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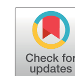
PCR-RFLP by *Alu*I for *coa* gene of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from burn wounds, pneumonia and otitis media



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ABSTRACT

Coagulase is considered as the main determinant to distinguish *Staphylococcus aureus*. The 3′ end coding region of the coagulase gene (*coa*) has a series of 81 bp tandem repeats varying in number and sites of restriction enzyme among diverse isolates. The polymorphism of the coagulase gene among 45 MRSA isolated from burn wounds, otitis media and pneumonia in Basra city, Iraq, were investigated using PCR-RFLP analysis for 3′ end region with *Alu*I enzyme revealing unique restriction patterns (18 patterns) not described in any previous studies. Particularly, pattern 1 was predominant. Different RFLP patterns of *coa* were shared among isolates from the different sampling sites.

The present study used the polymorphism of Coagulase gene to identify MRSA subtypes, estimate the efficiency of these methods in distinguishing the variable strains and compare these subtypes with the source of isolates.

1. Introduction

Staphylococci includes at least 40 species (Harris et al., 2002), are Gram-positive, commensal components of skin of human and animal and microflora of mucus membrane (McAdow et al., 2012a; Bonar et al., 2018). Depending on the capability to coagulate plasma, Staphylococci are subdivided into coagulase-negative staphylococci (CoNS) and coagulase-positive staphylococci (CoPS), involving *Staphylococcus aureus* (*S. aureus*), (Devriese et al., 2005; Taponen and Pyörälä, 2009; Bonar et al., 2018). *S. aureus* commensal of the human anterior nares, throat, skin folds, and gastrointestinal tract of humans (Crossley and Solliday, 1980; Peters et al., 2013). The pathogenic characteristics of *S. aureus* are chiefly due to several virulence factors such as coagulase, protein A, fiberonectin, heamolysin, nucleases, clumping factor, exfoliative toxins and enterotoxins (Gaurav, 2017). The secretion of coagulase is a significant characteristic can be used globally for the identification of *S. aureus* (Momtaz et al., 2011). Thus, the ability to clot the plasma by coagulase production is the first step in the identification of staphylococci bacteria (Bonar et al., 2018). Coagulase testing is done by either slide coagulase or the tube coagulase methods (Kateete et al., 2010). There are some issues associated with coagulase tests, firstly, some human CoNS secrete clumping factor and could be wrongly positive with the slide coagulase test, secondly, some animal origin staphylococci are slide coagulase test negative and tube coagulase positive, these may be misidentified as *S. aureus* unless addition tests are utilized (Kateete et al., 2010). Molecular typing methods, like *spa* (staphylococcal protein A) typing of gene, multilocus sequence typing (MLST), pulsed field gel electrophoresis, multilocus enzyme electrophoresis and coagulase gene genotyping are commonly used to investigate the epidemiology of the strains of *S. aureus* (Yang et al., 2017; Izadpanah and Asadpour, 2018). Analysis DNA sequence of the 3′ end of the *coa* gene that encodes Coagulase enzyme appeared heterogeneity in the 81 bp tandem repeats region encoding repeated 27-amino-acid residues in the C-terminal region. The amplification of this region by PCR revealed DNA amplicons of diverse size and number that may be additionally distinguished by Restriction Fragment Length Polymorphism (RFLP) method with *Alu*I as a restriction enzyme (Goh et al., 1992). Hence, this method (PCR-RFLP) can give a simple and exact subtyping method of *S. aureus* strains (Abdulghany and Khairy, 2014). On the other hand, the resistance of MRSA strains to methicillin is facilitated by a protein, identified as penicillin-binding protein 2a (PBP2a), which shows a low affinity for beta-lactams. PBP2a is encoded

Abbreviations: coa, coagulase-encoding gene; CoNS, coagulase-negative staphylococci; *S. aureus*, *Staphylococcus aureus*; CoPS, coagulase-positive staphylococci; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; MRSA, methicillin-resistance *Staphylococcus aureus*; hr, hour; min, minute; sec, second; UV, ultra-violate ray; V, voltage; A, aer of otitis media; T, throat of pneumonia patient; B, burn wounds; n, number; TBE, Tris/Borate/EDTA; EDTA, ethylenediaminetetraacetic acid; BHIB, brain heart infusion broth

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by *mecA* gene, is a chromosomal gene located through a large gene cassette, identified as staphylococcal cassette chromosome *mec* (Ebrahim-Saraie et al., 2015).

2. Materials and methods

2.1. Bacterial isolates

One hundred and fifty-eight samples were collected 120 samples from burn wounds, 21 samples from otitis media and 17 samples from pneumonia patients of Al-Fayha'a hospital, Al-Basrah hospital, Al-Sadr teaching hospital and medical clinics, between Feb. 2015 to Aug. 2015. Swabs were placed in sterile tubes with brain heart infusion broth (BHIB) media, labeled and transported immediately to the Microbiology Laboratory for processing (Talan et al., 1989). All the samples were culture on mannitol salt agar and tested by standard microbiological procedures including Gram stain, catalase and tube coagulase test of human plasma (Brown et al., 2005).

2.2. Genomic DNA extraction

DNA was extracted by Genomic DNA Mini Kit (Blood\Culture Cell) from Geneaid (Korea); according to the manufacturer's instructions, the DNA were detected by gel electrophoresis, the samples were loaded in 0.8% agarose gel 1 × TBE (54 g Tris-base, 0.5 M EDTA, 1-l distilled water, pH = 8 and diluted with 400 ml of distilled water) and electrophoresed at 60 V for 30 min.

2.3. 16S rDNA gene

The bacteria were identified by PCR with the universal bacterial *16S rDNA* gene primers: 27 Forward 5′-AGAGTTTGATCCTGGC-3′ and 1492 Reverse 5′-GGTTACCTTGTTACGACTT-3′ (Miyoshi et al., 2005). The mixture of PCR reaction was contained 2 µl (30 ng) of target DNA, 2 µl (20 pmol) of each primer and the volume of this mix was adjusted to 50 µl with sterile water with PCR PreMix (Bioneer, Korea). Amplification was carried out in a thermal cycler (PCR Sprint thermal cycler, Thermo, USA). The PCR program comprised initial denaturation at 94 °C for 2 min, 30 cycles (denaturation at 94 °C for 30 Sec, annealing at 55.5 °C for 45 Sec and extension at 72 °C for 1.5 min) and final extension at 72 °C for 5 min. The amplified PCR mixtures were resolved by electrophoresis through 1% agarose gel at 60 V for 1 h and visualized under UV light.

2.4. coa PCR amplification

The 3′ end region of the *coa* gene was a was carried out using primers Coag2, 5′-ACCACAAGGTACTGAATCAACG-3′ and Coag3, 5′-TGC TTTCGATTGTTCGATGC-3′ (Alpha, Kaneda) described by Silva and Silva (2005). The PCR reaction mixture was prepared as the manufacture of *16Sr DNA* gene above. The PCR program comprised initial denaturation at 94 °C for 2 min, 30 cycles (denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s and extension at 72 °C for 1.5 min) and final extension at 72 °C for 5 min. The amplified PCR mixtures were resolved by electrophoresis through 1% agarose gel at 60 V for 1 h and visualized under UV light.

2.5. 16S rDNA and coa genes sequences

The unpurified PCR products of *16S rDNA* (*n* = 53) were sent to purify and perform the sequencing at Sangon Co. and Macrogen Co.

2.6. Detection of mecA gene

The detection of *mecA* gene to identify methicillin-resistance *Staphylococcus aureus* (MRSA) was carried out according to Del Vecchio et al. (1995). The *mec*A was amplified by PCR using primers, MR1 5′-TAGAAATGACTGAACGTCCG-3′ and MR2 5′-TTGCGATCAAATGTT ACCGTAG-3′. PCR product was electrophoresed in 1% agarose gel, stained with ethidium bromide, at 60 V for 1 h and visualized under UV light.

2.7. Restriction endonuclease digestion

Ten µl of PCR product of 45 isolates were incubated with 10 U of *Alu*I (Promega, USA) at 37 °C for 2 h. Ten microliters of the digested PCR product was electrophoresed in 1.5% agarose gel, stained with ethidium bromide at 60 V for 1 h. and visualized under UV light (Silva and Silva, 2005).

2.8. Bioinformatics

The sequences obtained were BLAST in National Centre for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast) for bacterial strains identification and similarity detection to the reported bacterial sequences. The phylogenetic relationships among *S. aureus* isolates were analyzed by rooted Neighbour Joining phylogenetic tree which constructed from concatenated sequences of 984 bp produced by a MAFFT alignment and visualized using forester version 1046. The Schematic representation of PCR-amplified *coa* gene was Created with SnapGene software version 4.2.5.

3. Results

3.1. Identification of bacterial isolates

Out of 158 isolates only 50 (23.69%) were identified as coagulase positive staphylococci, and 3 strains identified as coagulase negative staphylococci but phenotypically like *S. aureus*. All these isolates (*n* = 53) were identified as *S. aureus* by *16S rDNA* sequences.

3.2. Phylogenetic relationships

Rooted Neighbour Joining phylogenetic tree constructed from concatenated sequences of 984 bp shows the Phylogenetic relationship, distribution and similarity of *16S rDNA* of 53 isolates among *S. aureus* isolated (B: isolated from Burn wounds, T: isolated from throat of pneumoniae patients and A: isolated from ear of Otitis media patients) and identified in present study with ATCC.BAA-39 type strain are presented in Fig. 1. This distribution revealed that the strains of *S. aureus* were different even among the specimens isolated from similar sources.

3.3. Detection of MRSA isolates

mecA gene was detected in 45 from 53 (85%) *S. aureus* isolates. 16 (94%) isolates of MRSA were from throat (pneumonia) samples, 14 (93%) from burn wound and 15 (71.4%) from middle ear (Otitis media) samples (Fig. 2).

3.4. Coagulase gene typing

Only 45 (MRSA) of 53 *S. aureus* isolates were amplified with Coag2 and Coag3 primers by PCR, three of them were tube coagulase negative. The PCR products showed 6 bands of different sizes ranged approximately from 659 to 902 bp (Fig. 3). Seven MRSA isolates showed band at 902 bp, five isolates showed band at 821 bp, one isolate showed band at 761 bp, twenty-six isolates showed band approximately 740 bp, one isolate had one band at 717 and five isolates showed band at 659 bp.

Fig. 1. Rooted Neighbour Joining phylogenetic tree constructed from concatenated sequences of 984 bp produced by a MAFFT alignment and visualized using forester version1046. This tree shows the Phylogenetic relationship, distribution and similarity of ***16S rDNA*** of 53 isolates of *S. aureus* (B: isolated from Burn wounds, T: isolated from throat of pneumoniae patients and A: isolated from ear of Otitis media patients) identified in present study with ATCC.BAA-39 type strain. All lengths of horizontal branch were drawn to scale. Scale bar show the distance of evolutionary between sequences determined by calculating the lengths of the horizontal lines linking two isolates. Bootstrap values after 100 repetitions are indicated.

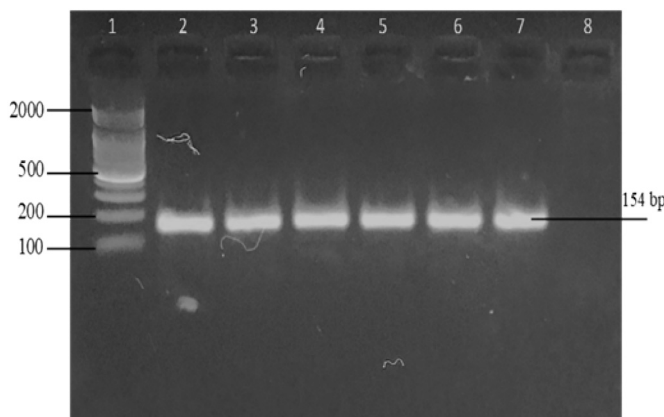

Fig. 2. Amplification of *mecA* gene form bacterial isolates detected by agarose gel electrophoresis, Lane 1: 100 bp Marker (Bioneer), Lane 2–7: *mecA* gene bands at 154 bp, and Lane 8: Blank.

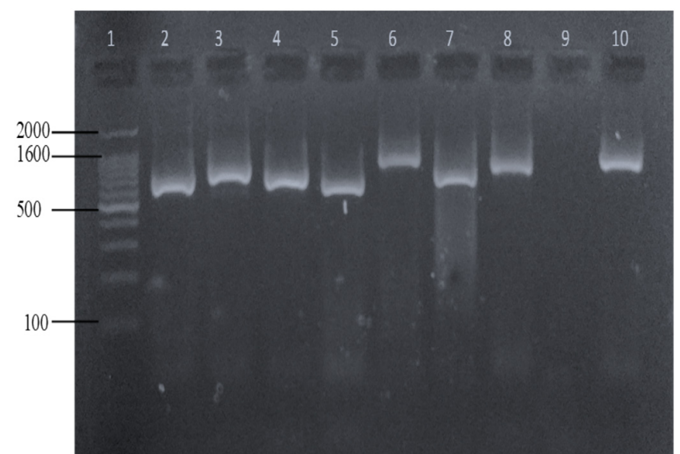

Fig. 3. PCR products of *coa* gene (amplified by coa-3 primer). Lane 1, 100-bp ladder; Lane 9, Blank; Lanes 2–8 and 10 *coa* gene bands at 650–900 bp.

3.5. Restriction fragment patterns

Digestion PCR amplified product of the 45 clinical isolates by *Alu*I restriction enzyme identified 18 different restriction enzyme patterns was showed in Figs. 4. The first pattern, 443 bp, 271 bp and 81 bp from product of 740 bp, was more frequent ($n = 15$) among other (Table 1). The comparison of the *coa* genotype of MRSA isolates and their sources were shown in Table (2), which appeared that the isolates shared genetic relatedness among the MRSA genotypes isolated from different sources, while some isolates have RFLP *coa* genotypes not resembled the profile of the other MRSA isolates from same and other sources.

4. Discussion

The coagulase test was performed with human plasma, because the human origin isolates have shared the property of coagulating human plasma with a higher percentage than other plasmas (Abd Al-Abbas, 2004; Bonar et al., 2018).

According to the susceptibilities of antibiotic, there are two types of *S. aureus*: methicillin-sensitive *S. aureus* “MSSA” and methicillin-resistant *S. aureus* “MRSA” (Khan et al., 2018). The use of methicillin has caused the presence of MRSA (Lakhundi and Zhang, 2018). It is related to significant morbidity and mortality, comprising soft tissue and surgical site infections (David and Daum, 2010; Leibler et al., 2017). Since the precise and rapid detection of antibiotic resistance is very important in the treatment of staphylococcal infections (Al-Khafaji and Flayyih, 2015). The MRSA isolates were detected by *mec*A gene amplification, which allows the bacteria promote antibiotics resistance including methicillin, penicillin, and other penicillin-like antibiotics (Del Vecchio

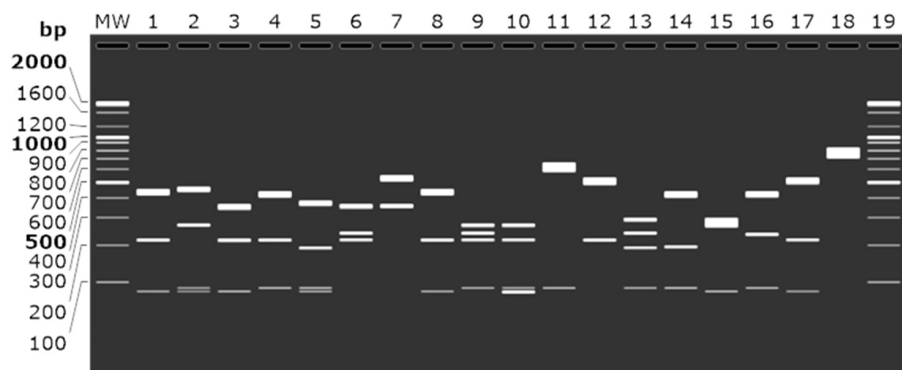

Fig. 4. Schematic representation (Created with SnapGene) of PCR-amplified coa gene digested with *Alu*I. Lane MW and 19: 100 bp DNA ladder (as Bioneer 100 bp Ladder); Lanes 1 to 18 showing the 18 *Alu*I patterns observed among the 45 isolates.

Table 1
Frequency of Coa genotype patterns in the 3-Terminal region of *coa* gene detected by *Alu*I.

Pattern	PCR products (bp)	RFLP pattern (bp)	Frequency no.
1	740	443, 271, 81	15
2	902	459, 273, 89, 81	5
3	740	435, 216, 89	4
4	659	362, 216, 81	4
5	740	378, 192, 89, 81	2
6	740	362, 216, 243	2
7	902	540, 362	2
8	740	443, 216, 243	1
9	821	273, 243, 216, 89, 81	1
10	821	273, 216, 89, 81*3	1
11	740	651, 89	1
12	740	524, 216	1
13	821	297, 243, 192, 89	1
14	717	435, 193, 89	1
15	659	297, 281, 81	1
16	761	435, 237, 89	1
17	821	524, 216, 81	1
18	821	No recognition site	1
Total		45	

et al., 1995; Shah et al., 2017; Tsai et al., 2017). The results of the present study displayed a high frequency of MRSA as 45 (85%) from 53 *S. aureus*, this agreed with other studies which appeared 82.47% of isolates were resistant to methicillin (Wolk et al., 2009; Thampi et al., 2015; Al-Khafaji and Flayyih, 2015; Lakhundi and Zhang, 2018). On the contrary, the Egyptian study by Hamza et al. (2015) showed that only 20% of coagulase positive were *S. aureus* were Methicillin- Resistance. This could be a guide for the clinician to use suitable antibiotics by using vancomycin for MRSA infected patients, while penicillin for MSSA infected patients (Japoni et al., 2004; Falcone et al., 2015).

The common causative bacteria in pneumonia is MRSA (Claeys et al., 2016), and the fast identification would be helpful for the early diagnosis of this disease (Huang et al., 2015). In the present study, out of 17 suspected pneumonia throat samples 16 (94%) were MRSA. This agreed with Hamdan-Partida et al. (2018).

The *mec*A gene was detected in 71.4% of *S. aureus* from ear discharges (from otitis media "OM" infection) which comes in line with the study of Rath et al. (2017). On the contrary, another published report recorded only 7% of MRSA (Kumar et al., 2016). Rising the rates of MRSA leads to increase the infection rates in hospitals that increasing of morbidity and mortality rates (Al-Ruwaili, 2018). However, the results of throat and ear discharge samples are in agreement with Lakhundi and Zhang's (2018) study showing that in addition to MRSA existing in hospitals, there are new strains which can invade community and infect people.

Burn wounds are a proper environment for multiplication of bacteria and are the richest infection sources than surgical wounds, chiefly due to the larger area of wound and the longer period of patient residency in the hospital (Church et al., 2006; Abdulazeem et al., 2016). In present study, MRSA represent to a high prevalence (93%) of *S. aureus* isolated from burn samples. This agreed with the Iraqi study by Ronat et al. (2014). Such ratio poses a threat to the patient life because of the sensitivity of MRSA to only limited antibacterial agents decreasing the options of therapy (Mohammed et al., 2015).

The produced coagulase from *S. aureus* is considered a chief virulence factor (Motamedi et al., 2015). Coagulase-positive bacteria could not be recognized phenotypically in all cases because some of the biochemical possessions were weak or uncharacteristic for specific species. Since, three isolates were CoNS but appeared resemblance to *S. aureus*, however genotypically reclassified as CoPS species by *coa* gene detection, similar result was obtained by Veras et al. (2008) that revealed the *coa* gene were detected in seven isolates of the coagulase-negative test. Therefore, there is no single phenotypic test (like tube coagulase) can give precise results for *S. aureus* identification (Kateete et al., 2010; Gharib et al., 2013; Hamza et al., 2015). Thus, molecular methods give a precise diagnosis for coagulase positive bacteria (Sleiniute and Siugzdaite, 2015).

Although the positive result of coagulase test would indicate the presence of *S. aureus* (Björkstén et al., 2001; McAdow et al., 2012b; Malachowa et al., 2016), and the *coa* gene is existed on all *S. aureus* chromosomes, (Watanabe et al., 2005; Watanabe et al., 2009), but the study by Hamza et al. (2015) appeared that only 30 strains of 70 *S. aureus* were coagulase positive by *coa* gene amplification. This result may be due to the absent of the precisely complementary primers to the *coa* gene, since the *coa* gene has a diversity among these isolates (Upadhyay et al., 2012; Dendani et al., 2016). Resembling to the result of the present study which appeared that *coa* gene was amplified in only 45 isolates.

The cause of this diversity among isolates of *S. aureus* is because of the deletion or insertion mutations in a part of the 3′ end region (Goh et al., 1992; Saei et al., 2009). By these results, it is suggested that there is no universal primer for *coa* gene especially to the 3′ end region.

Polymorphism of the *coa* is one of the molecular-based typing methods of *S. aureus* isolates (Izadpanah and Asadpour, 2018). The amplification of *coa* 3′ end region by coa-3 primer gave six classes of amplicons according to their sizes, ranging from 659 bp to 902 bp (Fig. 3), the 740 bp is the predominant amplicon. These results are in agreement with many studies that revealed variable amplicons resulted from amplification of this region although they used the same primers. Due to the different sizes of the *coa* alleles (Sindhu et al., 2010; Perillo et al., 2012; Al-Daghistani et al., 2017; Yadav et al., 2018), but the sizes of amplicons in the present study were different than the other studies because of the profiles virulence genes which are very heterogeneous and differ among countries (Silveira-Filho et al., 2014).

The resulted amplicons from *coa* digestion with *Alu*I (Fig. 4)

Table 2
Distribution of *Alu*I RFLP patterns and frequencies of coagulase according to the genotypes of MRSA isolated from the three sources of samples.

Origin	n	Genotype																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Burn wounds	16	12																	1
Otitis media	14	3	1	1	1	2	2		1					1	1				
Pneumonia	15		4	3	3			2		1	1	1	1			1	1	1	

revealed considerable genetic variability as in the categorizes with18 patterns, the pattern 1 (443, 271 and 81 bp fragments) was the predominant among *coa* alleles while the pattern 18 has no recognition site for *Alu*I enzyme (Table 1). The results were like those of other studies (Mullarky et al., 2001; Silveira-Filho et al., 2014) showed a significant degree of variability in RFLP patterns of *coa* gene amplified with the same primers after the digestion with the same enzyme, although the sizes of PCR products attained in the present study were differ from those in previous studies, but they have unique restriction patterns not described before. Su et al. (1999) studied *coa* gene variety in *S. aureus* isolates from four countries. They described 5 genotypes which were predominant for each country, though, predominant types altered based on the geographical regions.

Different genotypes were got among the isolates from the present study proposing *S. aureus* has heterogeneity in a certain degree among the isolates from different cases of patients (Table 2). Likewise, genotyping using spa PCR-RFLP of MRSA isolates of human and chicken meat origin, showing the genetic relation among these isolates (El Bayomi et al., 2016). The six different amplicons of PCR product and the 18 patterns of RFLP *coa* revealed that the *S. aureus* isolated strains have more than one different of the *coa* gene in addition to the distribution of *16S rDNA* sequences among the *S. aureus* isolates (Fig. 1), which comes in line with the study of Soltan-Dallal et al. (2016) which revealed that numerous coagulase genotypes were in charge for the majority of bovine mastitis cases from different countries but only some of genotypes were predominated in each country.

Data availability

The samples data used to support the findings of this study are included within the article.

Conflicts of interest

No conflicts of interest were declared by the authors.

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