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Detection of mecA Gene and Phage Typing in Nosocomial Outbreaks of Methicillin Resistant *Staphylococcus aureus* in Iran.

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ABSTRACT

Staphylococcus aureus is one of the major causes of community and hospital-acquired infections. Bacteriophage considered as a major risk factor acquires S. aureus new virulence genetic elements. A total number of 120 S. aureus isolated from different specimens obtained from Hospitals were distinguished by susceptibility to 19 antimicrobial agents, phage typing, and PCR amplification for mecA gene. All of MRSA isolates harbored mecA gene, except two unique isolates. The predominant phage group is belonging to the (mixed group). Phage group (II) considered as an epidemiological marker correlated to b-lactamase hyper producer isolates. Three outpatients MRSA isolates had low multiresistance against Bacitracin (Ba) and Fusidic acid (FD), considered as CAMRSA isolates. It was detected that group I typed all FD-resistant MSSA isolates. Tetracycline (TE) resistant isolates typed by groups (II) and (III) in MSSA. It could be concluded that (PERSA) S. aureus isolates from the wound that originated and colonized, and started to build up multi-resistance against the topical treatment. The changes in phage typing pattern showed that the increase was caused by the introduction and spread of many resistant strains. MRSA isolates reflected somehow of homogeneity concerning phage typing rather than MSSA.

Keywords: Staphylococcus aureus, Bacteriophage, mecA

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INTRODUCTION

Methicillin-Resistant Staphylococcus aureus (MRSA) is an important bacterial pathogen causing nosocomial and community onset infections[1-2].The prevalence of MSRA has increased in many parts of the world causing serious infections in hospitals that pose a serious burden in terms of medical and socio-economic costs and cause significant morbidity and mortality[3]. In the other wise, the fact that 90% of hospital staff are carriers of S. aureus portends serious for the epidemiology and pathogenesis of Staphylococcal infections [4]. The wide spread of antibiotic resistance among S. aureus strains is a major concern in the treatment of Staphylococcus infections. These strains often resistance to multiple antibiotics with attendant increased morbidity; the surveillance and control of these strains was highly desirable [5]. Molecular typing techniques have been used with increasing frequency in studies of the epidemiology MRSA and also for a better understanding of the evolutionary relationships among MRSA clones [6- 9]. One of the conclusions emerging from these studies was that a complete characterization of MRSA lineages requires not only identification of the genetic background of the bacteria but also identification mec element, which carries methicillin resistance determinant mecA [9-11].There is relatively little information on the diversity of strains causing infection. It is important to have knowledge of the most common strains associated with human infections and their sources in each episodes and environment in order to improveour understanding of the epidemiology of this pathogen and solve the problems. Although bacterial interaction is a well recognized phenomenon, there has been surprisingly little research with respect to MRSA and MSSA. The mechanisms responsible for this phenomenon is not readily apparent. Gopal Rao and Wong [12] concluded that there is a complex relationship between various strains of EMRSA and MSSA especially in the skin. This interaction may have an important bearing on colonization of patients with MRSA. It may explain some of the epidemiological and clinical observations as well as understanding the methods for the movement of resistant genes, like:Transduction (phages) – Plasmids – Integrons – Transposons.The resistance in MRSA is due to the expression of Penicillin binding protein (PBP2a) encoded by mecA genes [13, 14] which is located on the Staphylococcal cassette chromosome (SCC)[15], a large genetic mobile element which differs in size and genetic composition among different strains of MRSA [16]. Different types of SCC mec cassettes were extensively studied by PCR techniques [15].The resistance mechanism involves changes or defects brought about by mutation on mecA gene which results in the organism's resistance to antibiotics. In addition, other antibiotic resistance genes may also be present in the cassette rendering resistance to multiple antibiotics[15]. Detection of mecA gene by Polymerase chain reaction is considered as the gold standard [17, 18] for methicillin resistance as these genes are highly conserved among Staphylococcal species. Several investigations were done concerning the screening and emergence of MRSA in the Kingdom. Blouse et al identified that MRSA Saudi isolates all belonging to phage group III [19]. It was clear that consensus SmaI pattern observed for Saudi strains was different from other non-related isolates. Kishan et alreported for the most b-lactamase-producing isolates of S. aureus belonging to other phage groups [20]. Skov et al have reported that all phage group II isolates recovered prior to harbored blaz on the chromosome [21]. The final event in multiplication of phage was lysis of the host cell by murein hydrolyses of various substrate specificities [22]. The genetic studies that showed that drug-resistance genes were easily transduced among S. aureus cells by prophage [23]. This suggested a relationship between the intracellular state of the drug-resistance genes and temperate phages. Thus, they could show that some of the temperate phages transduced the drug-resistance genes. In the present study, the PCR method was used for the detection of mecA genes among the MRSA strains. The study aimed to set up a rapid and accurate detection procedure for methicillin resistance among Staphylococcal isolates through the amplification of specific gene determinants by PCR and examining phenotypic (resistance to antibiotics, phage typing) backgrounds mecA gene in order to treat clinical condition and in eradication of the pathogen. As well as defined the epidemic drift of phage-types within originated susceptible wild type S. aureus population as a microbial biomarkers for monitoring the usage of antibiotics. And analyze the contribution of the phage typing, as a phenotypic marker.

MATERIALS AND METHODS

Experimental

A total numbers of 120 isolates of S. aureus from different patients were collected over a period of one year from 2014 to 2015 from microbiology laboratory in Hajar and kashani Hospitals in Shahrekord ,Iran . The following reference strains of bacterial species were used as controls: Methicillin resistant S. aureus (MRSA) (NCTC 10442), methicillin sensitive S. aureus (MSSA) (ATCC 25923), Coagulase Negative Staphylococcus

epidermidis (CNS) (ATCC 12228). All isolates were streaked for purity growth on Blood Agar plates (Merck,Germany) for over night incubated at 37 °C and Gram stain smears from a Staph Culture showed gram positive cocci in clusters [24]. Catalase test detects the presence of cytochrome oxidase enzymes in Micrococcaceae to [25] Slide agglutination test (Staphurex) based on the detection of clumping factor and protein A. Coagulase test was performed [26]. Tube coagulase test measures the production of free coagulase (Staphylocoagulase) [27]. Then all of isolates were streaked onto Manitol Salt Agar (MSA) (Merck,Germany). If the manitol was fermented to produce acid, the phenol red in then medium changes color from red to yellow. If this color change exists, it can be presumed that the isolate is a strain of S. aureus. Deoxyribonuclease (DNase) test was performed by heavily spot-inoculating several colonies on nutrient agar media (Merck,Germany) containing DNA. Colonies of S. aureus that produced DNase were surrounding by a clear zone where the DNA had been depolymerized and hydrolyzed [28]. Detection of methicillin resistance in S. aureus was performed by Kirby–Bauer technique of disk diffusion method was used to detect the methicillin resistance. The isolates were tested by employing Mueller Hinton Agar. The 1 µg oxacillin (OX) and cefoxitin (FOX) 30 µg disk diffusion were used with Mueller Hinton Agar media supplemented with 4% NaCl was used to detect MRSA according to the NCCLS [29]. Disk diffusion testing by FOX disk was performed to simultaneously evaluate its value in predicting OX resistance. Commercial antimicrobial agents containing disks were placed on the plates by multidispenser. Triplex Polymerase Chain Reaction (PCR) method to amplify three different genes was used [30]. 16SrRNA: a specific gene for Staphylococci species. nuc: for detection of S. aureus. mecA: to detect methicillin resistance strains. For DNA templet extraction three to five colonies subculture of S. aureus strains were taken and resuspended into 200 ml of PCR water in an eppendorf tube, vortex and then centrifuged at 13,000 rpm for one min just to form a pellet then the DNA can be obtained directly from the supernatant. Oligonucleutide primers were obtained from MWG-Biotech AG (Berlin, Germany). For performation PCR total volume 25 µl of reaction mix was prepared by adding 1.5 µl of primer mix, the primer mix was done by adding 1 µl from each mecA primers, 0.25 µl from each nuc primers and from each 16srRNA primers. 4 mM MgCl2 , 12.5 µl Ready Mix^{TM}, {when Ready Mix^{TM} used as 1X it will contain a concentration of 1.25U Thermo prime plus DNA polymerase, 75 mM tris HCl [ph:8.8], 20 mM (NH4)2SO4, 2 mM MgCl2, 0.01% (v/v) Tween 20, 0.3 mM dNTPs, precipitant and red dye for electrophoresis} 3 µl PCR water and 4 µl Template DNA. Each run contained the MRSA, MSSA and CNS standard strains with and two negative controls (blank reagent and PCR water). The reaction was carried by using the Thermal Cycler (TC) from Gene Amp PCR 9700 . The cycling protocol consisted of one cycle for 5 min at 94 °C, followed by 35 cycles consisting of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and elongation for 2 min at 72 °C then one cycle of final elongation for 5 min at 72 °C. 10 µl of the product was loaded on to 2% Pulsed Field Certified Agarose and electrophoresis was done at 120 V for 2 h (GT UVTP Gel tray 15 · 25 cm. GT-Gel casting system. Viewed under UV transillumination (Bio-Rad) and photograph using gel documentation system, print image (Bio-Rad P68V paper. 110 mm · 21 mm high density type). The analysis of the DNA PCR products was evaluated according to the following controls: MRSA control NCTC 10442 (mecA, 16Sr RNA and nuc positive). MSSA control ATCC 25923 (mecA negative, 16SrRNA and nuc positive). CNS control ATCC 12228 (mecA and nuc negative, 16Sr RNA Mueller Hinton plates (without salt) were inoculated in the same way which was used for OX and FOX, by Kirby–Bauer technique. The sensitivity was recorded according to the NCCLS guidelines. Reading the reaction of the test organism to each antibiotic as 'sensitive' or 'resistant' Sensitive (S): Zone radius wider than, equal to or not morenthan 3mm smaller than the control. Resistant (R): No zone of inhibition or zone radius measure 2 mm or less.

Phage typing

An International Human Staphylococcal Phage Typing Set (IPS), containing 23 phages was used. Suspend the dried phage in 1.0 ml of broth or we can use 1.0 ml of stock phages, and dilute in 10-fold steps to 1 in 1,000,000. Apply one 0.02 ml drop of each dilution to a dried lawn of propagating strain and incubate overnight at 30 °C, then we determine the titer and RTD. The RTD (routine test dilution) is that dilution of phage which just fails to give confluent lysis (i.e. gives semi-confluent lysis) on its propagating strain. After that either soft agar or broth propagation methods might be used depend on the phage, as recommended in the phage set but two methods were performed for all the phages as follows: Broth propagation or soft agar propagation. In this study 2 hour, lightly inoculated, nutrient broth culture grown with shaking at 37°C was used for typing. The grown culture is used to flood a plate containing appropriate agar, and the excess culture removed. The flooded plate is dried open and this can take several hours, a minimum of 2h is necessary. Phages, appropriate for the species, are applied at 100· RTD by using multi-head inoculators. Lids are replaced

as soon as the phages suspension has been applied. After drying, the plates are incubated overnight at 30 °C. Phage reaction were read, by eye, and recorded on the conventional phage scales.

RESULTS

S. aureus on B.A plates showed smooth, slightly domed colonies with 1–2 mm diameters. All isolates were positive to catalase, Staphurex, manitol fermentation, tube coagulase and DNase tests. All isolates were contain the 16sRNA and nuc genes which identified as S. aureus by genotypic method in Fig. 1. OX and FOX disks diffusion methods were applied for detection of MRSA according to NCCLS, that recommended the use of the cefoxitin disk test as a surrogate marker for the detection of oxacillin resistance in Staphylococcal isolates. Eighty isolates were identified as MSSA strains and 40 isolates were characterized as MRSA strains. The results were confirmed by PCR detection for mecA gene. thirty eight out of 40 MRSA isolates were positive for mecA gene. The 80 MSSA isolates were classified into 14 various antibiograms .42 MRSA isolates were classified into 19 antimicrobial agents . For b-lactam antibiotic+b-lactamase inhibitor (AMC), all MSSA isolates demonstrated susceptible to it. While 88% of MRSA isolates were resistant. For other antibiotics, the resistance rates were significantly increased in MRSA isolates compared with the MSSA. In contrast, the resistance to Ba antibiotic was higher than that was found in MRSA which was 32.4% in the former and 19% in the latter . It was observed a group of 30 isolates emerged out of 80 of MSSA had resistant for two or three of the antibiotics.

Distribution of phage types

The distribution of studied S. aureus isolates in the different phage groups was shown. 76.4% of the total isolates were typable by IPS (83.1% in MSSA and 64.2% in MRSA strains) and 30.7% (16.8%) in MSSA – 35.7% in MRSA) were non typable. There were 55 different phage typing patterns among 64 MSSA isolates, while 21 various patterns of 27 typable MRSA isolates. The predominant phage group in the study belonged to the Mixed group (64.8%). It was observed identical phage typing patterns between MSSA and MRSA isolates: (81. No. 97 in MSSA_No. 15 and 48 in MRSA) and (54/85. No. 78 in MSSA_No. 85 in MRSA). In MSSA isolates, it was observed the PG resistance isolates were distributed in all phage lytic groups, but group III was the most common (52.3%). All nontypable isolates exhibited resistant to PG. In MRSA isolates, b-lactams resistant isolates were distributed in all phage lytic groups, but the most common one was III (40.4%). Also for the AMC, SXT, TE, CIP and MUP resistance isolates, it was observed that the group III was the most common. In MSSA isolates, both GN resistance isolates exhibited typability by groups II and M. TE resistant isolate typed by II and III. One isolate which resistant to SXT was typed by group III/V alone. Both E and Ba resistance isolates distributed in all lytic groups, but group III was the most in E resistant isolates (57.1%) whereas groups I, II and III had the similar high percentage (60.8%) in Ba resistance isolates. FD resistance isolates typed by I, II, III and V. Group I typed all FD resistant isolates. Whereas the group I (34.4%) and III (37.9%) were the most in FD resistance MRSA isolates. AK resistance MRSA isolates were typed by group III (36%) and II (32%) as a common lytic group. Ba resistant isolates typed by all groups except V lytic group and C resistant isolates typed by II and M groups. All nontypable isolates distributed among various antibiotics.

DISCUSSION

Early and accurate diagnosis of MRSA is crucial in effective management and control of spread of MRSA infections. PCR-based assays are considered as the gold standard for the detection of MRSA, due to the heterogeneous resistance by various phenotypic detection methods displayed by many clinical isolates. Genotypic methods are more accurate in detecting methicillin resistant Staphylococci as compared to conventional susceptibility methods. S. aureus could be one of the most dexterous bacteria in exchanging useful genetic information with other bacterial species, also with phages that have genomic DNA materials. the GC content of Staphylococcal Chromosomal Cassette mec (SCC mec) (which have the mecA gene that responsible for resistance to methicillin) is non Staphylococcal origin suggested that it may have been acquired long ago in evolutionary terms. S. aureus could be infected by both polyvalent or/and host restricted phages so-called staphages belonging to families: Myoviridae and Siphoviridae [31]. Useful transducer genetic elements or jumping genes could be transferred horizontally in S. aureus population. The originated and colonized bacteria from Hospital reflected the epidemiological phenotypic and genotypic determinant markers for S. aureus group in Iran. It could help for setting up an evaluated antimicrobial usage strategy. Clinical microbiology laboratories testing for antimicrobial agents susceptibility among S. aureus is important to monitor for emerging resistance patterns [32]. The emergence of antibiotic resistance was one of the most

serious phenomena of the last 20 years and several strategies have been proposed to try to tackle it. The selective pressure resulting from the extensive use of antibiotics has lead to the acquisition and spread of antimicrobial resistance determinants [33]. All MRSA strains have SCC mec which act as harbor for the different Staphylococcal genes, especially resistant encoded gene and Most MRSA isolates were from inpatients which may expose to different antibiotics causing emerging the resistance or by gain the resistance from the hospital strains. In the present study, it was clinically crucial to determine rapidly, whether S. aureus methicillin resistant or not. Data revealed that mechanism expressing mecA gene as an important one against methicillin in S. aureus, differed among the studied 120 clinical isolates. A 80 of them were disseminated as MSSA lacking mecA and 40 were MRSA showed phenotypic resistance to methicillin. A 38 of them harbored mecA gene except three isolates. The structural gene for methicillin resistance, mecA, encodes a novel penicillin binding protein (PBP)-2', which has reduced affinity for b-lactam antibiotics [34]. SCC mec is found in another staphylococcus species from which is presumed to have been transferred; however the original donor of mecA to Staphylococci is unknown, as the element has not yet been identified outside this genus. *Staphylococcus sciuri* has an intrinsic PBP that shares 87.8% amino acid homology with PBP-2' [35]. It means that mecA gene mechanism is diverse, and the presented studied S. aureus group is heterogeneous and homogeneous population.Phage-typing data on MRSA strains indicated high special prevalence of phage type II. Phage type II on MSSA considered as an epidemiologic marker with frequent strong reaction typability (79.1%) individually or mixed with other phage groups compared to group III (67.2%) and phage group I (56%). Contrarily, Blouse et al found weak lytic reaction with group II phages. The increase of phage group II had been linked to a frequent useof b -lactams. Many MRSA strains showed heterogeneously resistance to b-lactams [36]. Study in (1961) showed successfully induced and isolated temperate phages from drug-resistance and proved the roll of the integrated prophage. Phage group III resembled the highest typability for S. aureus followed by group I that started to appear. Remarkably, phage group II did not exist mostly in that previous study. The investigators suggested that the conflict findings could be due to a variable and complex expression of resistance among the different MRSA sub-populations or strains [37]. Besides, some MRSA isolates may have incomplete regulator genes (mecI and/or mecR1) and/or high genomic diversity as was recently reported. Seems to have few isolates in the beginning within MSSA population exhibited horizontally distributed resistance only to one antibiotic and continued building up this resistance to be more than one vertically in MRSA population for the same of them. Another study in (2001) showed that two of the early MSSA strains exhibiting resistance only to PG, were genetically similar to the properties of two internationally spread MRSA. Also, showed resistance only to b-lactams and another contemporary multi resistant MRSA widely spread [38]. The pattern of multi drug resistance that characterizes the nosocomial strains may be result of the exposure to multiple antibiotics. Alternative drugs should be used when resistance is high [39]. In this study, the resistance rate for MSSA had increase to 9% for FD antibiotic comparing with the other studies [40]. Also in MRSA isolates, this rate was highly in present study 69%, while it was reported a decrease to 31% in another study. The reasons of this selection were due to the widely use FD in unreasonable quantities that led to the emergence of resistance rapidly. Manson and Howard (2004) found significant relationship between the use of topical FD and the isolation of MSSA resistant organism at the individual patient level and support the hypothesis that the observed increase in resistance is causally associated with the increased use of topical FD. The emergence and global spread of MRSA may be viewed as a process of accelerated evolution [41].Type 95 had emerged and another phage group II had noticed to be important in the present study. In addition, the importance of this type 95 was obviously noticed with a percentage of (18%) for MSSA had lowest existence comparing to the other phages. The change in phage type pattern showed that the increase was caused by the introduction and spread of many resistant strains. These could suggest the undergoing similar changes in the distribution of types, probably also influenced by selective pressures through the use of PG and other drugs [42]. The usage of TE for MSSA promoted the resistance in 2 strains (PG, TE and E) for antibiogram. As well as another isolate in antibiogram in 2 strains showed resistant for (PG, TE) and (PG, TE, B). These four isolates had different in phage types. Three of them showed that phage type (group I) was eradicated as a marker phage for frequency resistance of PG, except one isolate that typed by it. Among MSSA isolates, increased variability to antimicrobial agents. However, the persistence of one predominant clone of MSSA was shown several different strains of both MSSA and MRSA were capable of maintaining persistent colonization [43]. A Japanese study, conducted in 1999 and 2000, of 229 S. aureus isolates from a variety of skin infections in outpatients reported a 21% prevalence of MRSA [44].Sunderrajan and Kale (1984) concluded the correlation with the penicillinase production and the isolates phage groups. He noticed that multidrug-resistance and penicillinase production was higher in isolates from operation theatres [45]. Changing in phage type pattern showed that the increase was caused by the introduction and spread of many resistant strains. During the last decade, resistance to GN showed high performance through MRSA isolates 90% for MRSA and 5.7% for MSSA [36]

compared to ours which was 69% for MRSA while it was 2.5% for MSSA. The distribution of various phage types could be considered as an indicator for the outbreak of infection associated with a predominant phage type, this could demonstrate the spread of MRSA in Iran hospitals. Depending on a phage marker which was predominant in isolates. It was clear to discriminative isolates into a major group was typed by this phage indicator. In 64 typed MSSA isolates, was observed to lack the similarity of phage typing patterns within about 55. It means different distribution of number and type of phage for each isolate which was unrelated. Typically, the response to particular phage was consistent for isolates representing the same strain, and thus a panel of diverse phages could be used to identify and differentiate of S. aureus MRSA isolates reflected somehow of homogeneity concerning phage typing. The nontypable isolates were 15.1% in S. aureus may reflect a variety of circumstances, including the absence of an appropriate cell surface receptor for the phage restriction–modification systems that prevent replication of phage DNA, or the presence of an incompatible lysogenic phage. The phage type assigned to an isolate indicates those phages to which the isolate was determined to be sensitive. As well as studying the genetic back ground of MSSA isolates by combining the phenotypic as well as genotypic may reflect the behavior of S. aureus for acquiring the source of resistance from its origin. In addition of using each individual special isolates for the evaluation of antibiotic susceptibility. In recent years, there has been an alarming increase in nosocomial Staphylococcal infections by strains with multipledrug resistance. At present, this situation is leading to evaluation of Staphylococcal pathogens potentially resistant to any available antibiotic.

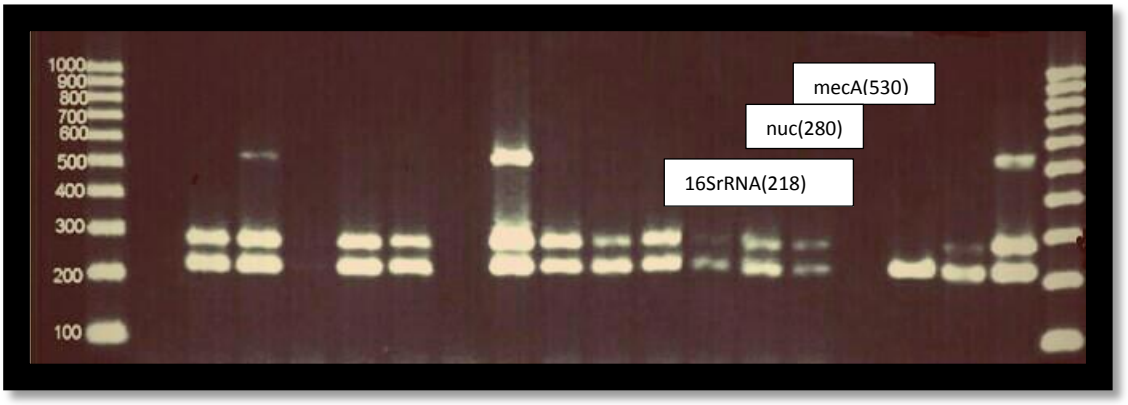
CONCLUSION

It is important for Clinical microbiology laboratories that testing for antibiotics among S. aureus to monitor for emerging resistance patterns (EMSSA).Phenotypic and genotypic analysis performed in this study revealed diversity in the epidemiologically between MSSA/MRSA studied isolates. Heterogeneity inside MSSA for having virulent factors was so wide, while it is homogenized for MRSA, i.e. the variations between MSSA isolates seemed to be horizontally, while in MRSA were vertically. Three MSSA isolates showed fully sensitive seemed to be native wild type. All MRSA showed fully resistant to b-lactams. Phage type Ø95 is a new phage type, strong and stabile colonizer.The changes in phage typing pattern showed that the increase was caused by the introduction and spread of many resistant strains. MRSA isolates reflected somehow of homogeneity concerning phage typing rather than MSSA.

Table 1: Sequences of oligonucleotide primers for identification of S. aureus and detection of MRSA strains.

Gene	Primer	Oligonucleotide sequence	bp	Quality control
mecA	mecA1 mecA2	5'CTT TGC TAG AGT AGC ACT CG 3' 5'GCT AGC CAT TCC TTT ATC TTG 3'	531	NCTC 10442
16s RNA	16sf 16sr	5'GTA GGT GGC AAG CGT TAT CC 3' 5'CGC ACA TCA GCG TCA G 3'	218	ATCC 12228
nuc	nuc1 nuc2	5'GCG ATT GAT GGT GAT ACG GTT 3' 5'AGC CAA GCC TTG ACG AACTAA AGC 3'	280	ATCC 25923

Figure 1: Amplification of three genes: mecA(530), nuc(280) and 16SrRNA(218) M: is a 1000 bp DNA marker



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