; 30 ug), Trimethoprim/Sulfamethoxazole (SXT; 25 ug), Chloramphenicol (CM; 30 ug) and Levofloxacin (LEV; 5 ug).

2.3. DNA Extraction

Total DNA was extracted from bacterial isolates cultured on nutrient agar medium, inoculated individually into brain heart infusion broth broth (BHI) and incubated at 37°C for 24h. The Gene Jet Genomic DNA purification kit (Thermo Scientific) was used for DNA extraction according to manufacturer's protocol, which consists of initial digestion of the staphylococcal cells with lysozyme (10 mg/ml) and proteinase K (20 mg/ml), 500 µl of the extraction solution was added and the mixture was centrifuged (5000 g/1 min). The supernatant was transferred to Gene Jet Genomic DNA purification column and centrifuged (5000g/1 min). The eluent was discarded, and 500 µl of extraction solution was again added to the column. After centrifugation and disposal of the collected eluent, 500 µl of the wash solution was added to the column and the column was centrifuged at 2080g/min. The column was then transferred to a 1.5/ml tube and 200 µl Milli-Q water heated at 70°C was used for elution under centrifugation (5000 g/ min).

2.4. PCR

PCR was conducted in a volume of 50 µl containing, 1X reaction buffer with $MgSO_4$, 200 µM each dNTPs (dATP, dTTP, dCTP and dGTP), 0.4 pmol of each primer, 0.5 units of Taq DNA polymerase, template and d.d.H_2O. PCR amplification was performed in a Perkin-Elmer (Gene Amp PCR System 2400) for 35 cycles after initial denaturation for 10 min at 95°C. Each cycle consisted of denaturation at 95°C for 35 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min. The primer extension was extended to 7 min at 72°C in the final cycle. Two specific primers were used for *ent* A gene detection, Ent A (F): 5'AGCGAGAAAAGCGAAGAAATAAATGAA'3 and Ent A (R): 5' GCCATAACTCGTGTATAAGTATATG TCAAT'3. Also, the primer of *mec* A gene were Mec A (F):5'GTAGAAATGACTGAACGTCCGATAA'3 and Mec A (R):5'CCAATTCCACATTGTTTCGGTCTAA'3.

2.5. Enterotoxin A production

Staphylococcal enterotoxin was produced using the dialysis bag method described by **Donnelly** [3], 30 to 40 cm dialysis bag (Cut-Off 12,000-16,000 MW, USA) previously washed with distilled water, closed at one end, filled with 50 ml of double-strength brain heart infusion broth (BHI), the other end closed. The bag was bend to a "U" shape and hanged in a 250 ml Erlenmeyer flask containing 18 ml of 0.02 M phosphate buffer pH 7.4 in 0.9% NaCl and autoclaved for 15 min at 121ºC. The flask was inoculated with *S. aureus* previously cultured in 5 ml BHI at 37ºC/18 h. After incubation at 37ºC, 200 rpm for 24 h, the buffer containing bacteria and toxin was centrifuged at 8000 g/ 10 min at 4ºC and the supernatants was used for enterotoxin detection.

2.6. Enterotoxin A detection by ELISA

The produced staphylococcal enterotoxin crud from isolates was tested for the presence of (SEA) by indirect ELISA [11]. 96-well ELISA plate (Corning, Lowell, MA, USA) were coated with 200 µl per well of rabbit polyclonal staphylococcal enterotoxin A antibody (SIGMA-ALDRICH, product CAT# S7656) specific to sea in coating buffer pH 9.6 (1 ul/ml), and then the plate was incubated overnight at 4°C. This was followed by washing four times (three min each) with washing phosphate buffer saline – Tween (PBS-T). The tested samples were prepared in a dilution of 1/10 ml sample buffer; pH 7.4 and then 200 µl were added per well, 2 wells per sample as replicates, followed by incubation overnight at 4°C. It is important to mention that to A1 well 200 µl of sample buffer were added to serve as a blank, the positive control represents the purchased ent A protein. After washing as mentioned before, Alkaline phosphatase-conjugate was added at a dilution of 1:3000 in conjugate buffer pH 7.4 (200 µl per well) and then incubated at 37 ºC for 3 h. After washing the substrate buffer at pH 9.8 prepared immediately before use and containing the p-nitro-phenyl-phosphate (substrate) at concentration of 0.75 mg/ml was added. 200 µl per well were added to each well. The plate was incubated at room temperature (RT) for 20-30 min. The reaction was stopped by adding 50 µl of 3M NaOH to each well. The ELISA values were determined at wavelength 405 nm using Bio-Rad ELISA Reader Model 3550.

3. Results

3.1. Isolation Source and Antibiotic Resistance

Sixty four *Staphylococcus* isolates were derived from wound pus, throat, sputum, conjunctiva, urine and stool of patients suffering from clinical infections. 60.9 % of the isolates were recovered from wounds, 20.3 % from throat, 6.2 % from conjunctiva, 6.2 % from stool, 4.6 % from sputum and 1.5 % from urine. Sixty two isolates were biochemically identified as *S.aureus* and two was identified as *S. epidermidis*. Forty one *S. aureus* and one *S. epidermidis* out of the sixty two were found to be methicillin resistant as tested by cefoxitin (MRS). The methicillin resistant isolates were also tested against twelve commonly used antibiotics (Table 1). All methicillin resistant isolates were showed 100 % resistance to ampcillin, amoxicillin/clavulanic acid as well as cefoxitin, oxacillin, on the other hand, levofloxacin and teicoplanin only affected one isolate each. Moreover, gentamicin,, amikacin,, rifampin, chloramphenicol, erythromycin, trimethoprim/sulfamethoxazole affected on 9.5 %, 11.9 %, 14.2 %, 16.6 %, 19.0 % and 23.8 % of methicillin resistant isolates each respectively. Finally, 47% of the isolates were vancomycin resistant (VRSA).

3.2. Enterotoxin A production

The extracted DNA from the 64 staphylococci isolates were investigated for enterotoxin A (*ent* A) encoding gene by PCR alongside the reference strain as a control. Figure 1 shows the presence of the amplified products after agarose gel of twenty positive isolates from the 64 including the

reference strain. Nineteen bands were for *S.aueus* isolates and one band for *S.epidermidis* (No.52).

Table 1. Multiple resistance patterns for MRSA *S. aureus* isolates to the most common antibiotics in Egypt.

Isolate Source	Number of Isolates	Number of Resistant Isolates												
		FOX	OX	AMC	VA	AN	AP	E	GM	RA	TEC	SXT	CM	LEV
Wound	39	22	22	22	7	18	22	15	18	19	21	17	16	22
Throat	13	12	12	12	6	12	12	11	12	10	12	11	9	10
Conjunctiva	4	4	4	4	3	4	4	4	3	4	4	3	3	4
Stool	4	1	1	1	1	1	1	1	1	1	1	1	0	1
Urine	1	1	1	1	1	0	1	0	1	1	1	1	1	1
Sputum	3	3	2	3	2	2	3	3	3	3	3	2	2	3
Total	64	42	42	42	20	37	42	34	38	36	41	32	35	41

FOX; Cefoxitin, **OX;** Oxacillin, **AMC;** Amoxicillin/Clavulanic acid, **VA;** Vancomycin, **AN;** Amikacin, **AP;** Ampcillin, **E;** Erythromycin, **GM;** Gentamicin, **RA;** Rifampin**, TEC;** Teicoplanin, **SXT;** Trimethoprim/Sulfamethoxazole**, CM;** Chloramphenicol, **LEV;** Levofloxacin.

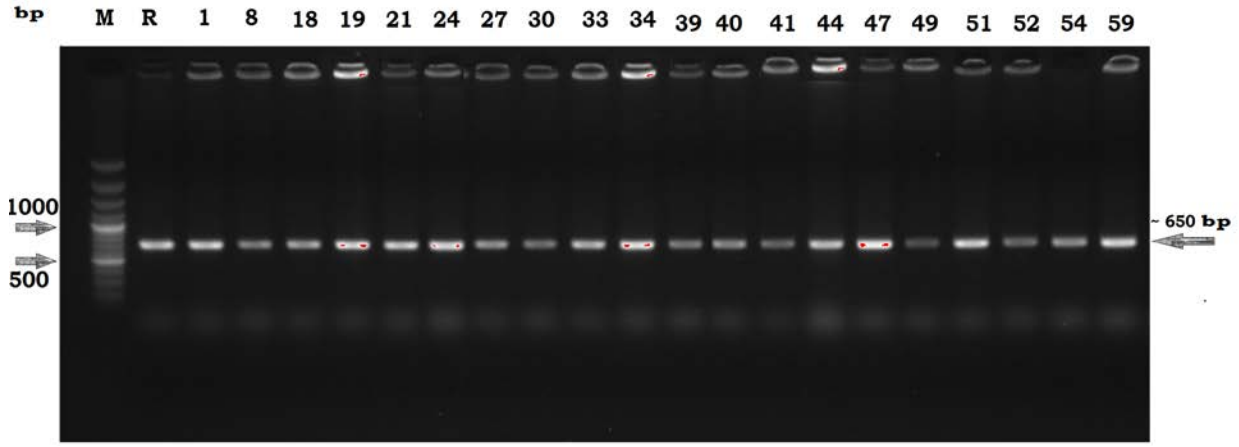

Figure 1. PCR detection for staphylococcal *ent* A gene for positive samples on 1.5% agarose gel of PCR amplification of *ent* A genes, Lane 1; Marker (100 bp), Lane 2; Reference Strain, Lanes 3:17, 19 and 20; *S.aureus* and Lane 18; *S.epidermidis*)

3.3. ELISA and PCR Detection

The ELISA was used to confirm staphylococci enterotoxin production for the twenty *ent* A carrier gene isolates. Twelve isolates were to ent A protein producter, seven of were isolated from wound pus, three from throat, one from conjunctiva and one from urine. Whilst, eight isolates were negative for ent A protein production. Moreover, all of the 20 isolates were methicillin resistant, 13 of them were vancomycin resistance **(table 2)**. Agarose gel in figure (2) proved the existence of *mec* A gen within the 12 enterotoxin A positive isolates. Isolate no; 34 exhibited 2 bands during PCR amplification of *mec* A gene one is the target gene but the other is considered as an unspecific PCR (e.g for a pseudogene sharing same sequence but with a longer or shorter sequence). Or more possibly it can be an insertion or a deletion that is found at a heterozygous state.

Table 2. Staphylococcal enterotoxin A detection using ELISA

Isolate Number	Isolates	Source of Isolation	Ent A Detection	VRS Resistance	MRS Resistance
Reference Strain	*S. aureus*	ATCC 13565	+	S	S
1	*S. aureus*	Throat	-	R	R
8	*S. aureus*	Wound pus	+	R	R
18	*S. aureus*	Conjunctiva	-	R	R
19	*S. aureus*	Conjunctiva	+	R	R
21	*S. aureus*	Wound pus	+	R	R
24	*S. aureus*	Throat	+	R	R
27	*S. aureus*	Wound pus	-	R	R
30	*S. aureus*	Wound pus	+	R	R
33	*S. aureus*	Throat	-	R	R
34	*S. aureus*	Throat	+	S	R
39	*S. aureus*	Wound pus	-	S	R
40	*S. aureus*	Urine	+	R	R
41	*S. aureus*	Wound pus	+	R	R
44	*S. aureus*	Throat	-	S	R
47	*S. aureus*	Wound pus	+	S	R
49	*S. aureus*	Wound pus	+	S	R
51	*S. aureus*	Wound pus	+	S	R
52	*S.epidermidis*	Throat	+	S	R
54	*S. aureus*	Wound pus	-	R	R
59	*S. aureus*	Wound pus	-	R	R
Total	19; *S. aureus*	20	12	13	20

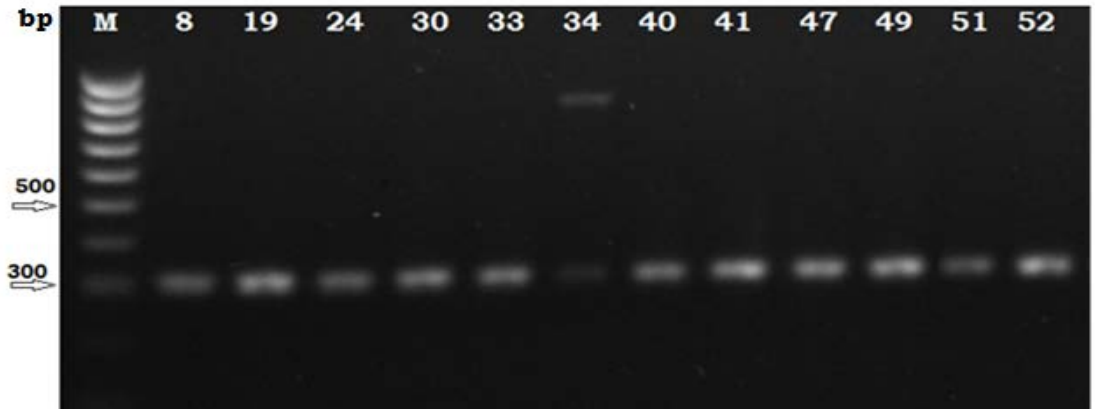

Figure 2. *Mec* A gene PCR image for 12 enterotoxin A producer isolates with 100 bp marker.

4. Discussion

The pathogenicity of staphylococci depends on their ability for toxin production and antibiotic resistance. In the present study; the majority of *S.aureus* isolates were isolated from wound pus, throat, conjunctiva, stool and urine respectively. *S. aureus* most commonly colonises under the anterior nares. The nostrils, rest of the respiratory tract, open wounds, intravenous catheters, and the urinary tract can also be infected by *S.aureus* [12]. We found that 42 isolates (65.5 %) were methicillin resistant; the high MRSA percentage may be a result of mistreatment or unnecessary administration of antibiotic in Egypt. The inappropriate use of antibiotics can increase risk to patient safety, reduce the efficacy of these drugs and drive up avoidable healthcare costs [13]. Vancomycin-resistant *S. aureus* tend to be multidrug resistant against a large number of currently available antimicrobial agents, compromising treatment options and increasing the likelihood of inadequate antimicrobial therapy and increase in morbidity and mortality [14]. In the present study, VRS showed resistance to a wide range of antimicrobial agents. This may be due to the increased use of antibiotics in the intensive care units. Linezolid and quinupristin/dalfopristin were recently approved by the Food and Drug Administration and are antimicrobials with activity against glycopeptides-resistant Gram positive microorganisms such as VRSA [15]. The genetic mechanism of vancomycin resistance in VRSA is not well understood. Several genes have been proposed as being involved in certain clinical VRSA strains [16,17]. The experimental transfer of the *van* A gene cluster from *E. faecalis* to *S. aureus* [18] has raised fears about the occurrence of such genetic transfer in clinical isolates of methicillin resistant *S. aureus*.The current study showed that cefoxitin and oxacillin had the strongest influence on the investigated isolates (42 isolates from the original 64), which reclassified theses isolates as MRSA. Cefoxitin is more sensitive for the detection of *mec* A-mediated resistance than oxacillin [19,20]. Other antibiotics investigated gave lower results when compared with those results of MRSA, for instance; 41 isolates were resistant to teicoplanin (belongs to glycopeptides) which represents 97% of MRSA, while; only 20 isolates were resistant to vancomycin (belongs to glycopeptides) which represents 47.6% of MRSA. Also, by the same way 85.6 % 88% and 90% of MRSA isolates were resistant to the following aminoglycosides antibiotics amikacin, rifampin, and gentamicin, respectively. Whilst; 97% of them were resistant to the Levofloxacin (belongs to fluoroquinolone). Finally, most of MRSA isolates can be considered as multiple drug resistant. Pathogenesis of *S. aureus* is mainly related to a combination of toxin-mediated virulence, invasive capacity, and antibiotic resistance [1]. Also, 12 out of twenty isolates *ent* A gene mediated could produce ent A toxic protein. These 12 isolates also possess *mec* A gene which responsible for antibiotic resistance of *S. aureus [21]*. Hyper production of β-lactamases which may lead to phenotypic expression of oxacillin resistance [4]. The spread of resistance depends in a subtle way on the rates of gene transfer and antibiotic inflow [22]. During cells replication high-frequency horizontal transfer pathogenicity island-encoded virulence factors, such as plasmids, bacteriophages. These pathogenicity islands carry genes for virulence determinants such toxins. fluoroquinolones and trimethoprim have also been implicated in similar DNA damage induction in staphylococci. These damage elements also play a crucial role in spreading antibiotic resistance and virulence genes among bacterial populations [21]. *Ent* A gene and *mec* A gene in *S. aureus* (MRSA) isolates in three different ovine milk/dairy product samples [23]. In conclusion, the results of this study showed the occurrence of MRS in Egypt. Also, this MRSA could produce ent A toxic protein. The increase of vancomycin resistance among MRSA and excessive use of antimicrobial agents have worsened the sensitivity, which call for further epidemiological studies.

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