RAPID DETECTION OF METHICILLIN RESISTANT STAPHYLOCOCCI FROM THE NASAL SCREENING SWABS BY MANNITOL SALT AGAR WITH CEFOXITIN AND OXACILLIN DISKS

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ABSTRACT

Objective: To assess the reliability of Manitol salt agar (MSA) for directly identifying Methicillin Resistant Staphylococcus aureus (MRSA) and Methicillin Resistant Coagulase negative Staphylococci, (MRCoNS) in nasal swabs for screening purposes using cefoxitin and oxacillin disks.

Study Design: Descriptive and Quasi-experimental

Place and duration of Study: The study was done in the two surgical units of Combined Military Hospital, Rawalpindi and all the samples were processed at the Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi during July 2007.

Material and Methods: A total of eighty four duplicate swabs were taken from the anterior nares of various staff members of the two surgical units and were directly inoculated on Mannitol salt agar with Cefoxitin disc 30µg (MSAFOX) and oxacillin disc 1µg (MSAOX). All the samples were simultaneously inoculated on blood and MacConkey agar for conventional testing, using standard conditions, and confirmed as MRSA or MRCoNS by oxacillin disk diffusion technique. The staphylococcal isolates were later confirmed as MRSA/ MRCoNS by polymerase chain reation (PCR) for mecA gene analysis.

Results: There were 45 staphylococci which revealed mec A gene (40 MRCoNS and 5 MRSA) by PCR. Both the disks with MSA effectively identified the methicillin resistance. MSA with cefoxitin could identify 40 methicillin resistant staphylococci (35 MRCoNS and 5 MRSA) where as MSA with oxacillin could identify 39 methicillin resistant staphylococci (34 MRCoNS and 4 MRSA). There was no significant difference between the two disks in sensitivity, specificity, positive and negative predictive values and overall efficacy of the procedures.

Conclusion: MSA with cefoxitin 30µg and oxacillin 1µg appear to be highly accurate, easy to perform and beneficial for quick and reliable detection of methicillin resistant staphylococci from the nasal carriers in a routine microbiology laboratory.

Keywords: Nasal carriage, screening swabs, mannitol salt agar, methicillin resistant Staphylococci.

INTRODUCTION

Humans are the natural reservoir for Staphylococcus aureus, and asymptomatic colonization is far more common than infection. Colonization may be transient or persistent and can last for years. Persons colonized with Staphylococcus aureus are at increased risk of subsequent infections [1]. The most common sites for carriage are the nose, axilla and perineum [2, 3].

Since the discovery of methicillin resistant Staphylococcus aureus (MRSA) in 1960, the incidence of infections caused by this isolate is on the rise. Transmission usually occurs by direct contact with a colonized carrier [2]. Many studies have shown that there is a vast population of asymptomatic carriers in the hospitals most of whom are unrecognized. They constitute the institutional reservoir of MRSA and act as vectors for the spread of these multidrug resistant organisms to uncolonized but susceptible patients leading to various infections, thus not only causing increase in length of hospital stay but also the health care cost, morbidity and mortality [4].

Epidemiological surveys and effective control measures are the need of the hour to control MRSA infections in the hospitals as well as from the community. As no such survey has been done in Pakistan so the

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actual picture is still gloomy. Rapid screening, implementation of barrier nursing along with timely and accurate identification of MRSA has a pivitol role in preventing its spread.

Molecular techniques like PCR is only restricted to the reference laboratories. Their role as a direct method for the detection of MRSA from the screening swabs is yet to be established. Most of the public health laboratories rely on easy to perform and cost effective phenotypic detection methods. For this purpose, only those media should be used for screening specimens which enhance the recovery of MRSA [5, 6]. Numerous reports in literature suggesting various screening media for MRSA isolation stress upon the fact that oxacillin or methicillin based culture media have never been able to achieve the sensitivity and specificity that is acceptable to all.

Mannitol salt agar has long been used as a selective and differential medium for S. aureus. It requires standard conditions of incubation and is supplemented with salt and mannitol. Staphylococcus aureus colonies appear as yellow and Staphylococcus coagulase negative colonies as pink or whitish on this media. It has been suggested that if cefoxitin or oxacillin is added in this media, this will further differentiate among Staphylococcus aureus as Methicillin resistant (MRSA) or otherwise. Recovery of MRSA using Mannitol salt agar with cefoxitin or oxacillin would take 24 hours as compared to other phenotypic methods taking about 48 hours. Sometimes it is difficult to procure base powder of cefoxitin or oxacillin to be used in agar. We have introduced a simple procedure to avoid this problem. We used disks of cefoxitin 30µg and oxacillin 1µg on Mannitol salt agar (Fig 1).

Health care workers in ICUs and high dependency units are being screened monthly for MRSA carriage. Swabs are received at our faculty on routine basis. Mainstay of screening is rapid diagnosis so that outbreaks and epidemics are timely controlled. Keeping this in view, a study was conducted to evaluate the performance of MSA with cefoxitin and MSA with oxacillin disks at temperature and time defined by Clinical and Laboratory Standards Institute (CLSI) (formerly NCCLS) for the rapid diagnosis of MRSA carriage from the nasal screening swabs in terms of sensitivity and specificity.

MATERIALS AND METHODS

Eighty-four duplicate nasal swabs were taken from various personnel of two surgical units of Combined Military Hospital, Rawalpindi for screening purposes collected in the month of July 2007. All the samples were processed at the Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi. Dry un-moistened cotton swabs were collected from the anterior nares. No transport media was used as the swabs were inoculated within one hour of collection. Two swabs were taken per person; one swab was plated on Blood (BA) and MacConkey agar (MA) and the inoculated plates were incubated for 24-48 hrs at 35±2°C in ambient air. Later they were screened for typical staphylococcal colonies by Gram's staining, catalase, coagulase, and DNase tests [7]. On the basis of positive and negative slide and tube coagulase tests, they were identified as S.aureus and Coagulase negative staphylococcus (CoNS). All these isolates were tested for oxacillin susceptibility according to the CLSI criteria [8] and were subjected to PCR for mecA gene analysis. The second swab was used for plating on two plates of Mannitol salt agar. On one of these plates cefoxitin disk 30µg (MSAFOX) and on the second plate oxacillin disk1µg was applied (MSAOX). The procedure was adopted as already suggested previously for primary antibiotic sensitivity testing [9]. The methicillin susceptible S. aureus strain ATCC 29213 (i.e., CLSI quality control strains for routine MIC testing) and ATCC 25923 (i.e., CLSI quality control strain for routine disk diffusion testing) were used as controls8. Media were also tested for support of growth using the known dilution equivalent to 100 CFU/ml numbers each of S. aureus and Coagulase negative Staphylococcus.

Cefoxitin and Oxacillin Susceptibility Testing

For MSAOX and MSAFOX, oxacillin disk of 1µg and cefoxitin disk of 30µg (Oxoid, Basingstoke, UK), were applied to the MSA after direct inoculation with nasal swabs, on an area of maximum expected growth. Plates were kept at 33oC±2 and 35°C±2 in ambient air for identification of MRSA respectively. Zone diameters were measured at 24-48 hrs, using transmitted and reflected light. According to CLSI a zone diameter of ≤19mm was taken as resistant and a zone diameter of ≥20mm as susceptible for cefoxitin disk while a zone diameter of >13mm taken as susceptible, 11-12 mm as intermediate and < 10 mm as resistant for oxacillin disk. Using calipers, inhibition zone diameters were measured to the nearest millimeter at the inner zone edge [8].

PCR for mec A gene

Presence of mecA gene was considered as the the'gold standard' method for establishing the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for each of the techniques studied. The PCR for mecA gene was performed in accordance with the procedure described by Sakoulas et al [10]. Bacterial DNA was extracted from the suspected colonies on MSA and Staphylococcal colonies on BA using DNA extraction kit by Symbiosis Asti, Italy.

The primer pair used was (5--CTCAGGTACTGCTATCCACC-3-) and (5--CACTTGGTATATCTTCACC-3-; (Gene link, Hawthorne, NY) as described by Ryffel et al. Amplification of reaction mixture was carried out in thermal cycler (Master cycler, Eppendorf, Hamburg Germany). The 50µl PCR mixture consisted of 5µl of the extracted bacterial DNA, each nucleotide (MBI, Fermantas, USA) at a concentration of 0.2 mM, 2.5 mM MgCl2, 0.25µM of each primer and 0.25U of Taq polymerase (MBI, Fermantas, USA). The PCR program consisted of DNA denaturation of 5 minutes at 950 C; 30 cycles with a 30 seconds denaturation step at 940 C; 30 seconds annealing step at 420 C; 30 seconds extension at 720 C and a final 10 min extension step at 720 C. The amplification product of mecA gene (448-bp DNA fragment) was detected by 1% agarose gel electrophoresis with ethidium bromide staining and observing under UV light (Fig. 2).

Data Analysis

Data was analysed using SPSS version 11 (SPSS Inc; Chicago, IL; USA). P values of <0.05 were considered significant. Sensitivity, specificity, positive predictive values and negative predictive values were also

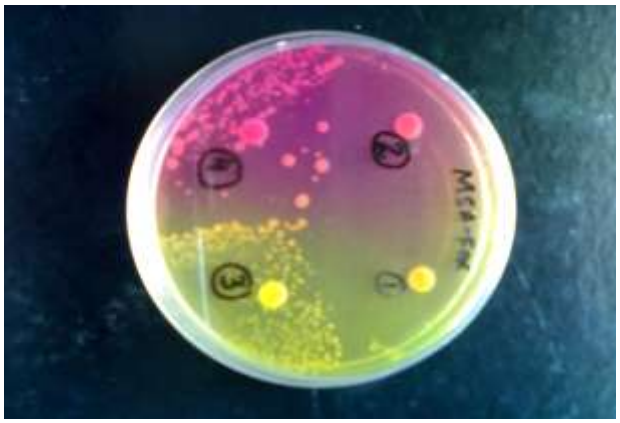

Fig.1: Growth of MRSA (yellow) and MRCoNS (Pink) colonies on Mannitol Salt agar from nasal swabs along with control Staphylococcus aureus (1) (Methicillin Sensitive, MSSA) and Coagulase negative Staphylococcus (Methicillin sensitive, MSSE) using disks of cefoxitin 30µg.

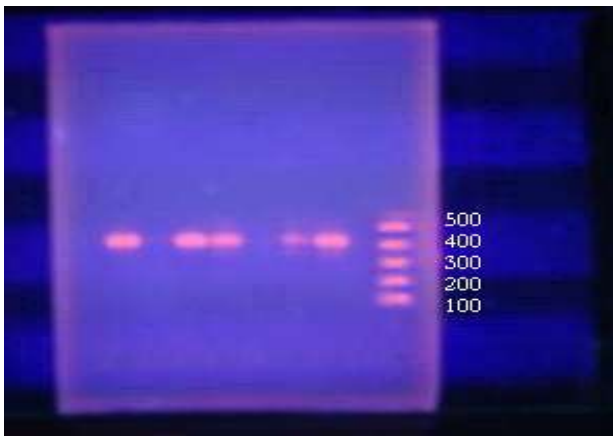

Figure 2: Agarose gel electrophoresis patterns showing single PCR amplification product of S.aureus mecA gene (448-bp) from MRSA (Methicillin resistant Staphylococcus aureus) isolates. Lane 1, positive control; Lane2, negative control; Lane 3, MRSA; Lane 4, MRSA; Lane 5, S.aureus; Lane 6, MRCoNS (Methicillin resistant Coagulase Negative Staphylococci); Lane 7, MRCoNS; Lane M, DNA molecular size marker (100-bp ladder). A schematic representation of the amplified fragments is shown on the left side of the figure.

calculated using appropriate formulae. Overall efficiency of each medium was also calculated.

RESULTS

A total of 84 (duplicate) nasal swabs were screened for MRSA with the abovementioned methods. The Staphylococcus aureus had given yellow colonies while CoNS had yielded colourless colonies (pink) on MSA media. There were 12 Staphylococcus aureus and 72 Coagulase negative staphylococci identified through conventional methods of culture on blood agar and then Gram staining, catalase, coagulase and DNase tests. Two nasal swabs from two individuals out of total 84 nasal swabs had given no yield at all on any of the media used. Therefore, they were excluded from the study. However, there were two nasal swabs from two individuals, which yielded both Staphylococcus aureus and CoNS. Thus the total number of isolates remained 84. By the use of PCR (Fig 2), among them five MRSA (6.0%) and forty MRCoNS (47.6%) were found to be positive for mecA gene amplified product of 448-bp DNA after overnight incubation in brain heart infusion, thus confirmed as MRSA and MRCoNS. The MRSA isolates had their breakpoint MICs within the CLSI defined range i.e., Resistant ≥4µg/ml and Sensitive ≤2µg/ml. Five MRSA strains (6.0%) and 35 MRCoNS (41.7%) were detected by MSAFOX whereas 4 MRSA (4.8%) and 34 MRCoNS (40.5%) were detected by MSAOX media (Table 1). However, one MRCoNS strain each was falsely detected by both the methods. Thus 39 and 37 isolates resistant to methicillin were picked by MSAFOX and MSAOX respectively out of total 45 mec A positive (Methicillin resistant) strains (5 MRSA & 40 MRCoNS).

In the case of MRSA and MRCoNS the colonies had grown up to the edge of the disk (6mm). The overall sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) have no significant difference between the two methods used (Table-2).

The conventional methods took 18-24 hours for routine culture and then further 24 hours for susceptibility testing for identification of MRSA and MRCoNS whereas direct testing from nasal swabs using Mannitol Salt Agar used 24 hours for identification of MRSA and MRCoNS from the specimens (Fig 1). Moreover, mec A gene identification only detect methicillin resistance, but does not differentiate between Staphylococcus aureus and Coagulase negative Staphylococcus. However, there was no significant difference between use of oxacillin and cefoxitin disks on MSA in identifying methicillin resistance (p=0.5608). Nevertheless, both the methods correctly picked 38 mecA negative strains (methicillin sensitive) out of total 39 mecA negative strains.

DISCUSSION

Staphylococcus aureus is the most common cause of surgical site infections (SSIs) and second only to coagulase-negative staphylococci as a cause of nosocomial bloodstream infection and community acquired infections [11]. Thirty to 40% of all

Table-1: Number of Staphylococci detected using Mannitol Salt Agar with cefoxitin (MSA_{FOX}) and oxacillin (MSA_{OX}) disks, out of all those staphylococci identified by conventional methods and confirmed by mec A gene presence (n=84).

Isolates of Staphylococci identified using conventional method and mec A gene	Number of Staphylococci identified using MSA_{FOX}	Number of Staphylococci identified using MSA_{OX}
MSCoNS (n=32)	n= 37	n= 38
MSSA (n=7)	n= 7	n= 8
MRSA (n=5)	n= 5	n= 4
MRCoNS (n=40)	n= 35	n= 34

MSCoNS= Methicillin sensitive Coagulase negative Staphylococci, MRSA= Methicillin resistant Staphylococcus aureus, MRCoNS= Methicillin resistant Coagulase Negative Staphylococci, MSSA= Methicillin Sensitive Staphylococcus aureus. MSA_{FOX}= Mannitol Salt Agar with disk of cefoxitin 30µg, MSA_{OX}= Mannitol Salt agar with oxacillin disk 1µg.

Table-2: Specificity, senitivity, positive predictive value, negative pridictive value and overall efficacy of the two methods using Mannitol Salt agar with cefoxitin (MSA_{FOX}) and Manitol Salt agar with oxacillin (MSA_{OX}) disks

Parameters	MSA_{FOX}	MSA_{OX}
Specificity	97.4	97.4
Sensitivity	86.7	82.2
Positive Predictive value	97.5	97.4
Negative Predictive value	86.4	82.6
Efficacy	91.7	89.3

the S.aureus infections are due to MRSA. Most cases of nosocomial infections occur through exposure to the hands of health care workers after they have been transiently colonized with MRSA from their own reservoir or from contact with an infected patient. Outbreaks may also result from exposure to a single long-term carrier or environmental sources [12]. Screening high-risk patients and health care workers is a prerequisite to limit the spread, but its cost effectiveness is yet to be ascertained.

In our study we have used 1µg disk of oxacillin according to CLSI criteria, but as from 2005 onwards CLSI has claimed cefoxitin as preferred method8 for the detection of methicillin resistance so it was decided to apply cefoxitin disk as well, to find out which one is better before introducing it into the laboratory routine for screening swabs. For primary plating of clinical samples one seldom gets to choose the inoculum, but improved results were seen in one of the study with high inoculum [13]. Mannitol salt agar has long been used as a selective medium for the isolation of S.aureus but it has some inhibitory effects as well. However, no microbiological standards exist that define the most appropriate medium for the enhanced recovery of MRSA from the screening swabs [14].

One study had evaluated selective broth and real time PCR for rapid screening and identification of MRSA achieving a sensitivity of 93.3% and a higher specificity of 89.6% [15]. Another study by Nasia et al evaluated four different media MSA, MSAOX, Mueller-Hinton agar (MHAOX) consisting of oxacillin and MSA with lipovitellin and oxacillin disc 1µg (MSALOX). MSALOX had given the highest yield by detecting 90% of MRSA carriers and misdiagnosed only four as false positives thus achieved a sensitivity of 90% and specificity of 93% [16]. When broth enrichment was compared with primary plating on solid media, sensitivity was increased with no loss of specificity. In our study similar specificity was observed with MSAFOX and MSAOX, discriminating very well between S.aureus and CoNS through colour as well as picking up the methicillin resistance through cefoxitin and oxacillin disks.

Study by Perry et al evaluated CHROM agar MRSA (CMRSA), Staph aureus ID (SAID) and two other media supplemented with 4µg/ml of cefoxitin for the detection of MRSA. All these studies stated that growth inhibition was observed in several strains of MRSA [17].As these studies had used laboratory saved isolates which are unlike the isolates directly cultured from human carriers so the results are not comparable with the surveillance specimens. Moreover, all the MSA media are not alike and have different salt content, which may affect the growth of certain strains [8]. Two studies have evaluated CHROM agar S. aureus with 4µg/ml of oxacillin for better detection of MRSA, however no recommendations were made for screening swabs [12, 18].

None of the S. aureus strain in our study was found to be mannitol negative but one strain of CoNS was mannitol positive and such strains of CoNS are reported in the literature [19]. It is suggested that yellow colonies on MSAFOX and MSAOX prior to PCR should be tested for coagulase test.

Time saving was observed with MSA as colour of the colonies was suggestive of S. aureus. Studies conducted to detect MRSA directly from clinical specimens by PCR technique had shown increased sensitivity and specificity of 100% and 97% respectively [20]. However, PCR cannot differentiate between MRSA and MRCoNS.

In summary the appropriate, accurate and time saving phenotypic methods for the detection of nasal carriage of MRSA are MSAFOX and MSAOX; yellow colonies giving resistant zone of inhibition can be directly taken as MRSA particularly in set ups where PCR facility is not freely available. Although no significant difference was noted between the use of oxacillin and cefoxitin disks on MSA, but MSAFOX may have advantage over MSAOX in that it would not require specific incubation temperature of 33 + 2^{O}C and a full 24 hours incubation.

CONCLUSION

MSA with cefoxitin 30 ug oxacillin 1 ug appear to be highly accurate, easy to perform and beneficial for quick and reliable detection of MRSA from the nasal carriers in a routine microbiology laboratory.

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