New Rapid Method for Differentiation of MRSA and SSA by PCR Restriction Analysis of 920 bp of *dnaJ* gene

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Abstract: Methicillin-resistant *Staphylococcus aureus (*MRSA) and sensitive *Staphylococcus aureus* (SSA) are responsible for a high proportion of nosocomial infections, which makes difficulty in treatment. MRSA infections are responsible for increased mortality rates, longer lengths of hospital stay, and higher rates of treatment failure compared to SSA infections. Detection of MRSA by conventional method is time consuming, influenced by culture medium, concentration of NaCl, temperature, time of incubation and antibiotics. Various PCR methods had been applied for the rapid detection and identification of *Staphylococcus* species. The *dnaJ* gene sequence is potentially useful for the identification of genetically related *Staphylococcus* species and subspecies. While, with other bacterium PCR-restriction analysis is preferred, as a simple and cost-effective method that does not involve radioisotopes. For that reason in this study, we established and evaluated a rapid new protocol of identifying clinically relevant MRSA species by PCR- restriction analysis of the *dnaJ* gene. SSA and MRSA strains were isolated during a one year period from patients with bacterimia. Identification of *S. aureus* was performed by standard laboratory methods. Resistance to methicillin was detected by disc diffusion susceptibility test. DNA extraction was performed for both clinical blood samples as well as from isolated SSA and MRSA. Primers were designed to amplify specific *dnaJ* gene target and confirming the presence of *S. aureus*. MRSA was speciated by PCR-restriction analysis of *dnaJ* gene using XapI restriction enzyme. Our results showed two distinguished patterns of PCR-restriction analysis for SSA and MRSA. Only SSA is known to have a XapI restriction sites. Using our protocol, we were able to demonstrate the existence of staphylococcus and to identify their methicillin resistance. Therefore, we suggest that it would be very useful to apply PCR amplification restriction analysis to *dnaJ* gene directly to clinical specimens early in the diagnostic process. This would save several days that are required for conventional culture. Thus, this established protocol is suggested as a simple and useful method for the rapid detection and simultaneous identification of MRSA in primary clinical specimens or for the identification of culture isolates. This rapid detection would allow clinicians initially to avoid potentially inappropriate treatment options.

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1. Introduction

Bacteremia was defined as one or more positive blood cultures accompanied by systemic manifestations of infection such as fever, chills, and sweats, with or without local signs and symptoms. Bacteremia caused by *Staphylococcus aureus* (*S. aureus*) continues to be a common problem worldwide which makes treatment difficult (**Perez-Roth, *et al.*, 2001; Tiemersma, *et al*., 2004 and Brown**, ***et al*., 2005)**. Also *S. aureus* remains a common cause of bloodstream infections of community onset. Increasing numbers of these community-onset infections are being caused by methicillin resistance *S.aureus* (MRSA) and capable of causing life-threatening diseases (**Carvalho**, ***et al*., 2010** and **Welsh *et al*., 2010)**. MRSA bacteremia is associated with an approximate mortality rate of 50% (**Marchaim**, ***et al.*, 2010)**. Factors contributing to MRSA bacteremia include the use of invasive devices, concentration of very sick patients, high work-load, microorganisms' cross-transmission, and widespread use of antibiotic therapy (**Dellit**, ***et al*., 2007).**

The clinical impact of MRSA infections is significant, regardless of the origin of infection. Active screening and compliance to appropriate infection control activities have been shown to play an important role in the control of MRSA (**Welsh, *et al.*, 2010)**.

Detection of MRSA by conventional method is time consuming, influenced by antibiotics, culture medium, NaCl concentration, temperature and time of incubation. There are many published assays for the detection of *S. aureus* and MRSA by PCR (**Jaffe, *et al*., 2000, lem, et *al*., 2001, Grisold**, ***et al*, 2002**, **Jonas, *et al*., 2002, Louie, *et al.*, 2002 and Malathi, *et al*., 2009)**. Rapid diagnostic tests have the potential to make efforts even more effective. Thus, infection prevention has taken a step forward with the introduction of various tests for rapid identification of MRSA carriers. Most molecular methods for identification of *S. aureus* have been PCR-based. A

range of primers designed to amplify species-specific targets have now been developed. Such targets include the nuclease (*nuc*), coagulase (*coa*), protein A (*spa*), *femA* and *femB*, *Sa442*, 16SrRNA and surface-associated fibrinogen-binding protein genes. More recently, PCR based methods have been used routinely by reference laboratories as their standard method for detecting the *mecA* gene. PCR enables an earlier determination of methicillin resistance by detecting *mecA*, which has been called a "gold standard" for the detection of the presence of methicillin resistance in staphylococci (**Francois, *et al*., 2003**, **Hardy, *et al.*, 2004**), and confirming the presence of *S. aureus*.

The *dnaJ* gene encodes the DnaJ protein, which is also known as Hsp40, and is a member of the heat shock protein (Hsp) family; and the *dnaJ* gene sequence is more discriminative than the sequences of other conserved, housekeeping genes used in the taxonomy of staphylococci. The *dnaJ* gene sequence is potentially useful for the identification of genetically related species and even subspecies **(Hauschild and Stepanovic, 2008 and Ho *et al*., 2009).**

In this study, we established and evaluated a rapid new method of identifying clinically relevant MRSA species by PCR-RFLP based analysis of the *dnaJ* gene. This method can be used to rapidly detect MRSA species in isolates or in blood using a previously described *dnaJ* gene-specific primer pair **(Shah, *et al*., 2007)**.

2. Materials and Methods

Exclusions criteria included initiating therapy prior to admission, lack of signs of infection (considered to represent contamination). Each patient was counted once. Demographics, clinical characteristics, implicated source, therapy, and outcome were recorded.

Microbiological methods

Patient Samples

Non duplicate clinical specimens from blood were collected from patients at the National Cancer Institute (Cairo, Egypt). For each specimen, only non duplicate isolates were included (the first isolate per species per patient). Data collected on each patient consisted of demographic data including age, admission date, ward, hospitalization duration, and sites of positive culture. Patient consent was obtained before collection of specimens. All infections were hospital-acquired, i.e. isolated 72 h after patient admission to the hospital.

(i)Species identification and susceptibility tests

One hundred and fifteen consecutive staphylococcal isolates recovered from blood clinical specimens.

Screening of Staphylococcus spp. from clinical specimens

The clinical isolates were identified morphologically and biochemically by standard laboratory procedures. Also the identification was confirmed by Microscan Positive Identification (PID) panel type 20 (Dade Behring, West Sacramento, USA)

Antimicrobial susceptibility testing

Both automated and manual methods were used to detect the antimicrobial susceptibility pattern of the isolates. Susceptibility testing for methicillin resistance and other antibiotic resistance phenotypes was carried out by the Kirby- Bauer methods **(CLSI, 2009).** MIC of methicillin was determined by E-test kits (AB Biodisk, Solna, Sweden). The results were categorized according to CLSI standards (CLSI, 2009). Reference strains used as controls were *S. aureus* (ATCC 33591).

Microscan PBPC 20 automated system was also used for antimicrobial susceptibility testing of staphylococcal isolates. Standard quality control ATCC (Manassas, Virginia) strains with known minimum inhibitory concentration (*S. aureus* ATCC 29213, methicillin-sensitive *S. aureus* ATCC 6538 and methicillin-resistant *S. aureus* ATCC 33591) were used as a reference strains.

(ii) Molecular methods:

DNA extraction:

DNA was extracted from blood specimens and from clinical isolates culture. Frozen isolates were grown on Trypticase soy agar with 5% sheep blood overnight at 35°C. One to three isolated colonies were used for DNA extraction. DNA was extracted using Genomic DNA extraction Kit (Fermentas lifescience, EU) in accordance with the manufacturer's instructions.

PCR-RFLP analysis of *dnaJ* gene

PCR (thermal cycler brocycler TCS, BOECO, Germany) was used for detection and amplification of *dnaJ* gene. The previously described **Shah, *et al.* (2007)** *dnaJ* degenerate primers SA-F (5'-GCC AAA AGA GAC TAT TAT GA-3') and SA-R (5'-ATT GYT TAC CYG TTT GTG TAC C-3') were used to amplify the *dnaJ* gene fragment. The primers were synthesized from the Biotechnology Facility at the University of Connecticut, USA. The PCR mixtures were first incubated for 3 mins at 94°C, followed by five cycles at 94°C for 30 s, 45°C for 30 s, and 72°C for 60 s. The mixtures were then subjected to a series of 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s; these were completed with a final extension at 72°C for 3 min. The presence of a PCR product was confirmed by 1% (wt/vol) agarose gel electrophoresis and visualized with ethidium bromide.

Restriction analysis:

Ten microliters of the amplified PCR products were transferred to a clean micro-centrifuge tube and digested with restriction enzymes according to the supplier's instructions (Fermentas lifescience, EU). Briefly, 1 unit of XapI digestive enzyme was added to 1 ug of the genomic DNA. Then the mixture was incubated at 37°C for 5 mins, followed by analysis on an agarose gel. Gels were stained with ethidium bromide, and visualized by ultraviolet light and photographed.

Statistical analysis:

We used the chi-square test for categorical variables, a Student *t* test, or analysis of variance for continuous variables; the log-rank test for time-dependent variables; and stepwise forward logistic regression for multivariate analysis of dichotomous outcomes. All tests were performed using SPSS computer software release 10. A *P* value of 0.05 was considered to indicate statistical significance.

3. Results:

We encountered 115 patients with positive blood culture for *Staphylococcus* spp. fourteen of these individuals were excluded. These patients did not meet our inclusion criteria: recurrent disease (*n* _ 6), no clinical signs of bacteremia (considered to have contamination; *n* _ 5), and three have received prior antibiotic therapy. All remaining 101 patients were included. 44 isolates were identified as *S.aureus* by conventional method and Microscan semi-automated test, (Table 1). MRSA accounted for 26 (%) of cases. MRSA was associated with higher mortality (23.9% versus 8.9% in MSSA). These isolates were also associated with longer durations of bacteremia (4.7 ± 6.5 versus 2.7 ± 2.9 days; *P* _ 0.01).

All blood specimens that were positive for *S.aureus* and their corresponding isolated species were subjected to DNA extraction. The extracted DNAs were used as a template for PCR-restriction analysis of *dnaJ* gene using XapI restriction enzyme. A total of 44 clinical isolates of SSA (18) and MRSA (26) were tested to validate the results of PCR-restriction analysis of the *dnaJ* gene in a clinical setting.

The *dnaJ* gene was amplified by PCR with a Staphylococcus specific pair of primers, the specificities of which have been examined previously **(Shah, *et al.*, 2007 and Hauschild and Stepanovic, 2008).** The 920-bp products amplified from these species by PCR showed in figure 1. PCR products were digested with XapI, and the resulting fragments were separated by agarose gel electrophoresis (figure 2). Four distinctive patterns were obtained for all SSA and reference strain. MRSA had no XapI restriction sites for all the studied species.

4. Discussion

The emergence of *Staphylococcus* spp. not only as human pathogens, but also as reservoirs of antibiotic resistance determinants, requires the development of methods for their rapid and reliable identification in medically important samples. A variety of manual and automated methods based on phenotypic characteristics have been developed for the identification of staphylococci, including conventional identification methods and several methods that use commercial kits. Unfortunately, the overall accuracies of these systems are low and range from 50 to 70% (**Perl, *et al.*, 1994; Ieven**, ***et al.*, 1995**; **Roisin, *et al.*, 2012).** Moreover, conventional reference methods are too laborious and time consuming to be used in clinical laboratories. Several problems associated with the systems mentioned above result from the variability in the expression of metabolic activities and/or the morphological features of some staphylococcal species (**Couto**, ***et al.*, 2001)** thus, if the strain has atypical characteristics, it may be difficult if not impossible, to precisely assign the strains to the species level. Furthermore, commercial systems may offer two or more suggestions as to the species identification with comparable levels of safety. Due to the limited number of stable features that can be used for species discrimination, many taxa remain difficult to distinguish from one another and are misidentified by phenotypic tests. To solve these problems, restriction fragment length polymorphism (RFLP) analysis of PCR products and a number of PCR amplicon sequencing-based methods have been reported for use for the identification of staphylococci (**Mellmann, *et al.*, 2006**; **Barros *et al.*, 2007; Shah, *et al.*, 2007; Esther, *et al.*,** 2012).

The use of nucleic acid targets, with their high sensitivity and specificity, provides an alternative technique for the accurate identification SSA and MRSA. We confirmed that the PCR-RFLP method is a highly specific, simple and time-effective. The PCR-RFLP test can be achieved with just one pair of primers and one restriction enzyme, and more than 100 samples can be processed in a few hour. The cost and efficiency of the PCR-RFLP method are comparable to those of the quantitative PCR method published previously (**Francois, *et al.*, 2004, and Malathi, *et al.*, 2009**). Especially, it seems to be a very useful tool for rapid identification of MRSA in clinical laboratories, since serious MRSA infection has been increasingly reported in persons without identified predisposing risk factors, including recent healthcare exposure. Results reaffirming the analytical advantages of *dnaJ* DNA have been demonstrated. Not only can *dnaJ* PRA be applied to culture isolates, but it is also applicable directly to specimen. The uses of our protocol on specimens, will save time and expense. Thus, this protocol is

suggested as a simple and useful method for the rapid detection and simultaneous identification of MRSA in primary clinical specimens or for the identification of culture isolates. In addition, this study highlights the usefulness of the *dnaJ* gene as a single alternative marker for Staphyllococci.

Table (1): Number & percentage of species of *Staphylococci* isolated from blood specimens:

Staphylococalisolates	No.	%
S. aureus	44	43.6
S. hyicus	3	2.9
S. simulans	6	5.9
S. haemolyticus	7	6.9
S. auricularis	6	5.9
S. epidermidis	11	10.9
S. xylosus	2	2.0
S. hominis	20	19.8
S. cohnii	1	0.09
S. warneri	1	0.09

Table (2): MIC for *Staphyllococcus sp.* against tested antibiotics:

	CoNS n=57				*S.aureus* n=44			
Antibiotic	Breakpoint	S	I	R	Breakpoint	S	I	R
Amikacin	32	15	10	32	32	15	1	28
Amoxicillin/K clavulanate	16/8	-	-	57	16/8	3	-	41
Ampicillin	16	-	-	57	16	3	-	41
Ampicillin/sulbactam	16	-	-	57	16	5	-	39
Azithromycin	2	9	-	48	4	19	-	25
Cefazolin	32	9	11	37	32	7	-	37
Cefepime	32	12		45	32	10	-	34
Cefoperazone	32	13	10	34	32	31	-	13
Cefotaxime	32	-	9	48	32	5	-	39
Cefotetan	16	17	-	40	16	23	-	21
Ceftriaxone	2	8	7	42	2	9	-	35
Cefuroxime	4	7	10	40	4	7	-	37
Cephalothin	8	-	-	57	8	6	-	38
Chloramphenicol	4	19	12	26	4	2	-	42
Ciprofloxacin	64	21	-	36	64	16	-	28
Clindamycin	8	15	13	29	8	10	7	27
Erythromycin	16	-	27	30	4	7	11	26
Gatifloxacin	32	25	8	24	32	24	3	17
Gentamicin	32	16	-	41	32	9	1	34
Imipenem	8	-	-	57	8	5	-	39
Levofloxacin	64	29	-	28	64	25	-	19
Linezolid	16	57	-	-	32	38	-	6
Moxifloxacin	32	39	18	-	16/8	25	6	13
Ofloxacin	64	35	14	8	8	16	2	26
Oxacillin	32	-	-	57	2	6	2	36
Penicillin	32	-	-	57	32	1	-	43
Rifampin	8	57	-	-	64	28	2	14
Sulfamethoxazole/trimethoprim	64	34	-	23	16	16	-	28
Tetracycline	16	57			16	9	-	35
Tobramycin	16	9	12	36	16	3	-	41
Vancomycin	32	57	-	-	32	28	2	14

CoNS: coagulase negative staphylococci; S: sensitive; I: intermediate; R: resistant

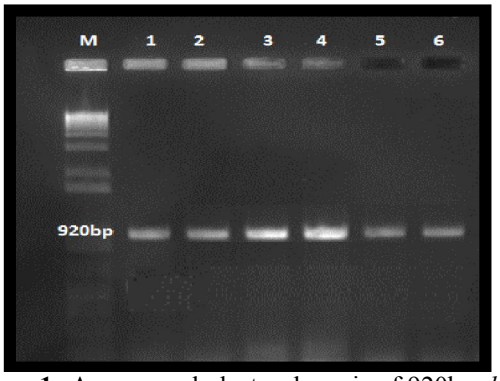

Figure 1: Agarose gel electrophoresis of 920bp *dnaJ* gene amplification product of *Staphylococcus* species, lanes 1to 6 random samples of *Staphylococcus* species and M: DNA marker, 1kbp ladder.

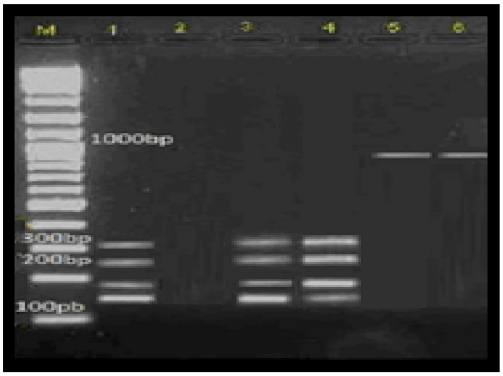

Figure 2: 920 bp PCR products of dnaJ gene digested with XapI, lane 1: reference SSA strain, Lane 2: negative control: lane 3 and 4: SSA studied species, lanes 5and 6: MRSA studied species with no XapI restriction site, and M: DNA marker, 50-bp ladder.

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