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Validation of a Multiplex PCR for the Identification of Methicillin Resistant *S.aureus* from Uncultured Clinical Specimens

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Abstract: ***Methicillin Resistant Staphylococcus aureus (MRSA) has become endemic in many countries including India. It is a dangerous pathogen and often challenges the management of hospital acquired infections. We demonstrate that a multiplex PCR targeting nuc, mecA and 16s rRNA is accurate and reliable for the identification of MRSA, with a simple sample processing protocol for PCR based diagnosis of MRSA directly in a variety of uncultured clinical samples. The PCR results were quite comparable to the clinical microbiology results. This study demonstrates the usefulness of a simple sample processing protocol and a multiplex PCR method for rapid and reliable detection of MRSA from uncultured clinical specimen. Though several commercial kits are available for the direct detection of MRSA from swabs, it is probably the first time to the best of our knowledge that a multiplex PCR has been designed and demonstrated to identify MRSA from varied uncultured clinical samples including pus, blood, urine, respiratory samples, drain fluids etc.***

Keywords: MRSA, multiplex PCR, *16s rRNA, nuc, mecA, PCR*

1. Introduction

S.aureus is a gram positive cocci(1) and is one of the most common causes of nosocomial infections including post-surgical wound infections. It is estimated that 30% of the human population are long term carriers of *S.aureus.* It can cause a number of infections, from superficial or deep skin and soft skin infections to life-threatening conditions like endocarditis, bacteraemia, sepsis and device related infections (2, 3). There are 32 species and 8 sub-species in the genus *Staphylococcus*, most of which preferentially cause infections in humans(1). MRSA infections may no longer be regarded as just a healthcare associated problem but are also community acquired. Therefore, global measures need to be taken in order to reduce the mortality, morbidity due to MRSA and also its spread (4).

S.aureus is a remarkable pathogen. Worldwide, the prevalence of MRSA infection varies from 2.3 to 69.1% (5, 6). In a study conducted in 15 Indian tertiary care centres, the MRSA prevalence was recorded as 41%. According to the study, the prevalence of MRSA varied from 25% in Western India to 50% in the Southern parts of India(7). Based on data from Canada, Australia and Scandinavia, prevalence of MRSA infections showed an increase during the years 2000-2008. Another study conducted in India involving 17 tertiary care Hospitals reported a prevalence rate of MRSA as 41% (30-85%) (Clinical and Laboratory Standards Institute, 2012) and it seems to have become endemic in India (8-10). The increase was mainly attributed to CA-MRSA infections, indicating that community associated MRSA infections are emerging as a severe threat to the world (7). No wonder, MRSA has been labelled as a "superbug" What is more, the molecular epidemiology of MRSA is also continuously changing in different demographics (11-14).

In most of the developing countries, the diagnosis of *S.aureus* infections is based on phenotypic tests, among which, coagulase test is considered as confirmatory. However, there are problems associated with the coagulase test; some Coagulase Negative (CoNS) *Staphylococcus* species which are retrieved from human infections, produce clumping factor and may be misinterpreted as *S.aureus* (15).

The threat and difficulties posed by MDR-MRSA need to be addressed without delay especially for the critically ill patients. We consider that rapid identification of species and detection of *mec*A directly in specimens bynucleic acid based assays would enable the clinicians to make informed decisions quickly compared to culture based phenotyping.An ideal molecular test for MRSA therefore should not only help to differentiate *S.aureus* from the rest of *Staphylococcus*genus in addition to detecting *mec*A, the genetic marker for methicillin resistance.

The *nuc* gene encoding for thermonuclease has been reported to be very specific for *S.aureus*(2) This PCR assay, specific for *S.aureus*, targeting the *nuc* gene was originally reported by Brakstad et al (16). Other structural genes like the *fem* genes (A, B and X) have shownvariable sensitivities (2, 17-19).

The gene responsible for resistance to methicillin and different β-lactam antibiotics is *mecA* which encodes a modified penicillin-binding protein 2a (PBP2a). Louie et al reported the direct identification of MRSA from blood cultures bottles thus displaying 99.2% sensitivity and 100% specificity(20). Real time PCR assays have also been developed which identified MRSA from several different species of *Staphylococcus*(21) targeting different regions of SSC*mec* cassette in addition to *mec*A. Though there are a

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few commercial kits available (22-26), none of them have been validated on clinical samples other than swabs. We have tried to address this unmet need by validating a simple home brew protocol for sample processing and a multiplex PCR for reliable detection of MRSA in a variety of clinical specimen including swabs, urine, endotracheal secretions, tissue, sputum, ascitic fluid, pancreatic fluid, CAPD fluid, drain fluid and also blood cultures.

This study reports the results of validation of *16s rRNA, nuc and mec*A as PCR amplification targets from a variety of uncultured clinical samples using a simple sample processing protocol (27) against various conventional clinical microbiology methods.

2. Materials & Methods

Microbiological and Phenotypic methods:

A total of 116 clinical samples comprising of swabs (n=96), urine (n=11), Ascitic fluid (n=02), Pigtail fluid (n=01), Blood culture (n=01), Sputum (n= 03), CAPD fluid (n=01) and Drain fluid (n=01) were collected from the Clinical Microbiology Laboratory of Global hospitals. Standard microbiological tests, which comprised of isolation on Mannitol Salt Agar (MSA) and slide coagulase test, were used to reconfirm the identity of *S.aureus* isolates. To obtain single colonies, the *S.aureus* isolates from MSA were sub-cultured onto Mueller Hinton Agar (MHA) and screened with Cefoxitin (30µg, Hi-media) and Oxacillin (1µg, Hi-media) discs.

DNA isolation and PCR amplification:

Cell free DNA lysates were prepared from all 116 samples by TEX (100mM Tris buffer pH 8.0, 50 mM EDTA, 10% Triton X-100) method (20) and used for PCR amplification. The scheme for clinical sample processing for multiplex PCR is depicted in Fig.1:

10µL of cell free DNA lysate was used for the Multiplex PCR. The amplified products were screened by agarose gel (2%w/v) electrophoresis. ATCC 6538P-MRSA was used as positive control (PC). Other gram positive and gram negative ATCC cultures were used as negative controls (NC). Gram positive ATCC cultures included-ATCC 27626 *Staphylococcus epidermidis* (*mec*A positive), ATCC 6305 *Streptococcus pnuemoniae*, ATCC 29212 *Enterococcus faecalis*, and ATCC 700221 *Enterococcus faecium*.Gram negative ATCC cultures comprised of- ATCC 19606 *Acinetobacter baumanii*, ATCC 10418 *E.coli*, ATCC 70060 *Klebsiella pnuemoniae*, ATCC 13048 *Enterobacter aerogenes*. The PCR reaction mixture included the following: 1.5 mM $MgCl_2$, 20pmol of each primer set of *mec*A, *nuc* and *16s rRNA*, 2mM of dNTP (Fermentas) along with 1 U of Taq (New England Biolabs) and 10 µL of cell free lysate as DNA template in 30 µL final volume. Primers used in the study and the thermal cycling conditions are listed in Table 1.

Table 1:

Gene	Sequence 5'-3'	PCR conditions	Product size	References
16s rRNA F	GTGCCAGCAGCCGCGGTAA	94°C X 5mins 94°C X 30s 55°C X 30s 72°C X 50secs 72°C X10mins (35 cycles)	886bp	(28)
16s rRNA R	AGACCCGGGAACGTATTCAC			
nuc F	GCGATTGATGGTGATACGGT	94°C X 5mins 94°C X 30s 55°C X 30s 72°C X 50secs 72°C X10mins (35 cycles)	270bp	(16)
nuc R	AGCCAAGCCTTGACGAACTAAAGC			
mecA F	TCCAGATTACAACTTCACCAGG	94°C X 5min 94°C X 30s 55°C X 30s 72°C X 50s 72°C X10 min (35 cycles)	162bp	(29)
mecA R	CCACTTCATATCTTGTAACG			

3. Results

Out of 116 clinical samples, 63 were MRSA and 25 were identified as MSSA. In genotyping also, when the amplified PCR products were analysed on agarose gel, 63 isolates were MRSA (*mecA* positive), 25 were MSSA (*mecA* negative). The remaining 28 isolates reported as non-*S.aureus* by both microbiology and genotyping (16s rRNA negative) (*E.coli, Streptococcus spp., K. pneumoniae, Candida spp., Enterobacter spp., and Pseudomonas spp*. MedCalc software (MedCalc Statistical Software version 15.6.1, MedCalc Software, Ostend, Belgium; https://www.medcalc.org; 2015) was used for statistical analysis of data; Sensitivity 100%; Specificity, 97.5%; Positive predictive value (PPV), 98.86%; Negative predictive value, 100% (table 2). Our protocol was able to correctly identify coagulase positive and *mec*A negative MSSA in addition to coagulase negative mecA positive *S.epidermidis*. The Multiplex PCR result for a few selected isolates and clinical specimen is shown in Fig 2 and 3.

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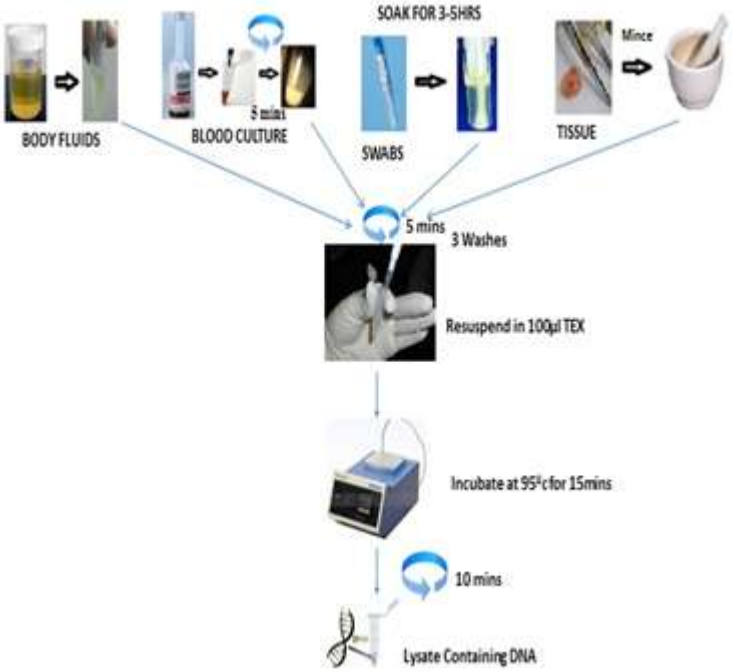

Figure 1: Flow chart for processing of Clinical samples

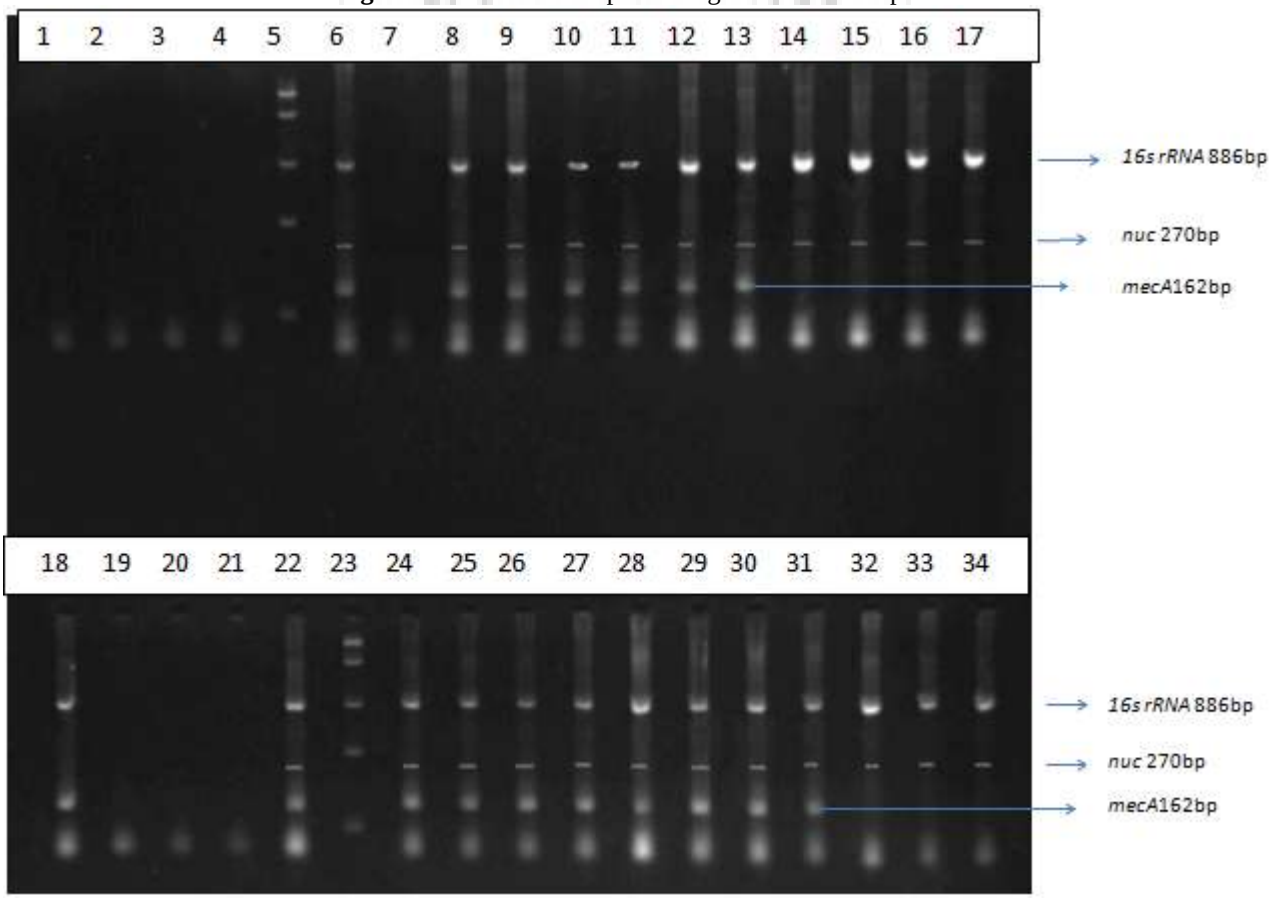

Figure 2: Multiplex PCR Results of clinical samples. Agarose gel analysis of PCR products from uncultured clinical samples. (1)ATCC 19606-*Acinetobacter baumanii*, (2)10418-*E.coli*, (3)70060-*K.penumoniae*, (4)*Enterobacter aerogenes*-ATCC 13048, (5)Molecular marker (100bp), (6)Positive Control, (7)Negative Control, (8)& (9)Nasal swab, (10&11)Groin swab, (12&13)Axilla swab, (14)Throat swab,(MSSA) (15)Wound swab,(MSSA) (16 & 17)Pus swab,(MSSA) (18) ATCC 27626 *Staphylococcus epidermidis mec*A positive, (19)*Streptococcus pnuemoniae*-ATCC 6305, (20)*Enterococcus faecalis*-ATCC 29212, (21)*Enterococcus faecium*-ATCC 700221, (22)ATCC 6538 P-MRSA, (23)Molecular marker (100bp), (24 &25)Dorsum of hand swab, (26)Wound swab, (27)Pus swab, (28)CAPD fluid, (29)Throat swab, (30)Pus swab, (31)Groin swab, (32)Rectal Swab, (MSSA)(33)Nasal swab,(MSSA) (34)Axilla swab, (MSSA)

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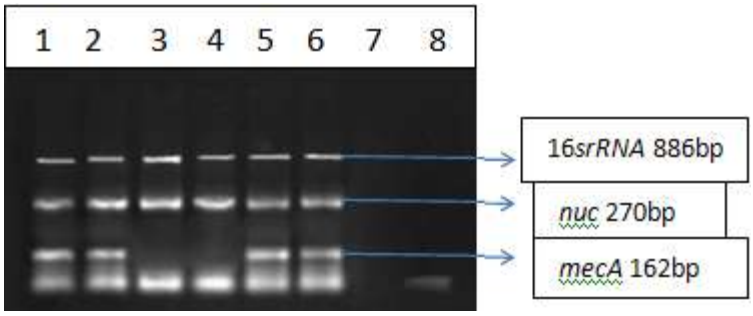

Figure 3: Multiplex PCR Results of body fluids. Agarose gel analysis of PCR products from uncultured clinical samples. (1)Urine, (2)Blood Culture, (3)Drain Fluid, (MSSA) (4)Pig tail Fluid, (MSSA) (5)CAPD Fluid, (6)Positive Control, (7) Blank, (8)Negative Control.

Table 2: Sensitivity and Specificity of the PCR assay compared to culture methods for detection of *Staphylococcus aureus*from Uncultured Clinical Samples

Molecular	Microbiology		Sensitivity	Specificity	PPV	NPV
	Positive	Negative				
Positive	87	0	100%	97.5%	98.86%	100%
Negative	1	28				

4. Discussion

Culture isolation and phenotyping are the main stay in the identification of MRSA and the reporting time depends on automation employed in the laboratory. The rich experience with various phenotyping methods has shown their limitations (22, 23) some of which are: protracted turn-around time, quality and performance of plasma used for the coagulase test (30, 31) particularly when the sample contains *S.intermedius, S.hyicus, S.delphini and S. schleiferi*subsp. *coagulans* which may be misdiagnosed as *S.aureus* and also falselyidentified on MSA plates (15) as mannitol positive, CoNS (*Staphylococcus caprae*, *S.hemolyticus* and *S.saprophyticus*) have been reported in Nigeria and Japan (32, 33). Therefore no single phenotypic test can absolutely guarantee identification of *S.aureus*. Simultaneous isolation on MSA and testing for coagulase activity are thus recommended to accurately identify *S.aureus*. However, there are situations when identification of MRSA is urgently required for critical care patients and a molecular method is required which can directly detect MRSA or non-*S.aureus* from clinical samples the same day and help the clinician make important therapeutic decisions. Any delay in diagnosis of MRSA and initiating appropriate antibiotic therapy could severely affect the outcome in the management of patients with sepsis and bacteraemia (34, 35). Worse outcomes have been demonstrated using a case study, which reported a 2.35 fold increase in mortality in MRSA sepsis patients when treatment was delayed by more than two days (36, 37). Rapid diagnosis and interventions would prevent an adverse outcome when the focal point of infection is apparent and suggestive of staphylococcal origin. Considering the fact that commercial molecular tests capable of identifying MRSA from varied clinical specimens are not available, our study which shows that a simple, economical method to process varied uncultured clinical specimen can be adopted for rapid identification of MRSA and reported the same day. We believe this is important value addition to the management of infectious diseases and it is possible that same sample processing protocol could be adopted for detection of any bacterial pathogen irrespective of the nature and quality of the specimen. Existing commercial kits (22-26)are not only expensive (22, 26) but also are closed systems using their own reagents, cartridges and software; further they are applicable only to nasal swabs, swabs of skin and tissue infections and blood cultures (38, 39).

There is scope for improvement of our sample processing protocol to completely eliminate any inhibition of PCR reaction. Our study included a number of varied uncultured clinical samples (urine, swabs, drain fluids, CAPD fluid, etc.) and revealed absolute sensitivity and exceptional correlation when compared to standard phenotyping results for direct detection of MRSA. Together, *16s rRNA* (genus specific), *nuc* (species specific), and *mecA* (methicillin resistance gene) offer dependable genomic targets for simultaneous detection and species affirmation of MRSA. In a laboratory where several Nucleic acid Amplification tests are being performed for genotyping of pathogens, our sample processing protocol may be easy to adopt. The approach presented here takes approximately 6h for reporting after receiving the specimen. Implementing this protocol would help the clinician to make evidence based therapeutic decision the same day.

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References

[1] Harris LG, Foster SJ, Richards RG. An introduction to staphylococcus aureus, and techniques for identifying and quantifying s. aureus adhesins in relation to adhesion to biomaterials: review. European Cells and Materials. 2002;4:39-60.
[2] Martineau F, Picard FJ, Roy PH, Ouellette M, Bergeron MG. Species-specific and ubiquitous-DNA-based assays for rapid identification of Staphylococcus aureus. J Clin Microbiol. 1998;36(3):618-23.

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[3] Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin Microbiol Rev. 2015;28(3):603-61.
[4] Stefani S, Chung DR, Lindsay JA, Friedrich AW, Kearns AM, Westh H, et al. Meticillin-resistant Staphylococcus aureus (MRSA): global epidemiology and harmonisation of typing methods. Int J Antimicrob Agents. 2012;39(4):273-82.
[5] Mendes RE, Hogan PA, Jones RN, Sader HS, Flamm RK. Surveillance for linezolid resistance via the Zyvox(R) Annual Appraisal of Potency and Spectrum (ZAAPS) programme (2014): evolving resistance mechanisms with stable susceptibility rates. J Antimicrob Chemother. 2016;71(7):1860-5.
[6] Nickerson EK, West TE, Day NP, Peacock SJ. Staphylococcus aureus disease and drug resistance in resource-limited countries in south and east Asia. Lancet Infect Dis. 2009;9(2):130-5.
[7] Mediavilla JR, Chen L, Mathema B, Kreiswirth BN. Global epidemiology of community-associated methicillin resistant Staphylococcus aureus (CA-MRSA). Curr Opin Microbiol. 2012;15(5):588-95.
[8] Anjali Kulshrestha VA, K. Mrithunjay,V. Himanshu, K. Manish and A.S Dalal. A Prospective Study on the Prevalence and Antibiotic Sensitivity Pattern of Methicillin Resistant Staphylococcus aureus isolated from Various Clinical Specimen at a Tertiary Care Post Graduate Teaching Institute. IntJCurrMicrobiolAppSci. 2017;6(3):1859-69.
[9] Indian Network for Surveillance of Antimicrobial Resistance group I, Joshi S, Ray P, Manchanda V, Bajaj J, Chitnis DS, et al. Methicillin resistant Staphylococcus aureus (MRSA) in India: Prevalence & susceptibility pattern. The Indian Journal of Medical Research. 2013;137(2):363-9.
[10] Y.C.Huang CJCa. New epidemiology of Staphylococcus aureus infection in Asia. Clin Microbiol Infect. 2014;20(7):605-23.
[11] Ansari S, Nepal HP, Gautam R, Rayamajhi N, Shrestha S, Upadhyay G, et al. Threat of drug resistant Staphylococcus aureus to health in Nepal. BMC Infect Dis. 2014;14:157.
[12] Kshetry AO, Pant ND, Bhandari R, Khatri S, Shrestha KL, Upadhaya SK, et al. Minimum inhibitory concentration of vancomycin to methicillin resistant Staphylococcus aureus isolated from different clinical samples at a tertiary care hospital in Nepal. Antimicrobial resistance and infection control. 2016;5:27.
[13] Kumar M. Multidrug-Resistant Staphylococcus aureus, India, 2013–2015. Emerg Infect Dis. 2016;22(9):1666-7.
[14] Laxminarayan R, Chaudhury RR. Antibiotic Resistance in India: Drivers and Opportunities for Action. PLoS Med. 2016;13(3):e1001974.
[15] Kateete DP, Kimani CN, Katabazi FA, Okeng A, Okee MS, Nanteza A, et al. Identification of Staphylococcus aureus: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. Ann Clin Microbiol Antimicrob. 2010;9:23.
[16] Brakstad OG, Aasbakk K, Maeland JA. Detection of Staphylococcus aureus by polymerase chain reaction amplification of the nuc gene. J Clin Microbiol. 1992;30(7):1654-60.
[17] Chikkala R, Oommen George N, S. Ratnakar K, Natarajan Iyer R, Sritharan V. Heterogeneity in <i>femA</i> in the Indian Isolates of <i>Staphylococcus aureus</i> Limits Its Usefulness as a Species Specific Marker. Advances in Infectious Diseases. 2012;02(03):82-8.
[18] Kobayashi N, Wu H, Kojima K, Taniguchi K, Urasawa S, Uehara N, et al. Detection of mecA, femA, and femB genes in clinical strains of staphylococci using polymerase chain reaction. Epidemiol Infect. 1994;113(2):259-66.
[19] Mohanasoundaram KM, Lalitha MK. Comparison of phenotypic versus genotypic methods in the detection of methicillin resistance in Staphylococcus aureus. Indian J Med Res. 2008;127(1):78-84.
[20] Louie L, Goodfellow J, Mathieu P, Glatt A, Louie M, Simor AE. Rapid detection of methicillin-resistant staphylococci from blood culture bottles by using a multiplex PCR assay. J Clin Microbiol. 2002;40(8):2786-90.
[21] Huletsky A, Giroux R, Rossbach V, Gagnon M, Vaillancourt M, Bernier M, et al. New Real-Time PCR Assay for Rapid Detection of Methicillin- Resistant Staphylococcus aureus Directly from Specimens Containing a Mixture of Staphylococci. Journal of Clinical Microbiology. 2004;42(5):1875-84.
[22] Ellem JA, Olma T, O'Sullivan MV. Rapid Detection of Methicillin-Resistant Staphylococcus aureus and Methicillin-Susceptible S. aureus Directly from Positive Blood Cultures by Use of the BD Max StaphSR Assay. J Clin Microbiol. 2015;53(12):3900-4.
[23] Kelley K, Cosman A, Belgrader P, Chapman B, Sullivan DC. Detection of methicillin-resistant Staphylococcus aureus by a duplex droplet digital PCR assay. J Clin Microbiol. 2013;51(7):2033-9.
[24] Peterson LR, Liesenfeld O, Woods CW, Allen SD, Pombo D, Patel PA, et al. Multicenter evaluation of the LightCycler methicillin-resistant Staphylococcus aureus (MRSA) advanced test as a rapid method for detection of MRSA in nasal surveillance swabs. J Clin Microbiol. 2010;48(5):1661-6.
[25] Reischl U, Linde HJ, Metz M, Leppmeier B, Lehn N. Rapid identification of methicillin-resistant Staphylococcus aureus and simultaneous species confirmation using real-time fluorescence PCR. J Clin Microbiol. 2000;38(6):2429-33.
[26] Warren DK, Liao RS, Merz LR, Eveland M, Dunne WM, Jr. Detection of methicillin-resistant Staphylococcus aureus directly from nasal swab specimens by a real-time PCR assay. J Clin Microbiol. 2004;42(12):5578-81.
[27] Sritharan V, Barker RH, Jr. A simple method for diagnosing M. tuberculosis infection in clinical samples using PCR. Mol Cell Probes. 1991;5(5):385-95.
[28] Poulsen AB. Detection of methicillin resistance in coagulase-negative staphylococci and in staphylococci directly from simulated blood cultures using the EVIGENE MRSA Detection Kit. Journal of Antimicrobial Chemotherapy. 2003;51(2):419-21.

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[29] Oliveira DC, Lencastre Hd. Multiplex PCR Strategy for Rapid Identification of Structural Types and Variants of the mec Element in Methicillin-Resistant Staphylococcus aureus. Antimicrobial Agents and Chemotherapy. 2002;46(7):2155-61.
[30] Bello CSS, Qahtani A. Pitfalls in the routine diagnosis of Staphylococcus aureus. African Journal of Biotechnology. 2005;. 4 (1):83-6.
[31] McDonald CL, Chapin K. Rapid identification of Staphylococcus aureus from blood culture bottles by a classic 2-hour tube coagulase test. J Clin Microbiol. 1995;33(1):50-2.
[32] Kawamura Y, Hou XG, Sultana F, Hirose K, Miyake M, Shu SE, et al. Distribution of Staphylococcus species among human clinical specimens and emended description of Staphylococcus caprae. J Clin Microbiol. 1998;36(7):2038-42.
[33] Shittu A, Lin J, Morrison D, Kolawole D. Identification and molecular characterization of mannitol salt positive, coagulase-negative staphylococci from nasal samples of medical personnel and students. J Med Microbiol. 2006;55(Pt 3):317-24.
[34] Kumar A, Roberts D, Wood KE, Light B, Parrillo JE, Sharma S, et al. Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Crit Care Med. 2006;34(6):1589-96.
[35] Paul M, Shani V, Muchtar E, Kariv G, Robenshtok E, Leibovici L. Systematic review and meta-analysis of the efficacy of appropriate empiric antibiotic therapy for sepsis. Antimicrob Agents Chemother. 2010;54(11):4851-63.
[36] Marchaim D, Kaye KS, Fowler VG, Anderson DJ, Chawla V, Golan Y, et al. Case-control study to identify factors associated with mortality among patients with methicillin-resistant Staphylococcus aureus bacteraemia. Clin Microbiol Infect. 2010;16(6):747-52.
[37] Shime N, Kosaka T, Fujita N. The importance of a judicious and early empiric choice of antimicrobial for methicillin-resistant Staphylococcus aureus bacteraemia. Eur J Clin Microbiol Infect Dis. 2010;29(12):1475-9.
[38] Kilic A, Muldrew KL, Tang YW, Basustaoglu AC. Triplex real-time polymerase chain reaction assay for simultaneous detection of Staphylococcus aureus and coagulase-negative staphylococci and determination of methicillin resistance directly from positive blood culture bottles. Diagn Microbiol Infect Dis. 2010;66(4):349-55.
[39] Louie L, Goodfellow J, Mathieu P, Glatt A, Louie M, Simor AE. Rapid Detection of Methicillin-Resistant Staphylococci from Blood Culture Bottles by Using a Multiplex PCR Assay. Journal of Clinical Microbiology. 2002;40(8):2786-90

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