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Surveillance of methicillin resistant *Staphylococcus aureus* in school going children and antimicrobial activity of *Rhizophora mucornata, Avicennia alba* and *Avicennia officinalis* against isolated MRSA

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Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections worldwide and outbreaks caused by MRSA are common. The present study investigated the prevalence of MRSA amongst school going children between the age group of 7 to 15 years. This study was carried out in Government Middle School. A total of 150 samples were collected and standard microbiological procedures were employed for isolation and screening of *Staphylococcus aureus*. The isolates were then subjected to antibiotic susceptibility testing using disc diffusion technique against 6 antibiotics (ampicillin, vancomycin, methicillin, gentamycin, oxacillin and clindamycin). The prevalence of MRSA was reported as out of the 150 collected isolates 39 were found to be colonised with *Staphylococcus aureus* out of which 79.4% were MSSA and 20.5% were MRSA. Majority of the *S.aureus* isolates showed resistance towards ampicillin (94.87%), oxacillin (37.00%) and clindamycin (80.00%).

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Introduction

Staphylococcus aureus is facultative anaerobic, Gram-positive cocci which appears as grape-like clusters when viewed under microscope and has large, round, yellow colonies, often with beta haemolysis, when grown on blood agar plates. It carries a wealth of pathogenic determinants, which promote tissue colonization, tissue damage, and distant diseases (Matthews et al., 1997). Methicillin Resistant *S. aureus* (MRSA) includes those strains that have acquired a gene

giving them resistance to methicillin and essentially all other beta- lactam antibiotics (Chambers, 2001).

Emergence of MRSA

MRSA was first identified in the 1960's and was mainly found in hospitals and nursing homes. This occurred because antibiotics were being given to people when they were not needed, and patients were not taking antibiotics as directed. MRSA infections were traditionally associated with exposure to a health care environment however; MRSA has newly evolved to include bacterial strains affecting persons without previous exposure to health care environments. MRSA is contagious and can spread through direct contact. MRSA is becoming a problem in paediatric population. CA-MRSA is increasing in the community and children are becoming more susceptible toward it as they are more likely to exhibit minor scrapes, cuts, bruises and respiratory infections than adults (Miller *et al.* 2010*)*due to their unhygienic practices such as improper washing of hands, touching things that could be possible reservoirs of contaminants or coughing, sneezing without covering their mouths. The fact that MRSA is prevailing in children should not be ignored because they have a higher tendency to transmit the infection than adults due to their ignorance towards hygiene.

Antibiotic Resistance

Resistance in MRSA is related to a chromosomal mecA gene that specifies the production of an abnormal penicillin binding protein called PBP2a or PBP2b. Penicillin–binding proteins are membrane-bound enzymes, which are the targets for all β-lactam antibiotics. PBP2a has a decreased affinity for binding β-lactam antibiotics resulting in resistance all β-lactams (Weems, 2001). The mechanism of resistance to methicillin is mediated via the mecoperon, part of the *Staphylococcal* Cassette Chromosome mec (SCCmec). Five different SCCmec types have been identified in methicillin-resistant *S. aureus* (MRSA) strains. SCCmec types I, II and III are mainly found in hospital-acquired MSRA (HA- MRSA), whereas SCCmec types IV and V are mainly associated with community- acquired MRSA (CA-MRSA). SCCmec contains the mec complex

Medicinal plants

Use of medicinal plants by man has been known for centuries, and therapeutic efficacy of several herbal species has been widely described (Natarajan et al., 2003).

In recent years, antimicrobials derived from the plants have received increased attention, as several synthetic antibiotics have shown ineffectiveness against pathogenic organisms Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and wellbeing. The rise of antibiotic resistant microorganisms is one of the severe problems in health care systems therefore; new drugs need to be found, in order to combat such diseases. Concerning the facts, it is worthwhile to screen plant species which have the anti bacterial properties. The increasing prevalence of multidrug resistant strains of microorganisms and the recent appearance of strains with reduced susceptibility to antibiotics raises an urgent need to search for new sources of antimicrobial agents. One of such species of plant which promises to possess antimicrobial properties is mangroves.

Mangroves

Mangroves are usually found only in tropical climates, as they need consistently warm conditions for development and survival. They occur in approximately 112 countries and territories and are largely confined to the regions between 30º north and south of the equator. (Bandaranayake, 2002). In general, mangroves are trees that grow in saline coastal habitats. Mangroves are used in traditional medicine for the treatment of many disease and they have also been proved for antiviral, antibacterial and antiulcer properties (Chandrashekar *et al*., 2009).

The purpose of the study is to investigate the prevalence of MRSA amongst school going

children and report the prevalence of MRSA colonization and to analyse the activity of *Rhizopora mucornata, Avicinnia alba* and *Avicennia officinialis* against isolated MRSA.

Materials and methods

Methodology for the surveillance of MRSA

Sample Collection

One hundred and fifty children between 7 to 15 years of age studying in three different corporation schools in Chennai were chosen as the study group. Nasal and wound swabs were collected aseptically using sterile cotton swabs (Hi media). Samples were labeled properly for easy identification and brought in sealed cartons to the laboratory and were streaked on sterile blood agar plates.

Isolation and identification of MRSA

The collected samples were streaked on sterilized Blood Agar plates and incubated at 37°C for 24 hours. After incubation, yellow coloured colonies with β haemolysis were selected for further characterisation. Screened isolates were then streaked on sterilized Mannitol Salt Agar (MSA) plates and incubated at 37ºC for 24-48 hrs (Cheesbrough, 2002).

Gram's staining test

Gram-positive cells, the first stain, the crystal violet-iodine complex imparts purple-black colour to the cells. This complex binds to the magnesium- ribonucleic acid component of the cell wall, forming complex, which is difficult to remove. The intensely stained cells are then washed with ethanol. This serves as a lipid solvent and a dehydrating agent for protein.

The Gram-positive bacteria contain low lipid content; hence the low amount of lipid is easily dissolved by alcohol. This makes minute pores in the cell wall that are closed by dehydration effect of alcohol. If the smear is then counterstained with safranine, the Gram-positive cells retain the blue colour of the primary stain.

Procedure

The bacterial smear on a clean glass slide was prepared and fixed using a spirit lamp. A few drops of crystal violet were poured on the smear, left it for 1 minute and then excess stain was removed with tap water. The smear was flooded with Gram's iodine for 1 minute and again washed with water. The stain was decolourised with 95% ethanol by dropping the reagent drop-wise until violet colour came off (30-50 seconds). The stain was washed with tap water and counter stained with safranine for 45 seconds and washed again with water. After drying, the slide was examined under oil immersion of a microscope.

Motility test

Hanging drop method

This method was used to determine the motility of the living organism without staining them.A Hanging-drop slide was cleaned and flamed using a spirit lamp and then placed it on the table with the depression uppermost. Petroleum jelly was spread around the cavity of the slide and four corners of the cover slip, using a match stick. The cover slip on a clean paper was placed with the petroleum jelly side up. A loop full of culture was transferred in the centre of the cover slip. The depression slide was placed on the cover slip, with the cavity facing down. The slide was pressed gently to form a sealed between the cover slip and the slide. And the culture drop was suspended on the cover slip. The slide was examined under oil-immersion objective and viewed that the movement of the bacteria

Catalase test

The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase expedites the breakdown of hydrogen peroxide (H2O2) into water and oxygen (2H2O2 + Catalase → 2H2O + O2). This reaction is evident by the rapid formation of bubble. Two sterilized glass slides were taken. One labelled as 'control' and the other as 'test'. A small amount of normal saline was added to each

slide. Using a sterilized and cooled inoculating loop, a small amount of overnight grown culture of bacteria was picked from mannitol salt agar plate were emulsified on each slide and mixed well. A few drops of Hydrogen peroxide were added to the test sample alone. The formation of bubbles indicates that the strain is catalase positive.

Coagulase slide test

Bound coagulase is attached to the bacterial cell wall and can enzymatically convert fibrinogen in plasma to form fibrin clot that deposits on the cell wall. As a result, individual cocci stick to each other and clumping is observed. The glass slide was cleaned with ethanol using sterile cotton and divided into two sections. One labelled as 'control' and the other as 'test'. A few drops of distilled water were added to each section. Using a sterile inoculation loop, a small amount of overnight grown culture of bacterial sample was picked from the mannitol salt agar plates and emulsified to make a smooth smear on each section. A few drops of blood plasma was added to the test sample and mixed well using sterilized applicator stick. After 5-10 seconds, the clumping of bacterial sample in the slide indicates the presence of catalase positive bacteria.

Antimicrobial susceptibility test (Kirby-Bauer disc diffusion method)

The antimicrobial susceptibility pattern of *Staphylococcus aureus* isolates from mannitol salt agar culture was determined by Kirby-Bauer-NCCLS modified disc diffusion technique (Cheesbrough, 2002). All the isolates were tested forantimicrobial sensitivity to different antibiotics discs. Such as, Methicillin (5 µg), Oxacillin (1 µg), Vancomycin (30 µg), Gentamycin (10 µg), Ampicillin (10 µg), and Clindamycin (2 µg). About 100 ml of Mueller Hinton agar was prepared using 4% NaCl and poured on sterile Petri plates; the overnight culture of MRSA isolates was taken aseptically using sterile cotton swab and swabbed over the Mueller Hinton agar plates. The plates were allowed to dry for five minutes.

Standard Antibiotics discs were taken aseptically and placed over the lawn culture on Mueller Hinton agar plates. The discs were pressed gently for better contact with agar plates. The plates were incubated at 35C for 18 to 24 hours, so as to favor the growth of methicillin resistant strains (BSAC 2002). After incubation antibiotic sensitivity pattern of respective organisms were identified with control strain ATCC 25923.

The diameter of the zone of inhibition produced by each antibiotic disc was measured, recorded and the isolates were classified as "resistant", "intermediate" and "sensitive" based on the standard interpretative chart updated according to the current National Committee for Clinical Laboratory Standards (NCCLS, 2002; Cheesbrough, 2004; Margaret, 1997). Thus the Methicillin- Resistant *Staphylococcus aureus* was confirmed by Kirby-bauer disc diffusion method.

Methodology for the activity of *Rhizophora mucornata, Avicinnia alba* and *Avicennia officinialis* against MRSA

Collection of plant sample

The leaves of *Rhizophora mucronata, Avicennia alba* and *Avicennia officinalis* were collected from the Mangrove forest at Pichavaram (11°24' N and 79°44' E) located in Tamil Nadu, India in the month of December 2013.

Preparation of plant powder

The collected leaves of *Rhizophora mucronata, Avicennia alba* and *Avicennia officinalis* were washed thrice with running tap water in order to remove the soil particles and one time washed in the sterile distilled water (Sivaperumal et al., 2010). The leaves were then shade dried for 15 days with occasional shifting in order to prevent photolysis and thermal degradation. The dried leaves were then chopped into small pieces and ground coarsely to powder form in a mechanical grinder and they were stored in air tight container. The obtained plant powder was used to prepare crude extracts by using Soxhlet extraction method.

Preparation of extract

Soxhlet extraction

The powdered plant material, 10 gm was placed inside a thimble made from thick filter paper and loaded into the main chamber of the Soxhlet extractor. The 200 mL of various solvents like ethanol, acetone and chloroform were taken into a distillation flask and the Soxhlet extractor was placed onto this flask and then equipped with a condenser. The solvent was heated to reflux. The solvent vapour travels up a distillation arm and the condenser cools the solvent vapour and the solvent drips back into the chamber housing the plant powder. The chamber containing the solid material is slowly filled with warm solvent.

Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber got full, the chamber was automatically emptied by a siphon side arm, with the solvent running backdownto the distillation flask. This cycle was allowed to repeat many times, over 6 hours with each solvent. During each cycle, a portion of the non-volatile compound dissolves in the solvent. So, after the completion of cycles the desired compound is concentrated in the distillation flask.

After extraction, the solvent is removed; the final extract was then evaporated to dryness on waterbath. The resulting extract was transferred into vials and preserved in room temperature for further study. The non- soluble portion of the extracted solid that remained in the thimble was discarded.

Antimicrobial study

The effects of various plant extracts on the bacterial strains were assayed by Agar well diffusion method. Muller Hinton Agar Medium were prepared and the bacterial inoculum (isolated MRSA) was swabbed over the surface of the Muller Hinton Agar media using sterile cotton swab to ensure the confluent growth of the organisms. After swabbing of the culture, four holes were made on the plate using gel punch and were labelled as E (for ethanol extract), A (for acetone extract), Ch (for chloroform extract) and C for control .Gentamycin, Cephalosporin and Vancomycin was used as a positive control to determine the sensitivity of the tested strains and Dimethyl Sulfoxide (DMSO) was used as negative control. The extracts were added in the respective holes and inoculated plates were incubated at 37°C for 24 h and the antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well.

Results and discussion

Ethanolic, acetone and chloroform extracts of *Rhizophora mucornata, Avicinnia alba and Avicennia officinialis* were tested against MRSA. It was observed that ethanolic extracts of *Rhizophora mucornata* and *Avicennia officinialis* had intermediate activity against MRSA whereas acetone extracts of all the three had activity against MRSA out of which *Avicennia officinalis* had a better activity (Table 1; Fig. 1).

Table 1. Comparative analysis of the activity of various extracts of *Rhizophora mucornata* (Plant A), *Avicennia alba* (Plant B) and *Avicennia officinialis* (Plant C) against MRSA.

Sample	Zone of Inhibition in mm								
	Plant A			Plant B			Plant C		
	Ethanolic Extract	Acetone Extract	Chloroform Extract	Ethanolic Extract	Acetone Extract	Chloroform Extract	Ethanolic Extract	Acetone Extract	Chloroform Extract
1	11	6	0	3	13	0	14	20	0
2	12	5	0	0	14	0	16	22	0
3	10	7	0	2	12	0	18	18	0
4	13	7	0	1	13	0	13	18	0
5	11.5	6	2	0	13	0	11	15	0
6	9	4	3	0	11	1	11	21	0
7	12	5	2	2	14	2	12	20.4	0
8	11	7	0	0	13	0	10	17	2

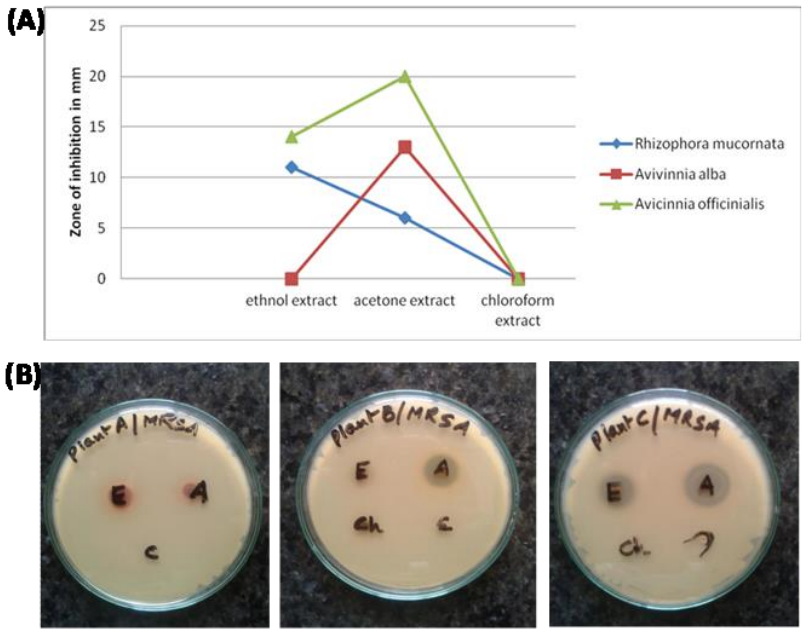

Fig. 1: (A) Graphical representation and (B) antibacterial assay plates of the comparative analysis of the activity of various extracts of *Rhizophora mucornata, Avicinnia alba* and *Avicennia officinialis* against MRSA.

The study was conducted in the different Government Schools in various localities of Chennai to survey the prevalence of MRSA amongst the school children. Nasal wound and skin samples were collected from students and were analysed for the presence of MRSA. A total of 150 samples were collected from different age groups ranging from 7 to 15 years from both the gender and was observed that 8 samples were colonized with MRSA. Chi square analysis was performed to calculate significant difference between gender, age, sample type and respiratory infection against MRSA colonization and it was found that there was no significant difference ($p>0.05$) amongst the variables.

Conclusions

The prevalence rate of MRSA was found to be nearly 5%. But to obtain a substantial result more number of samples need to be analysed, family and friends of the positive children need to be correlated. The MRSA positive children and school authorities were informed about the infection. Infected children were advised to kept in isolation and undergo treatment at any nearby primary health care center. Since few of the isolates (6 out of 8) were multi drug resistant action of three medicinal plant (*Rhizophora mucornata*, *Avicinnia alba* and *Avicennia officinialis*) were evaluated against MRSA. It was observed that *Avicinnia alba* and *Avicennia officinialis* had activity against MRSA.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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