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Drug resistance of *Staphylococcus aureus* in sinusitis patients

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Abstract

In this study on Sinusitis patients, we obtained 45 strains of *Staphylococcus aureus*. Antibiotic pattern of *Staphyloccus aureus* showed that resistance to Quinolones was 21% and 33% towards ciprofloxacin and oflaxacin respectively. Resistance to cephalosporins was 50% to cefuroxime, 41% and 50% to cefaperazone and cefotaxime respectively. Least resistance was noticed against aminoglycosides viz. Amikacin 47% and Gentamicin 21%. Resistance to Ampicillin and amoxicillin was 60% and 64% respectively. Oxacillin resistance was seen in 26% of the strains. Of the 45 isolates, 6 were found to be resistant for oxacillin . All these six isolates were subjected to Polymerase Chain Reaction (PCR) and they possessed the *mec*A gene. Correlation existed between the presence of *mec*A gene and oxacillin resistance in *Staphylococcus aureus* and these strains can be considered as MRSA and the patients can be advised for vancomycin therapy. Oxacillin resistance determination by phenotypic methods takes 24 hours to infer whereas PCR for *mec*A gene took only 6 hours. So the PCR techniques for the detection of *mec*A gene can be considered as gold standard (Rapid, Quick and accurate diagnosis) method for the detection of MRSA in spite of the cost involved.

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Introduction

Sinusitis is defined as inflammation of one or more of the paranasal sinuses caused by bacterial or viral infection; air-filled cavities in facial bones lined with pseudo stratified ciliated columnar epithelium and mucous goblet cells (Nord *et al*., 1995). There are several paired paranasal sinuses, including the frontal, ethmoid, maxillary and sphenoid sinuses. Maxillary sinuses are located behind the check bones and inflammation causes pain or pressure in the cheek (maxillary) area. They are present at birth and continue to develop as long as teeth erupt. Tooth roots in some cases, can penetrate the floor of these sinuses. Frontal sinuses are located on both sides of the forehead and inflammation causes pain or pressure in the frontal sinus cavity. These sinuses are late in developing and so infection here is uncommon in children (Orobello *et al*., 1991). Ethmoid sinuses are located between the eyes and inflammation causes pain or pressure pain between eyes. They resemble a honeycomb and are vulnerable to obstruction. Sphenoid sinuses are located behind the eye and inflammation causes pain or pressure behind the eyes, but often refers to the vertex of the head. They are usually present at the age of 3 and are fully developed at the age of 12 (Nord *et al*., 1995).

The symptoms are generally the same in both acute and chronic rhinosinusitis. The symptoms include- nasal symptoms (facial congestion, facial pain-pressure fullness and headache), Oropharyngeal symptoms (halistosis, dental pain, cough and ear pain, pressure fullness) and, systematic symptoms (fever and fatigue). The symptoms in single or combine occur. Acute and chronic sinusitis may be accompanied by thick purulent nasal discharge (usually green in colour, with or without blood) and localized headache (toothache) are present and it is these symptoms that can differentiate sinus related (or rhinogenic) headache from other headache phenomena such as tension headache and migraine headache (Salord *et al*., 1990).

It is important to diagnose nasal complaints accurately, because sinusitis requires antibiotics for rapid resolution. Untreated sinusitis can lead to serious and possibly life threatening complications. The clinical diagnosis of sinusitis is difficult because of the overlap in the symptoms of rhinitis and sinusitis.

Several studies in adults have shown a good correlation between cultures of the middle meatus and the sinus aspirates in patients with acute sinusitis, especially when purulence is seen in the middle meatus (Walder *et al*., 1981). In many geographic areas, amoxicillin is a reasonable first-line antibiotic. Although trimethoprim- sulfamethoxazole and erythromycin- sulfisoxazole have traditionally been used as first line antibiotic for patients with acute bacterial sinusitis, surveillance studies indicate the development of significant pneumococcal resistance from alteration of penicillin binding proteins. Erythromycin alone provides unsatisfactory coverage and is effective against β -lactamase producing organisms. When first line agents have failed or there is a high prevalence of β–lactamase resistance, amoxicillin or clavulanate or second or third-generation cephalosporins (e.g., cefuroxime, cefpodoxime, cefprozil) provide broader coverage. First-generation cephalosoprins (eg-cephalexin) and second generation cephalosporins (eg, cefaclor) provide improved coverage. Several quinolones (eg, ciprofloxacin, gatifloxacin, levofloxacin, moxifloxacin) have specific indications for the treatment of sinusitis, but these should be reserved for second or third time use or for more serious infections.

MRSA stands for methicillin resistant *Staphylococcus aureus* and also multi-resistant *Staphylococcus aureus*. *S. aureus* strains which are resistant to the normal antibiotics were successfully treated with Vancomycin (Mark et al., 2002). This is one of the antibiotics used to treat emerging multi-resistant

organisms. It has evolved an ability to survive treatment with beta-lactamase resistant beta-lactam antibiotics, including methicillin, dicloxacillin, nafcillin, and oxacillin. MRSA is especially troublesome in hospital-associated (nosocomial) infections. The methicillin resistance gene (*mec*A) encodes a methicillin resistant penicillin-binding protein that is not present in susceptible strains and is believed to have been acquired from a distantly related species. *mec*A is carried on a mobile genetic element. Many MRSA isolates are multiply resistant and are susceptible only to glycopeptide antibiotics such as Vancomycin and other investigational drugs (Mark et al., 2002). MRSA isolates have decreased susceptibility to glycopeptides. DNA fragments of *mec*A gene derived from MRSA are used as a probe and this has been reported to be a means of identifying methicillin resistance. More recently, several attempts to detect the presence of the *mec*A gene by the Polymerase Chain Reaction (PCR) have also been reported (Araj *et al.,* 1991**)**

The widespread emergence of methicillin resistant *Staphylococcus aureus* (MRSA), especially in various types of nosocomial infections, is a serious clinical problem worldwide. The incidence of methicillin resistance among nosocomial isolates of *S. aureus* is higher than 70% in some Asian countries such as Taiwan, China, and Korea. Recently, MRSA has also emerged in the community setting in some countries, including Asian countries (Duong,D *et al.*,). One of the cardinal features of the rapid emergence of MRSA in many parts of the world is the dissemination of specific clones; this has contributed to the accelerated increases in the incidence of MRSA. Therefore, it is important to investigate the genotypic characteristics and evolutionary pathway of MRSA clones as well as the genetic relatedness of the strains isolated in different geographic regions.

The aim of the present work is to evaluate the Antimocrobial activity of *Staphylococcus aureus* from sinusitis patients with respect to different antibiotics and to detect the Methicillin Resistant *Staphylococcus aureus* (MRSA) using genotypic method, rather using a phenotypic method. So the PCR techniques for the detection of *mecA* gene can be considered as gold standard (genotypic method). Accordingly, we disclose that *mec*A gene carrying *Staphylococcus aureus* were considered as MRSA and the patients who carry MRSA were advised to take Vancomycin therapy rather going with other antibiotics.

Materials and methods

Collection of samples

Pus samples collected from the middle meatus region of the maxillary sinuses by endoscopy from sinusitis patients were used in the study (Fig 1 & 2). The study group comprised patients, attending the Outpatient department of Government General Hospital, Chennai.

Isolation and identification

Gram staining was performed first on sample collected (Fig 4). The pus samples collected were plated on Nutrient agar, Blood agar (Fig 3), Chocolate agar and Macconkey agar. *S. aureus* was identified after performing a battery of standard tests like presence of coagulase, DNase and mannitol fermentation. The organisms showing the positive reactions in these tests were stored in Brain Heart Infusion agar in air-tight vials for further study.

Coagulase test

A drop of plasma was added to the colony of the test organism emulsified saline solution, and clumping within 10 seconds shows positive reaction.

DNase test

Deoxyribonulease hydrolyses deoxyribonucleic acid. (DNA) The test organism was cultured on a medium which contained DNA. After overnight incubation the colonies were tested for DNA production by flooding the plate with a weak hydrochloric acid solution. The

acid precipitates unhydrolysed DNA. DNase producing colonies are therefore surrounded by clear areas due to DNA hydrolysis.

Table 1. Primer sequence of mecA gene.

Target Gene	Nucleotide sequence 5'-3'	Expected size of amplicon (bp)	Reference
*mec*A (forward) primer 1282	AAA ATC GAT CGT AAA GGT TGG C	533bp	Unal etal 1994
*mec*A (reverse) primer 1793	AGT TCT GCA GTA CCG GAT TTG C		

Antibiogram

The antibiotic sensitivity test was carried out by Kirby-Buyer's Disc diffusion technique on Muller Hinton agar plates. (PH 7.2-7.4) Muller Hinton agar plates were prepared and inoculated with standardized inoculum (corresponding to 0.5 Mac farland tube) to form a lawn culture. With a sterile forceps, antibiotic discs were placed on the surface of the agar plate. The plates were incubated at 37°C for 24 hours. The diameter of the zone of inhibition for each antimicrobial was measured and recorded as resistance, intermediate or susceptible according to the standard CLSI interpretative criteria (Fig 6).

Detection of mrsa by oxacillin disc diffusion method

S.aureus isolates were tested for Methicillin resistance using 1µg of Oxacillin by Disc diffusion method. All the isolates of *S. aureus* were subjected to PCR assay for the presence of *mec*A gene.

Polymerase chain reaction

Forty five isolates which were biochemically confirmed as *S. aureus* were subjected to PCR assay for *mec*A gene. Amplification of the target gene was carried out using bacterial cell lysates as the source of template DNA. *S. aureus* cells were grown at 37°C on Luria Bertani agar (LB agar). Isolated colonies were picked up and inoculated into LB broth and kept for overnight incubation in the shaker incubator. The bacterial cells were pelleted by centrifugation at 10,000 rpm for 10 minutes. The cell pellets obtained were washed with Tris EDTA buffer and was resuspended in 200µl of Tris EDTA buffer and boiled for 10 minutes. Cell debris was removed by centrifugation at 10,000 rpm for 10 minutes and the supernatant containing the template DNA was used for PCR assay. The Primer Sequence and expected amplicon size are tabulated (Table 1).

Results and discussion

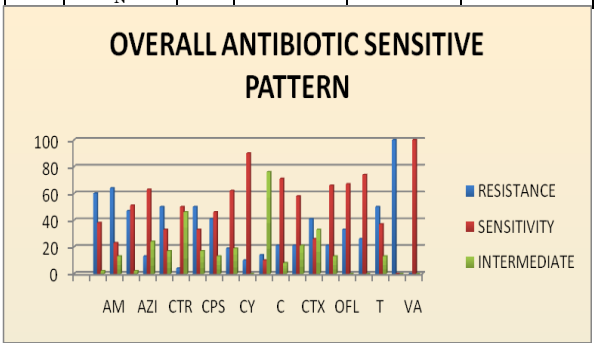
Out of 65 samples from cases of sinusitis, 45 strains of *Staphylococcus aureus* isolated were used in this study. All the strains were confirmed to be *Staphylococcus aureus* based on their colony characteristics, coagulase production and other tests. The colonies on Blood agar after 24 hours were beta haemolytic (Fig. 3), off white in colour, 3-4 mm in diameter with smooth surface and entire edge. The cells of *Staphylococcus aureus* are gram positive cocci, 0.5-1.5 µm in diameter that occur singly and in pairs ,short chains (3 or 4 cells), and irregular grape like clusters(Fig. 4). Methicillin resistant *Staphylococcus aureus* are significant pathogens that have emerged over the past 30 years to cause both nosocomial and community acquired infections. Resistance is primarily mediated by the production of an altered penicillin binding protein (PBP2) and gene encoding (*mec*A) have been found in all highly resistant Staphylococci.

The standard means of identifying methicillin resistance in the clinical microbiology laboratory is by antibiotic susceptibility testing, such as disc diffusion, agar or broth dilution methods described by CLSI. The performance of these tests has been erratic because many factors such as inoculum size, incubation time and temperature, pH of the medium, salt concentration of the medium and exposure to beta lactam antibiotics influence the phenotypic expression of resistance. Methicillin resistance is often expressed heterogeneously in that only 10^4 to 10^7

cells are phenotypically resistant. The empirical approach of most clinicians is to view all levels of methicillin resistance as being equivalent to intrinsic, high level resistance. Intrinsic methicillin resistant staphylococcal infections require vancomycin therapy and strict patient isolation procedures.

Table 2. Antibiotic sensitivity pattern of *Staphylococcus aureus* with different antibiotics.

S. NO.	Antibiotics	SYN	Resistance %	Sensitivity %	Intermediate %
1	ampicillin	A	60	38	2
2	amoxycillin	AM	64	23	13
3	amikacin	AI	47	51	2
4	azithromycin	AZI	13	63	24
5	cefuroxime	CFU	50	33	17
6	ceftriaxone	CTR	4	50	46
7	ceftaxidime	CTZ	50	33	17
8	cefperasone	CPS	41	46	13
9	cephaloridine	CA	19	62	19
10	cephodroxyl	CY	10	90	0
11	cephotaxime	CX	14	10	76
12	chloramphenicol	C	21	71	8
13	ciprofloxacin	CI	21	58	21
14	cotrimazole	CTX	41	26	33
15	gentamycin	G	21	66	13
16	OFLOXACIN	OFL	33	67	0
17	OXICILLLIN	OX	26	74	0
18	TETRACYCLINE	T	50	37	13
19	PENICILLIN	P	100	0	0
20	VANCOMYCIN	VA	0	100	0



Staphylococcal strains with borderline resistance, lacking PBP2a (Penicillin binding protein2a) or the *mec*A gene, have been effectively treated with beta lactam antibiotics and may not require expensive and inconvenient patient isolation procedures. Intrinsic resistance of Staphylococci appears almost exclusively to be due to PBP2a production, and techniques have been developed to identify the *mec*A genetic determinant that codes for this protein. PCR techniques show a high degree of correlation with oxacillin susceptibility susceptibility test and allow accurate classification of not only highly resistant but also borderline resistant strains.

Table 3. Distribution of methicillin resistant *Staphylococcus aureus* (MRSA).

Organism	MSSA %	MRSA	Total
S. aureus	39 (86.6%)	6 (13.3%)	45

In the study conducted by Geha *et al.,* 1993**,** out of 228 *Staphylococcus aureus* isolates tested, 40 isolates showed the presence of *mec*A gene and they were phenotypically resistant to oxacillin. So there was a 100% correlation between the *mec*A gene and oxacillin resistance. John Merlino *et al* conducted a similar study employing 90 clinical Staphylococcus isolates. 60 isolates were found to be methicillin resistant *Staphylococcus aureus* and all were found to be positive for *mec*A gene by multiplex PCR. In the study conducted by Griethuyse *et al.***,** 1991 out of 267 MRSA isolates all the strains were found to be PCR positive for *mec*A gene. In the study conducted on the microbiology of nosocomial sinusitis by (Bert *et al.,* 1999) the predominant pathogen was found to be *Staphylococcus aureus* (34%). A similar epidemiological study in sinusitis was conducted by (William sokol *et al*., 1984) and he observed *Staphylococcus aureus* (17.9%) in positive cultures.

In our study on sinusitis patients we obtained 45 strains of *Staphylococcus aureus***.** It is observed that resistance to quinalones was 21% and 33% towards ciprofloxacin and ofloxacin respectively. Resistance to cephalosporins was 50% to Cefuroxime, 50% to Ceftaxidime and 41% to Cefperasone. Oxacillin resistance was seen in 26% of the strains. Resistance to ampicillin and amoxicillin was 60% and 64%

respectively .Vancomycin shows 100 % sensitivity to all isolates (Table 2). Of the 45 isolates tested, 6 (13.3%) were found to be positive for oxacillin resistance (Table 3). All the 6 (13.3%%) isolates showed the presence of *mec*A gene amplified product containing 533 bp (Fig. 7). These strains designated as MRSA were found to be resistant to all the beta lactam drugs. These also showed resistance towards cephaloridine and cephotaxime. Rapid and reliable detection of MRSA is essential in order to secure the optimal treatment of patients with *Staphylococcus aureus* **i**nfections, as well as for infection control procedures. Phenotypic susceptibility testing often takes 1 to 2 days and sometimes even longer before a definite result is obtained.

Fig. 1. Endoscopic machine for specimen collection.

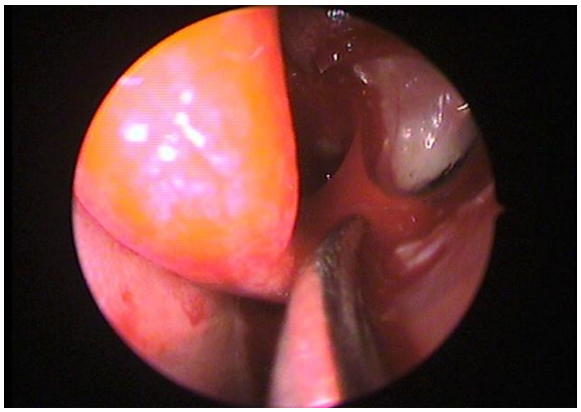


Fig. 2. Pus in middle meatus region.

Genotypic detection of drug resistance will undoubtedly become an important component of diagnostic armamentarium of the clinical laboratory.

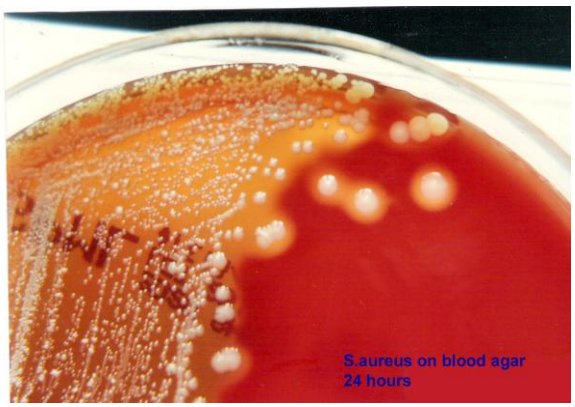


Fig. 3. Colony morphology in blood agar.

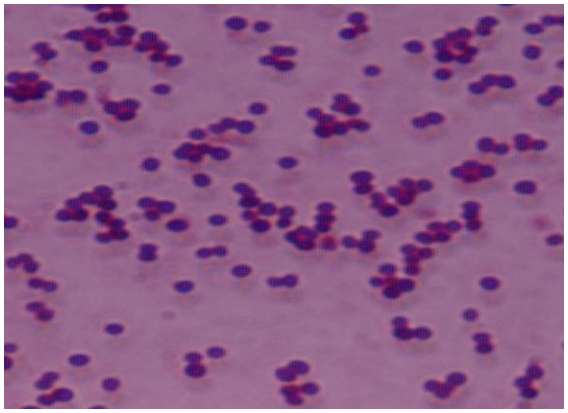


Fig. 4. Gram positive *S. aureus*.

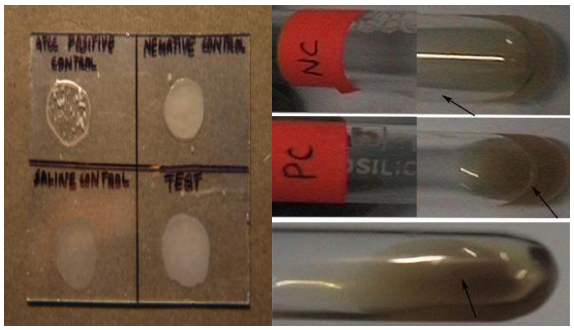


Fig. 5. A-Slide Coagulase reaction of *S. aureus*, B-Tube Coagulase reaction of *S. aureus*.

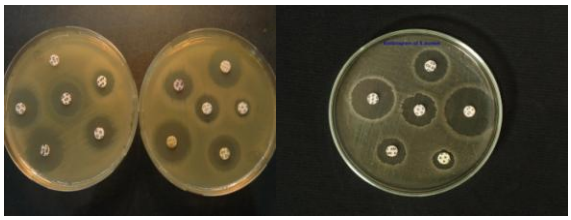


Fig. 6. Drug resistance patterns of *S. aureus*.

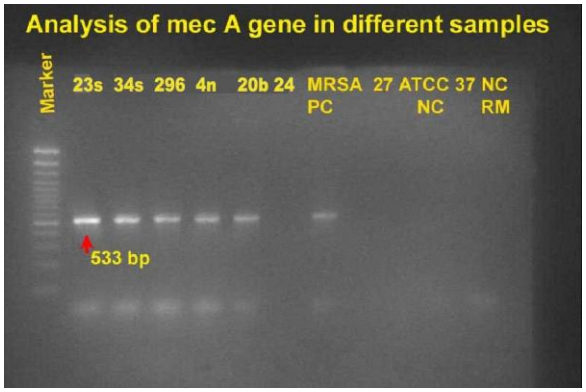


Fig. 7. PCR result of MRSA.

PCR assay for the determination of *mec* A gene in *Staphylococci* appear to be a beneficial adjunct to standard susceptibility testing and allow for the identification of intrinsic resistance in a timely and reliable manner. The cost of the laboratory depends on the volume of specimens tested. For this assay the time required to run an assay was approximately 3 hours. Since vancomycin continues to be only drug that reliably eradicates intrinsically methicillin resistance *Staphylococci,* preservation of this drug for only intrinsic resistance may help to control high cost associated with it's over use and slow the potential development of vancomycin.

Conclusion

Forty five strains of *Staphylococcus aureus* obtained from cases of sinusitis were used for this study. The overall susceptibility pattern showed that resistance to quinolones was 21% and 33% towards ciprofloxacin and oflaxacin respectively. Resistance to cephalosporins was 50% to cefuroxime, 41% and 50% to cefaperazone and cefotaxime respectively. Least resistance was noticed against amino glycosides viz. Amikacin (47%) and Gentamicin (21%). Resistance to Ampicillin and amoxicillin was 60% and 64% respectively. Oxacillin resistance was seen in 13.3% of the strains. All the *Staphylococcus aureus* were subjected to PCR assay. Six isolates of *Staphylococcus aureus* possessed the *mec*A gene (Fig 7). In our study there was a correlation between the presence of the *mec*A gene in *Staphylococcus aureus* and oxacillin resistance showed by them. So these strains can be considered as a MRSA and these patients can be advised for vancomycin therapy.

Oxacillin resistance determination by phenotypic methods takes 24 hours to infer whereas the PCR for *mec*A gene takes only 6 hours. So the PCR technique for the detection of *mec*A gene can be considered as "gold standard" method for the detection of MRSA in spite of the cost involved.

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