

Phenotypic and Genotypic Detection of Biofilm Producing Methicillin Resistant Staphylococcus aureus (MRSA) Isolated from Human

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Abstract

This study was aimed for phenotypic and genotypic detection of biofilm-producing Methicillin-resistant Staphylococcus aureus (MRSA) isolated from human. One hundred and fifty human nasal samples from different locations in Baghdad governorate, Iraq were collected during the period from October 2020 to April 2021. Samples were inoculated on mannitol salt agar (MSA) and incubated at 37°C for 24 hrs then, subcultured on blood agar. Detection of isolates was carried out according to colony characterization, Gram staining, conventional biochemical tests and analysis profile Vitek® 2, Bacterial resistance and MRSA was determined by disc diffusion. The biofilm synthesis was detected by Congo red agar method. The genotypic analysis of MRSA isolates using deoxyribonucleic acid (DNA) extraction to detect the 23srRNA, and mecA genes by polymerase chain reaction (PCR) assay. The MRSA was found in 18.6% (28/150) of human swabs, the biofilm positive MRSA of 28 of human isolates were 89.28%. The isolates showed high rate of multidrug resistance 82.14%, with high resistance to penicillin and methicillin (100%), azithromycin (85.71%), fusidic acid (75%) and oxacillin (71.42%). However, it was sensitive to: Bacitracin (100%), Norfloxacin (96.42%), Gentamycin and Clindamycin (85.71%), Vancomycin (71.42%). Genotypes identified by PCR showed that all isolate (100%) harboring 23srRNA and 76% harboring mecA genes. Our results revealed a correlation of mecA gene and biofilm synthesis in MRSA isolate.

Keywords: Staphylococcus aureus, 23srRNA, mecA gene, biofilm, Methicillin, Oxacillin

Introduction

Staphylococci are Gram and catalase positive, aerobic, non-motile, non-spore forming, cocci, arranged as pairs, tetrads or oftentimes irregular grape like clusters, Staphylococcus (S.) aureus is the major important human and animals' pathogen among the staphylococci and involved in the producing of a numerous clinical cases

(Markey et al., 2014). It has the capability to fastly develop antibacterial resistance practically to antibiotic used to treat of clinical cases, which lead to the emergence of MRSA (Rahmaniar et al., 2020), it is one of the major human pathogens; it is responsible for various diseases such as skin infections, invasive infections including pneumonia, infections of soft tissues, bones, and even fatal septicemia in human (Gordon and Lowy, 2008). MRSA are resistant to β -lactam antibiotics and mostly showed multidrug-resistant (MDR) to other antimicrobial agents, e.g., tetracycline, macrolides, aminoglycosides, and fluoroquinolones that are commonly used in the treatment of disease caused by these microorganisms (Ness, 2010). Biofilm synthesis by bacteria significantly participate in their survival in the host and has been considered as a key virulence factor responsible for serious chronic infections (Parrino, et al., 2019), the ability to produce biofilms is very important for *S. aureus* pathogenicity (Suresh, et al., 2019) and play a vital roles in its resistance against antimicrobial therapy and innate host defense mechanisms (Otto, 2008; Parrino, et al., 2018). Additionally, biofilm production by *S. aureus* and resistant to antimicrobial are functionally associated, as the phenotype of biofilm expression can be influenced by the occurrence of antimicrobial resistance (Stewart, 2002 and McCarthy, et. al., 2015). Numerous food- borne infections are associated with biofilms synthesis and regarded as an emergent public health concern (Srey, et. al., 2013) Biofilm-mediated disease have an adverse effect on the health of patient, and therefore the aim of this study was to phenotypic and genotypic detection of biofilm-producing MRSA isolated from human.

Materials and Methods

Sampling, isolation and identification of MRSA

This study was approved by The Committee of Ethics Research of the zoonotic unit, College of Veterinary Medicine, University of Baghdad, Ministry of High Education and Scientific Research-Iraq (No. 1177/ 23-9-2020). One hundred and fifty human nasal samples were collected from different locations in Baghdad governorate, Iraq during the period from October 2020 to April 2021. Samples were streaked on the surface of blood agar and MSA and incubated at 37°C for 24 hrs, identification of the isolates was carried out according to colony characterization, grape like clusters by Gram staining, conventional biochemical analysis including haemolysis, catalase, coagulase, oxidase, DNase and mannitol fermentation tests (Marky et al., 2014). In addition, to analysis profile Vitek® 2 compact system (BioMérieux, France).

Antimicrobial susceptibility testing

Bacterial resistance was determined by disc diffusion as previously reported (Bauer et al, 1966) against antimicrobial discs that are commonly used for treatment of human, the inhibitory zones around these discs were measured by millimeter (mm) unit by using a metric ruler, which showed that the isolate was interpreted as susceptible, intermediate, or resistant to particular antibiotic (CLSI, 2020). Phenotypic detection

of MRSA strains was performed by the disc diffusion assay, the genotypic confirmation of MRSA was performed to detect 23srRNA and *mecA* gene by PCR.

Biofilm detection assay

Biofilm synthesis of isolates were detected by Congo red agar (CRA) method as described by Freeman et al., (1989). Briefly, MRSA isolates were streaked on CRA, this medium was prepared by dissolving brain heart infusion broth (BHI) 37.9 g, sucrose 50g, Agar-Agar 10g and 0.8g Congo red powder. The BHI, sucrose, Agar Agar was dissolved in 990 ml of distilled water in flask, then heating at 100 C. The 0.8g of Congo red stain was dissolved in 10 ml distilled water in other flask and sterilize the two flasks by autoclave then the Congo red solution was added to other components after cooling 50 C, mixed all and poured in plates.

Molecular of identification for isolates MRSA

For PCR, genomic *S. aureus* DNA was extracted by Kit (Geneaid, Thailand). Concentrations and purity of extracted DNA was determined by Nanodrop spectrophotometer (Thermo, USA). Gel electrophoresis was used to detect DNA bands stained with red safe dye and visualized under UV light as described by Sambrook and Russell (2001). For DNA extraction 1g agarose was used. To generate PCR products 2g agarose was added to 100 ml 1XTBE buffer, boiled and cooled to 50°C. For polymerization, 2µl of Red Safe dye was poured and left 30 min at room temperature (Tsalet al., 2017). Gels poured into chamber then covered with 1 X TBE buffer. Then 10 µl samples were mixed with 5 µl dye buffer and loaded into wells with gel. DNA products were screened for the presence of 23sr RNA and *mecA* genes by PCR (Table 1), with specific primers (Bioneer, Korea) and PCR mixture of 20 µl with 2 µl premix of *Taq* DNA polymerase, MgCl₂, dNTPs, KCl, stabilizer tracking dye and tris-HCl, 1 µl of each primer (final concentration was 10 picomole /µl) and 2 µl of DNA template then the volume was completed with sterile distilled water (Long, et al., 2018).

primer selection

Two primers were used in this study were *mecA* and 23srRNA primers to detect the MRSA

Table 1. Types of primers were used in PCR assay and their designer

Code	5-3	Product length
MecA-F	GCCCGTGGATTAGTCGTTGA	623 bp
MecA-R	CGTCGAACCTGAGCTGTTACG	
23 srRNA-F	TCGGAATCTGGGAGGACCAT	350 bp
23 srRNA-R	AACGTAAGTCGGTTCGGTCC	

These primers that used (from Alpha DNA company, Canada) which were provide as

lyophilized form and were dissolved in sterile deionize D.W to gave a final concentration 100 Pico mole / μ l according to the recommendation of provider and then store in a deep freezer until use.

Amplification reaction

Table 2. Conditions of polymerase chain reactions

Final Extension	Extension	Annealing	Denaturation	No. of cycles	Initial denaturation	Gene
cycle 72 second 45	cycle 72 second 45	cycle 58 second 45	cycle 94 second 45	35	94 cycle 7min	MecA
cycle 72 second 45	cycle 72 second 45	cycle 58 second 45	cycle 94 second 45	35	94 cycle 7min	23srRNA

Before electrophoresis, PCR products for each well were loaded with 5 μ l sample. DNA ladders 100bp and 1kb (KAPA Biosystem/USA) were run concurrently with each electrophoretic run to detect size of amplified PCR product. Electrophoresis was performed at 70V for 1.5 hour for extracting genomic DNA and PCR products, then DNA bands visualized by UV trans illuminator (Scope-21/Japan)^[15] and photographed by using a digital camera.

Results

We found *S. aureus* 18.6% (28/150) human samples. Suspected colonies of *S. aureus* isolates appeared on mannitol salt agar as round, smooth, and fermenting or non-fermenting mannitol red-yellow as shown in Figures 1 and 2, below.



Figure 1-Fermentation of mannitolby *Staphylococcus aureus*on Mannitol Salt Agar.



Figure 2. Mannitol fermenting and non-fermenting *Staphylococci* on salt media

S. aureus appeared as Gram positive, cocci, single cell pairs and grape-like clusters. The isolates were positive for catalase, gelatinase and DNase (Figure 3), coagulase tests (Figure 4), and hemolysis (Table 3).

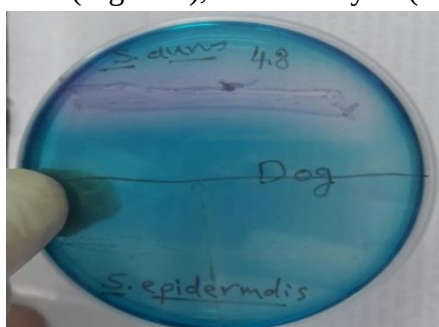


Fig 3- *Staphylococcus aureus* DNase test



Fig 4 -*Staphylococcus aureus* gelatinase test

Table 3: Biochemical reactions for *Staphylococcus aureus*.

Catalase	+
Oxidase	-
DNase	+
Coagulase	+
Gelatin liquefaction	+
Hemolysis	+

Table 4: Percentage of resistant and susceptible isolates of *S.aureus* in human

Antibiotic (mg)	Total samples	Sensitive	Intermediate	Resistant
Methicillin(5)mcg	28	0(0%)	0(0%)	28(100 %)
Oxacillin(1)µg	28	6(21.42 %)	2(7.14%)	20(71.42%)
Azithromycin(15)mcg	28	4(14.28%)	0(%)	24(85.71%)
Vancomycin(30)mg	28	20(71.42%)	1(3.57%)	7(25%)
Fusidic acid(30)µg	28	7(25%)	0(%)	21(75%)
Gentamicin(10)µg	28	24(85.71%)	0(0%)	5(17.85%)
Clindamycin(10)µg	28	24(85.71%)	0(%)	4(14.28%)
Bactricin(10)IU	28	28(100%)	0(%)	0(%)
Benzylpenicillin(10)IU	28	0(%)	0(%)	28(100%)
Norfloxacin(10)µg	28	27(96.42%)	0(%)	1 (3.57%)
Polymyxin pb300unit	28	16(57.14%)	0(%)	12(42.85%)

The biofilm positive MRSA of human swabs were 89.28%, the association of biofilm formation with *mecA* gene were 76.0%.



Fig 5. *S.aureus* positive to Congo red test change the color to black colony.

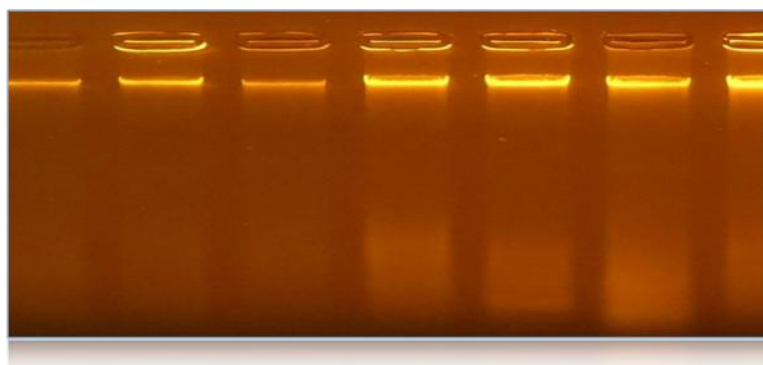


Fig 6: Genomic DNA bands of *S aureus* in human isolates by gel electrophoresis (1 % agarose, 90V, for 1.5 hour)

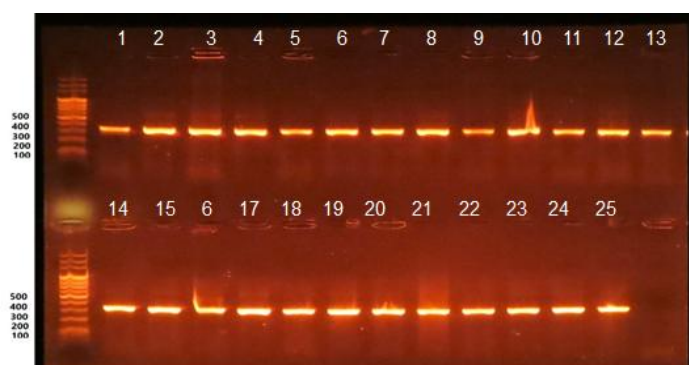


Fig 7.PCR product analysis of 23srRNA gene (350pb) in *S. aureus* isolates on agarose gel electrophoresis. where the marker (2000 -100bp) all the (28) positive isolates from human samples at PCR product.

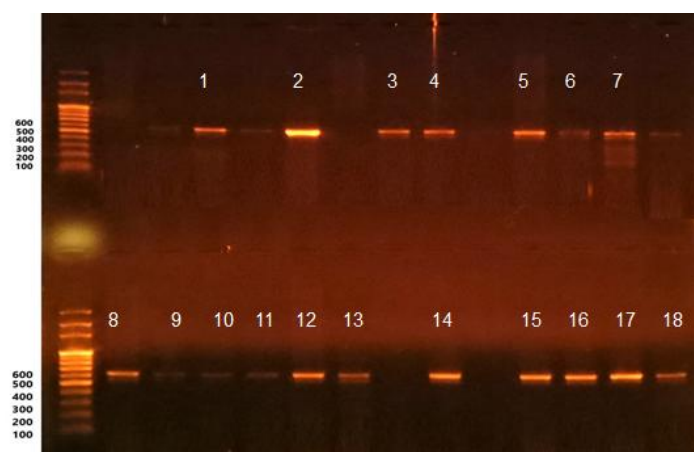


Fig 8.PCR product analysis of mecA gene (632pb) in *S. aureus* isolates on agarose gel electrophoresis. where M:marker(2000 -100bp)18 positive isolates from human samples at PCR product.

Discussion

The colony characterization, Gram staining and conventional biochemical tests in the current are similar to phenotypic characters for *S. aureus* as reported by Markey, et. al., (2014). In the current study, MRSA was found in 18.6% (28/150) nasal human samples, which is lower than that reported by Mohanty et al. (2018) who mentioned the percentage of 29.4% from nasal swabs in Egypt and less than the isolation rate of 69.5% reported by Alebachew et al. (2012) in Ethiopia. Yaseen et al. (2013) reported that the isolation rate 41% of *S. aureus* from patients with wound and burn infections in Al-Jumhuory Teaching Hospital in Mousl, Iraq.

Numerous techniques were developed for detection of MRSA including phenotypic (disk diffusion test) and genotypic methods such as, the mecA and blaZ genes, which are considered a genetic marker used for the rapid and direct detection of MRSA (Ostojić, et. al, 2015). Of the 28 MRSA isolated from human nasal swabs 25

(89.28%) were biofilm positive isolates. The growth of our isolates on Congo red agar showed its ability to form a slime layer; similar result was reported previously (Acheke, et.al., 2020), the Congo red agar was used in the detection of the slime (biofilm) production from *S. aureus* isolated from human and animals (Horiuk, et. al., 2019). Early identification of biofilm forming *S. aureus* in clinical isolates could be an important method in control and prevention of the infections (Horiuk, et. al., 2019). Monitoring the development of biofilm positive and multidrug resistant MRSA in human and animals are essential to prevent or control the spreading of this pathogen and the related zoonotic hazard.

In the present study, the antimicrobial susceptibility test of *S. aureus* isolated from human revealed high rate of multidrug resistance 82.14%, with high resistance to penicillin and methicillin (100%), azithromycin (85.71%), fusidic acid (75%) and oxacillin (71.42%). Several studies have shown that the *S. aureus* are resistant to one or more antimicrobial agent (Dweba et al., 2018; Akanbi et al., 2017). Alebachew et al., (2012) and Elhassan et al., (2015) reported that *S. aureus* isolated from burn wound and different clinical specimens was 77.3% and 100% resistant to methicillin respectively, while Spanu et al., (2012) and Rahman et al., (2018) recorded that 90% and 100% of *S. aureus* recorded from pus, wound infection was resistant to oxacillin respectively, and Tigabu et al., (2018) mentioned that isolated from school children in Ethiopia was 100% resistant to Cefoxitin. In Iraq, Lafi, (2019). Noticed that the human *S. aureus* isolates showed resistance (85.7%) to methicillin, (80.9%) to Oxacillin, (78.5%) to Cefoxitin, (52.3%) to Gentamycin, (47.6%) to Vancomycin, (42.8%) to Clindamycin (35.7%) to Chloramphenicol, (33.3%) to Trimethoprim. The incorrect usage of antimicrobial agents poses great hazards to both animal and human health due to the emergence of antimicrobial resistance (Okocha et al., 2018). The differentiation between antimicrobial susceptibility patterns among MRSA isolates may be back to the geographical location, and drugs approved, or back to multiple antimicrobial uses of MRSA treatments which lead to microbial adaptation to these antibiotics, or (Li and Webster, 2018).

Genotypes identified of the 28 MRSA isolated from human by PCR showed that all isolate (100%) harboring 23srRNA and 76% harboring *mecA* genes. which is higher than that has been observed in Iran by where the rate was 56% from patient's (Ghasemiam and Mirzaee, 2016). and lower than result reported by, AL-Ruaily and Khalil, (2011) they mentioned the rate of *mecA* 86.7% isolated from urine of patients in Bangladesh. The *mecA*-negative MRSA isolated in the current study; might be due to the presence of *mecA* homologues as *mec C* or other β lactam resistance mechanisms, such as *blaZ* gene (Elsayed et al., 2015; El-Ashker et al., 2015).

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