J Biochem Tech (2018) Special Issue (2): 107-110
ISSN: 0974-2328

Detection of *Staphylococcus aureus* Clinical Isolates by *Gene Sa442*: Improving the Clinical Diagnosis

Nasim Asadi Faezi, Alka Hasani*, Mohammad Ahangarzadeh Rezaee, Hossein Samadi Kafil, Akbar Hasani

Received: 05 Junuary 2018 / Received in revised form: 29 Jun 2018, Accepted: 06 July 2018, Published online: 05 September 2018
© Biochemical Technology Society 2014-2018
© Sevas Educational Society 2008

Abstract

Staphylococcus aureus is well known as a major pathogen, causing a variety of nosocomial and hospital-acquired infections. Rapid and reliable detection of methicillin-resistant *S. aureus* (MRSA) is important for initiation of appropriate antibiotic therapy and prevention of the spread of the organism. For rapid identification of methicillin-resistant *Staphylococcus aureus*, molecular methods are generally targeting *mecA* and species specific genes. *Sa442* DNA fragment is a popular species-specific target. However, recently, there have been few reports on *S. aureus* isolates that are negative for *Sa442* fragment; therefore, use of single gene or DNA-fragment–specific polymerase chain reaction (PCR) for identification of microbial isolate may result in misidentification. As a consequence, the use of one gene as single marker may lead to misidentification, while incorporation of an additional species-specific marker could increase strain coverage and assay robustness. Therefore, the present study was aimed at the development of a species-specific PCR assay for rapid identification of *Staphylococcus aureus* isolates, using both *nuc* and *Sa442* as target genes. Therefore, the present study was aimed at the development of a species-specific PCR assay for rapid identification of *Staphylococcus aureus* isolates, using both *nuc* and *Sa442* as target genes**.**

Keywords: *Staphylococcus aureus*, Clinical, Gene, Sa442.

Introduction

Staphylococci are ubiquitous colonizers of the skin and mucous membranes. Among them, *Staphylococcus aureus* is the mostpathogenic species and a leading cause of several life-threatening diseases (Diekema et al., 2001). The wide spectrum of clinical manifestations include superficial, deep-skin, and soft-tissue infections, osteomyelitis, pneumonia, endocarditis, and septicemia as well as a variety of toxin-mediated diseases, including food-borne gastroenteritis, staphylococcal scalded skin syndrome, and toxic shock syndrome, with ß-lactam antibiotics being the drugs of choice for therapy (O'Connor, 2006). Since the introduction of methicillin into clinical use in 1961, the occurrence of methicillin-resistant S. aureus (MRSA) strains has increased steadily, and nosocomial infections have become a serious problem worldwide(Barber, 1961; Lowy, 1998; Panlilio, 1992, Towneret al., 1998; Voss et al., 1994). MRSA strains are usually introduced into an institution by an infected or colonized patient or by a colonized health care worker. Thus, epidemiological surveys and control measures are particularly important for MRSA. Rapid screening followed by accurate and timely identification of MRSA becomes an elemental procedure in preventive measures (Fang & Hedin, 2003). In the clinical laboratory, S. aureus is identified by growth characteristics and the subsequent detection of catalase and coagulase activities or specific surface constituents. The DNase and thermostable endonuclease tests have been used as confirmatory tests for inconclusive or negative coagulase tests. Conventional susceptibility testing of S. aureus reliably detects resistance to methicillin or oxacillin if agar dilution or agar screening methods are used according to NCCLS standards (Kohner et al., 1999). Conventional MRSA screening takes up to three days, during which patients and staff carriers can spread MRSA. Development of rapid accurate detection techniques should contribute to the prevention of transmission. In clinical laboratories today, genotypic methods for species determination and detection of the methicillin-resistant gene mecA are being used more often to increase the accuracy of identification and to obtain reliable results more rapidly. Since the mecA gene is not

Nasim Asadi Faezi, Alka Hasani*, Mohammad Ahangarzadeh Rezaee, Hossein Samadi Kafil
Department of Bacteriology and Virology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.

Akbar Hasani
Department of Biochemistry and Clinical Laboratory, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

***E-mail**: dr.hasanialka@tbzmed.ac.ir; dr.alkahasani@gmail.com

exclusive to S. aureus, these methods include a species-specific detection test as well (Sütterlin et al., 2003). As a consequence, further investigation of suspected colonies using molecular techniques is often needed. Several species-specific markers have been described, to confirm *Staphylococcus aureus* isolates, including the coagulase gene (*coa*), the factor essential for expression of methicillin resistance gene (*femA*), the thermonuclease gene (*nuc*), and a chromosomal DNA insertion of 442 bp (*Sa442*). Both *coa* and *femA* show high polymorphism, and are unsuitable for diagnostic purposes (Schwarzkopf & Karch, 1994; Jayaratne & Rutherford, 1997). On the contrary, *nuc* is frequently used as a species-specific marker both in human (Costa et al., 2005) and veterinary isolates (Gao et al., 2011), being highly conserved among *Staphylococcus aureus* strains (Brakstad et al., 1992). Nonetheless, some isolates have been reported as nuc negative by partial deletion or mutation of the nuc gene (Costa et al., 2005; Van et al., 2008). Recently, *Sa442* targeting has been proposed as an alternative method to culture assays (Martineau et al., 1998). even though partial deletion is recognized in *Sa442* among a few human strains (Klaassen et al., 2003; Heilmann et al., 2004) As a consequence, the use of one gene as single marker may lead to misidentification, while incorporation of an additional species-specific marker could increase strain coverage and assay robustness (Van et al., 2008). Therefore, the present study was aimed at the development of a species-specific PCR assay for rapid identification of *Staphylococcus aureus* isolates, using both *nuc* and *Sa442* as target genes.

Methods and Materials

Sample for study, number and sampling method (if needed): 103 *Staphylococcus aureus* were obtained from the Sina Hospital of Tabriz during the October 2015 to March 2016, and identified in the microbiological laboratory. These isolates were collected from blood, urine, ulcer, cerebrospinal fluid, body fluids, catheter, tracheal tube, abscess, and nasopharyngeal and evaluated. Considering the 95% confidence and 80% test power, test 2 sequences and using the G power 3.2.1, sample size were at least 103 bacterial isolates. To determine identity after microscopic and morphological examination, the catalase tests, coagulase enzyme production test, reaction on mannitol salt agar and DNAase test were used to determine the identity of isolated gram-positive cocci (Tille, 2013) The confirmation of the identity of the bacteria examined as *Staphylococcus aureus* was investigated using the presence of nuc and sa442 genes by using specific primers and Polymerase Chain Reaction (PCR) (Sadeghi & Mansouri, 2014) Isolates identified as Staphylococcus aureus were kept at a temperature of -80 ° C in broth trapezoid (TSB) containing 15% glycerol for the further use (Lehmann et al., 1999; Mac Faddin, 1976).

DNA extraction

In the current study, DNA was extracted by DNA extraction kit. First, bacterium was inoculated in the LB (Luria-Bertani) liquid medium for about 24 hours. After the recommended incubation, ml enrichment culture was added into a 2 ml micro centrifuge tube and centrifuged at 13000×g for 5 min. Then the supernatant was discarded taking care not to disrupt the pellet. Added 400 µl Fast Lysis Buffer was added to the bacterial pellet, and the pellet re-suspended by brief, vigorous vortexing. In the next step, transferred the entire mixture was tranferred to a Pathogen Lysis Tube, vortexed at maximum speed for 10 min and then centrifuged at 13000 ×g for 5 min. At last, 100 µl of the supernatant was transferred to a fresh 1.5 ml micro centrifuge tube.

Molecular detection of Staphylococcus aureus using specific PCR

S. aureus strains were grown overnight at 37°C on trypticase soy agar (TSA). For confirmation of *S. aureus* species and detecting methicillin resistance a multiplex PCR was carried out for genes *nuc* with *nuc* forward primer 5 -AGT TCT GCA GTA CCG GAT TTG C- 3 and *nuc* reverse primer 3 -AAA ATC GAT GGT AAA GGT TGG C- 5. PCR conditions were the following: an initial temperature of 95∘C (5min), followed by denaturation at 95∘C (30s), annealing at 55∘C (30s), and elongation at 72∘C (1min), and a final elongation step at 72∘C (10min) as described by (Rushdy et al., 2007). Another single polymerase chain reaction (PCR) was used to detect the *Sa442* fragment with the *Sa442* forward primer 5 -AATCTTTGTCGGTACACGATATTCTTCACG- 3 and *Sa442* reverse primer 5 -CGTAATGAGATTTCAGTAAATACAACA- 3. PCR conditions were the following: an initial temperature of 96∘C (3min), followed by denaturation at 95∘C (1min), annealing at 55∘C (30s), and elongation at 72∘C (3min), and a final elongation step at 72∘C (4min). The PCR amplicons (6 ml) were visualized using UV light (Alpha Imager) after electrophoresis in a 1.8% (w/v) agarose gel containing 0.01 ml safe stain. Amplicons of the expected size for *nuc* and *sa442* (276 bp) and (108 bp) were obtained respectively (Rushdy et al., 2007; Martineau et al., 1998).

Results

In this study, 103 isolates of *Staphylococcus aureus* were identified from the patients was identified phenotypically and genotypically. The samples of our research were collected from various clinical specimens such as blood, ulcers (from the skin, surgery, internal, burn and infectious), urine and body fluids from patients referred to different parts of the hospital such as infections, burns, intensive care

units, skin , internal and surgical procedures. The average age of the patients was 40.3 years and 55.6% of the patients were male. From the 103 primary samples, one of them was excluded from the study due to lack of growth in sub culturing. Among 102 *Staphylococcus aureus*, 92 isolates were obtained from hospitalized patients and 10 isolates were outpatients. DNAase test was performed for confirmation of isolates for being *Staphylococcus aureus*. Of the 102 *S.aureus* isolates sub cultured from the stored frozen state on to Mueller Hinton agar plates, all were found to be coccoid, exhibiting grape like clusters in morphology under microscope. All of them were positive for catalase test too. All *S.aureus* were positive for slide coagulase test. When the tube coagulase test was performed, all of them possessed the coagulase enzyme. All isolates were positive for DNase enzyme. Furthermore, when isolates identified as *S.aureus* conventionally on a phenotypic test, were checked for the presence of nuclease test being, all of them produced a clear PCR product of the expected size (279 bp), confirming the speciation of isolates. Out of 102 *S. aureus* isolates, 99 isolates (97%) carried *nuc*, while 3 (3%) were negative. In the period of the study, all *S. aureus* isolates which tested were positive for the Sa442 fragment, resulting in a sensitivity of 100%.

Discussion

The emergence of methicillin resistance in *S. aureus* is of great concern because MRSA are often multidrug resistant (Chambers, 2001) Infections with MRSA are known to be associated with considerable morbidity and mortality (Cosgrove et al., 2003) Although *S. aureus* is not difficult to grow and is easy to identify, there is a need for the development of rapid and sensitive DNA-based assays which are suitable for the direct detection of *S. aureus* from clinical specimens to improve the rapidity and the accuracy of the diagnosis of *S. aureus* infections. Nucleic acid amplification by PCR has several applications in the detection of bacteria and other infectious agents in the clinical microbiology laboratory .Coagulase is produced by all strains of *S. aureus*. Its production is the principle criterion used in clinical microbiology laboratory for identification of *S. aureus* in human infections, and it is an important virulence factor. In routine diagnostic laboratory, a Gram stain is performed followed by the tube coagulase test. The tube coagulase test is checked after 4 h of incubation, and if the test is negative, a further 20 h of incubation is required; hence, the result of the test is only available after 24 h, thus, delaying definitive identification of S. aureus. Hence, in routine diagnostic laboratories, species identification is primarily based on biochemical characteristics of cultured organisms, which are time-consuming procedures. Molecular-based methods are becoming increasingly useful in clinical microbiology laboratories to increase accuracy of identification and to obtain reliable results more rapidly (Kearns et al., 1999). Species-specific 442-bp chromosomal fragment has been recently the specific choice for identification of *S. aureus* in multiplex PCR .The *Sa442* DNA fragment (an undefined 442-bp sequence), which is a popular DNA target for identification of *S. aureus* by polymerase chain reaction (PCR) methods, originally was described by (Martineau et al., 1998). There are few cases that state the absence of Sa442 fragment from *S. aureus* isolates in PCR assays. These isolates were coagulase positive and presented a pattern of biochemical reactions typical for *S. aureus* (Sütterlin et al., 2003). However, recently, there are few reports on *S. aureus* isolates that are negative for the Sa442 fragment (Klaassen et al., 2003; Sütterlin et al., 2003). These results proved that the use of single gene in the PCR assay for species identification may result in misidentification (Klaassen et al., 2003). Because *S. aureus* resembles CoNS on visual examination of agar plates and the coagulase status of an isolate is not always easily established in a timely fashion, the inclusion of Coa gene that can be used as a marker for identification of *S. aureus* along with Sa442 fragment is favorable. This has the advantage of improving specificity of the assay by checking for 2 determinants simultaneously for the identification of *S. aureus* and preventing misidentification of isolates lacking Sa442 fragment.

Hence, PCR assay for identification of *S. aureus* may result in misidentification when it is solely based on the Sa442 fragment. The primary mechanism of resistance to methicillin in *S. aureus* is the production of novel penicillin binding protein termed as PBP2a or PBP2V that has low affinity for beta-lactam antibiotics (Hiramatsu et al., 2001). Expression of methicillin resistance in clinical microbiology laboratory setting is subjected to environmental conditions (i.e., temperature, pH, salt concentration) (Geha et al., 1994). Conditional expression of PBP2a may cause ambiguities in susceptibility tests, thus, emphasizing the need to develop a rapid and sensitive method for the detection of methicillin resistance in staphylococci, which is not dependent on growth conditions. The results of the present study showed that 3 *S. aureus* isolates would not have been identified as such, if tested by single-gene PCR for *nuc*. Interestingly, one of these atypical strains had been isolated in a herd during a control programme for contagious microorganisms. In this herd, the strain detected at the beginning of the programme was a typical one, carrying both *nuc* and *Sa442*; thus, it was hypothesized that in the course of the programme a deletion of *nuc* gene occurred. There is a research which conducted the bacteriological misidentification of 4 *S.pseudintermedius* strains, which were morphologically and biochemically consistent with the species aureus, but tested negative for both *nuc* and *Sa442* (Pilla et al., 2013).

References

Barber M. Methicillin-resistant staphylococci. Journal of clinical pathology. 1961;14(4):385.

Brakstad OG, Aasbakk K, Maeland JA. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. Journal of clinical microbiology. 1992;30(7):1654-60.

Chambers HF. The changing epidemiology of *Staphylococcus aureus*? Emerging infectious diseases. 2001;7(2):178.

Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW, Carmeli Y. Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis. Clinical infectious diseases. 2003;36(1):53-9.

Costa A-M, Kay I, Palladino S. Rapid detection of mecA and nuc genes in staphylococci by real-time multiplex polymerase chain reaction. Diagnostic microbiology and infectious disease. 2005;51(1):13-7.

Diekema D, Pfaller M, Schmitz F, Smayevsky J, Bell J, Jones R, et al. Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. Clinical Infectious Diseases. 2001;32(Supplement_2):S114-S32.

Fang H, Hedin G. Rapid screening and identification of methicillin-resistant *Staphylococcus aureus* from clinical samples by selective-broth and real-time PCR assay. Journal of clinical microbiology. 2003;41(7):2894-9.

Gao J, Ferreri M, Liu XQ, Chen LB, Su JL, Han B. Development of multiplex polymerase chain reaction assay for rapid detection of *Staphylococcus aureus* and selected antibiotic resistance genes in bovine mastitic milk samples. Journal of veterinary diagnostic investigation. 2011;23(5):894-901.

Geha DJ, Uhl JR, Gustaferro CA, Persing DH. Multiplex PCR for identification of methicillin-resistant staphylococci in the clinical laboratory. Journal of Clinical Microbiology. 1994;32(7):1768-72.

Heilmann F, van der Zanden A, Reubsaet F, Wannet W. Identification of 2,600 clinical methicillin-resistant *Staphylococcus aureus* strains in The Netherlands yielded sporadic cases of strains negative for the species-specific Sa442 gene fragment. Journal of clinical microbiology. 2004;42(5):2350-.

Hiramatsu K, Cui L, Kuroda M, Ito T. The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. Trends in microbiology. 2001;9(10):486-93.

Jayaratne P, Rutherford C, editors. Association of femA gene heterogeneity with unusual phenotypic characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA), abstr. D-159. 97th General Meeting of the American Society for Microbiology; 1997.

Kearns A, Seiders P, Wheeler J, Freeman R, Steward M. Rapid detection of methicillin-resistant staphylococci by multiplex PCR. Journal of Hospital Infection. 1999;43(1):33-7.

Klaassen CH, De Valk HA, Horrevorts AM. Clinical *Staphylococcus aureus* isolate negative for the Sa442 fragment. Journal of clinical microbiology. 2003;41(9):4493-.

Kohner P, Uhl J, Kolbert C, Persing D, Cockerill Fr. Comparison of Susceptibility Testing Methods withmecA Gene Analysis for Determining Oxacillin (Methicillin) Resistance in Clinical Isolates of *Staphylococcus aureus* and Coagulase-Negative Staphylococcus spp. Journal of clinical microbiology. 1999;37(9):2952-61.

Lehmann PF. PR Murray, EJ Baron, MA Pfaller, FC Tenover and RH Yolken, eds. Manual of Clinical Microbiology. Mycopathologia. 1999;146(2):107-8.

Lowy FD. *Staphylococcus aureus* infections. New England journal of medicine. 1998;339(8):520-32.

Mac Faddin JF. Biochemical tests for identification of medical bacteria: Williams & Wilkins Co.; 1976.

Martineau F, Picard FJ, Roy PH, Ouellette M, Bergeron MG. Species-specific and ubiquitous-DNA-based assays for rapid identification of *Staphylococcus aureus*. Journal of clinical microbiology. 1998;36(3):618-23.

O'Connor L. Diagnostic bacteriology protocols: Springer Science & Business Media; 2006.

Panlilio AL, Culver DH, Gaynes RP, Banerjee S, Henderson TS, Tolson JS, et al. Methicillin-resistant *Staphylococcus aureus* in US hospitals, 1975–1991. Infection Control & Hospital Epidemiology. 1992;13(10):582-6.

Pilla R, Snel GG, Malvisi M, Piccinini R. Duplex real-time PCR assay for rapid identification of *Staphylococcus aureus* isolates from dairy cow milk. Journal of dairy research. 2013;80(2):223-6

Rushdy AA, Salama MS, Othman AS. Detection of methicillin/oxacillin resistant *Staphylococcus aureus* isolated from some clinical hospitals in Cairo using Meca/Nuc genes and antibiotic susceptibility profile. Int J Agric Biol. 2007;9(6):800.

Sadeghi J, Mansouri S. Molecular characterization and antibiotic resistance of clinical isolates of methicillin-resistant *Staphylococcus aureus* obtained from Southeast of Iran (Kerman). Apmis. 2014;122(5):405-11.

Schwarzkopf A, Karch H. Genetic variation in *Staphylococcus aureus* coagulase genes: potential and limits for use as epidemiological marker. Journal of Clinical Microbiology. 1994;32(10):2407-12.

Sütterlin K, Englert R, Schmidt-Wieland T, Schmitt J, Reischl U, Lehn N. Sporadic cases of *Staphylococcus aureus* organisms negative for a species-specific 442-base pair chromosomal fragment. Journal of clinical microbiology. 2003;41(7):3449-.

Tille P. Bailey & Scott's Diagnostic Microbiology-E-Book: Elsevier Health Sciences; 2013.

Towner K, Talbot D, Curran R, Webster C, Humphreys H. Development and evaluation of a PCR-based immunoassay for the rapid detection of methicillin-resistant *Staphylococcus aureus*. Journal of medical microbiology. 1998;47(7):607-13.

Van Leeuwen W, Roorda L, Hendriks W, Francois P, Schrenzel J. A nuc-deficient meticillin-resistant *Staphylococcus aureus* strain. Blackwell Publishing Ltd Oxford, UK; 2008.

Voss A, Milatovic D, Wallrauch-Schwarz C, Rosdahl V, Braveny I. Methicillin-resistant*Staphylococcus aureus* in Europe. European Journal of Clinical Microbiology and Infectious Diseases. 1994;13(1):50-5.