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Analysis of plasmid pattern and restriction digestion in methicillin resistant *Staphylococcus aureus* and methicillin resistant coagulase-negative *Staphylococci*

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Abstract
The present research study have been focused on the analysis of plasmid pattern and restriction digestion in Methicillin Resistant *Staphylococcus aureus* (MRSA) and Coagulase Negative *Staphylococci* (CoNS) in clinical isolates. A total of 72 Sputum Samples were collected from the hospitals in Namakkal and Salem District, Tamil Nadu, India. The collected samples were plated on different media, namely Nutrient agar medium, Mannitol salt agar medium, Blood agar medium, MacConkey agar medium, DNAse Agar medium and *Hi*chrome MeReSa agar medium and incubated at 37ºC for 12-24 hours. The incubated agar media plates were studied for Morphological characteristics, Staining reaction and Biochemical characteristics. The Plasmid DNA was obtained from the isolated Staphylococcal species and the restriction digestion was carried through *Eco*RI and *Hind* III restriction enzymes.

Keywords: methicillin resistance, coagulase negative, *staphylococcus aureus*, plasmid, restriction digestion

1. Introduction
Globalization has entailed a massive increase in trade and human mobility facilitating the rapid spread of infectious agents, including those that are drug resistant. Until recently, community strains have been reliably susceptible to most antibiotics, but the prevalence of Methicillin resistance is increasing. *Staphylococcus aureus* has been reported as a major cause of community and hospital acquired infections. *Staphylococci* are conditional pathogens and the genus consists of at least 32 species [1].

Staphylococcus epidermidis and some other species of Coagulase-negative *Staphlococci* are the major cause of catheter and foreign body associated infections. The heterogenicity of these diseases and the unique ability of *Staphylococcous aureus* to develop resistance to virtually any new antibacterial agent reflect the extraordinary capacity of this organism to adapt and survive in a great variety of environments [2]. Over the past 30 years, molecular and genetic dissection of *Staphylococcus aureus* has revealed a great number of target tissues, and secreted enzymes and toxins that are responsible for infection and distant disease [3].

Methicillin was introduced in 1959 to treat infections caused by penicillin resistant *Staphylococci.* In 1961, there were reports of artificial induction of Methicillin resistance in *Staphylococci* and by 1963 there appeared the first infections with methicillin resistanct *Staphylococus aureus* [4]. MRSA is now a problem worldwide in hospitals and is being increasingly recovered from nursing homes and other health care facilities. The gene responsible for methicillin resistance in is *Staphylococcus aureus mec*A with *mec*DNA. This is possible through the introduction of exogenous DNA into its genome and this region has been bound to be mobile is known as Staphylococcal chromosomal cassette mec [5].

A mutation in the *mec*A gene of *Staphylococus* will provide resistance to all beta-lactums. This gene codes for penicillin binding protein- a mutation in the gene prevents penicillin from effectively binding foreign particles. Coagulase-negative *Staphylococci* strains also have become a serious problem as they express methicillin resistance which involves all β-lactum antibiotics and leads to a significant limitation in therapeutic options [6].

2. Materials and Methods
2.1 Clinical Samples
A Total of 72 Sputum Samples were collected using sterile containers (Hi-Media, Mumbai, India) from the hospitals in Namakkal and Salem District, Tamil Nadu, India. The collected samples were immediately transferred to the laboratory and processed.

2.2 Isolation of *Staphylococcal* species
The collected samples were plated on different media, namely Nutrient agar, Mannitol salt agar, Blood agar, MacConkey agar, DNAse Agar and Hicrome MeReSa agar medium (Special Medium for Methicillin-Resistant *Staphylococcus species*) and incubated at 37ºC for 12-24 hours. The incubated plates were studied for Morphological characteristics, Staining reaction, Biochemical characteristics, Confirmation of Methicillin Resistance, Plasmid Profile and Restriction digestion pattern of the isolates includes, Methicillin-Resistant *Staphylococcus aureus* & Methicillin-Resistant Coagulase Negative *Staphylococci*.

2.3 Confirmation of Methicillin Resistant *Staphylococcus aureus*
A Colony from Nutrient agar medium was streaked on the

HiCrome MeReSa Agar medium and incubated for 18-24 hours at 37ºC. After the incubation, the results should be observed for bluish-green color colonies on the agar medium.

2.4 Confirmation of Coagulase Negative *Staphylococci*

2.4.1. Coagulase Test

2.4.1.1 Slide Test Method

A Loopful of plasma was placed on the clean glass slide and emulsify a colony of the isolated organism in the plasma to make thick suspensions and mix gently. Look for clumping of the organisms within 10 seconds.

2.4.1.2 Tube Test Method

0.5 ml of diluted citrated plasma in a small tube was incubated with heavy saline suspension of the organisms and was incubated at 37ºC for 1- 4 hours. It was examined for every 15 minutes for the formation of the coagulum compared with control.

2.5 Isolation and Purification of Plasmid DNA

For the isolation of plasmid DNA from MRSA and MR-CoNSA clinical isolates, 10 ml of overnight culture was taken in a centrifuge tube and centrifuged at 5000 rpm at 4˚C for 10 minutes. The pellet was resuspended in 200µl of solution I and 10µl of lysozyme. Mixed well and then incubated at 37˚C for 30 minutes. 200µl of solution II was added and the mixer was incubated at 37˚C for 10 minutes. 200µl of solution III was added and incubated at 4˚C for 10 minutes. The content was centrifuged at 12000 rpm for 15 minutes at 4˚C. Supernatant was taken (containing plasmid and protein) and equal volume of phenol: chloroform (1:1) mixture was added and mixed it well. The Content was centrifuged at 12000 rpm for 10 minutes at 4˚C. Aqueous layer was taken and 0.1 volume of sodium acetate was added followed by 0.7 volume of Isopropanol was added and centrifuged at 12000 rpm at 4˚C for 10 minutes. After centrifuge pellet was taken and 1 ml of 70 % ethanol was added and resuspend the pellet and centrifuged at 12000 rpm for 10 minutes at 4˚C. The pellet was obtained (plasmid DNA) and resuspend in 20µl of TE buffer and keep the plasmid at 4˚C. The isolated plasmid DNA was identified through Agarose gel electrophoresis with suitable 100bp ladder marker [7].

2.6 Restriction Digestion of Plasmid DNA

10µl of plasmid DNA was obtained in a sterile microfuge tube. 0.5µl of restriction enzymes, 2µl of 10X buffer and 0.2µl of BSA were added to the microfuge tube and made the volume 20 µl with sterile water. The mixture was incubated at 37^0C for 3 hours the temperature-controlled water bath. After incubation reaction wsa stopped by heat activation at 65^0C for 20 minutes. The samples were loadded in a 1.5% agarose gel electrophoresis. After running the gel was visualized under UV transilluminator. Restricted plasmid DNA were electrophoresised on 1.5% of agarose gel [8].

Table 1: Restriction Digestion Reaction Mixtures

Reaction Mixture I	
Reagents	**1X Concentration**
10X Buffer	2 µl
BSA	0.2 µl
EcoRI	0.5 µl
DNA	10 µl
Nuclease free Water	7.3 µl
Reaction Mixture II	
Reagents	1X Concentration
10X Buffer	2 µl
BSA	0.2 µl
Hind III	0.5 µl
DNA	10 µl
Nuclease free Water	7.3 µl

3. Results

A Total of 72 Wound swab samples were collected and cultured, out of this 29 samples yielded Methicillin-Sensitive *Staphylococcus aureus* (MSSA), 21 samples with Coagulase-Negative *Staphylococci* (CNS), 4 samples with Methicillin-Resistant *Staphylococcus aureus (*MRSA), 3 samples yielded Methicillin-Resistant Coagulase-Negative *Staphylococcus aureus* (MR-CoNS) and 15 samples were sterile (Table. 2). The identification of Methicillin Sensitive *Staphylococcus aureus*, Methicillin resistant *Staphylococcus aureus* and Coagulase Negative Methicillin Resistant *Staphylococcus aureus* on the basis of Colony morphology with different media includes, Nutrient agar medium, Mannitol salt agar medium, MacConkey agar medium, Blood agar medium, DNAse agar medium and HiCrome MeReSa agar medium, Coagulase Test and biochemical analysis (Table.3). From all the Staphylococcal isolates, the plasmid DNA were obtained and it was confirmed by agarose gel electrophosis (Fig. 1). All the plasmid DNA isolates were subjected for restriction digestion using EcoRI and HindIII restriction enzymes and reports were conformed by agarose gel electrophosis (Fig. 2).

Table 2: Isolation of *Staphylococcus* species from wound swab samples

No. of Samples	**Isolation of *Staphylococcus* species**				
	MSSA	**CoNS**	**MRSA**	**MRCoNS**	**No Growth Identified**
72	29 (36.7%)	21 (29.1%)	4 (5.5%)	3 (4.1%)	15 (20.8%)

(Note: MSSA-Methicillin Sensitive *Staphylococcus aureus*; MRSA-Methicillin Resistant *Staphylococcus aureus*; CoNS-Coagulase Negative *Staphylococci*; MRCoNS-Methicillin Resistant Coagulase Negative *Staphylococci*)

Table 3: Morphological and Biochemical characteristics of MRSA and MRCoNS

S. No	**Tests**	**Results and observation for MRSA**	**Results and observation for MRCoNS**
1.	Preliminary Test Gram Staining	Gram Positive Cocci in clusters	Gram Positive Cocci in clusters
2.	Catalase Test	Positive	Positive
3.	Oxidase Test	Positive	Positive
4.	Coagulase Test Slide Test Method Tube Test Method	Positive	Negative
5.	Indole Test	Positive	Negative

6.	Methyl Red Test	Positive	Positive
7.	Voges-Proskauer Test	Positive	Positive
8.	Carbohydrate fermentation Test Sucrose Mannitol Lactose Maltose	 Acid Production - No gas Acid Production - No gas Acid Production – No gas Acid Production – No gas	 Acid Production - No gas No acid and gas production Acid Production – No gas Acid Production – No gas
9.	Citrate Utilization Test	Positive	Negative
10.	Triple Sugar Iron Agar Test	A/A	A/A

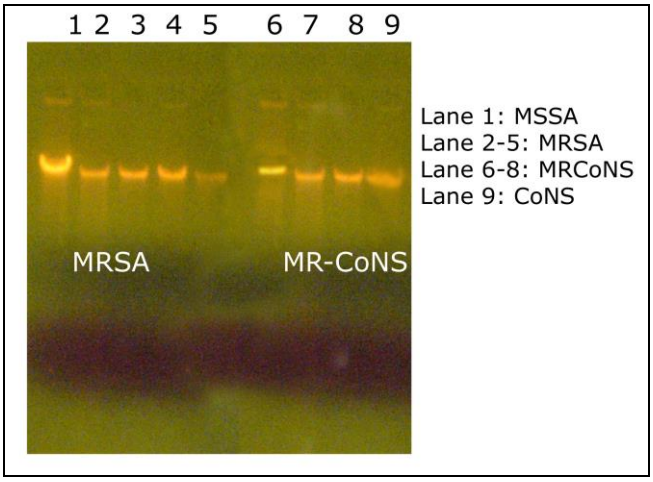

Fig 1: Visualization of Plasmid DNA from Staphylococcal isolates

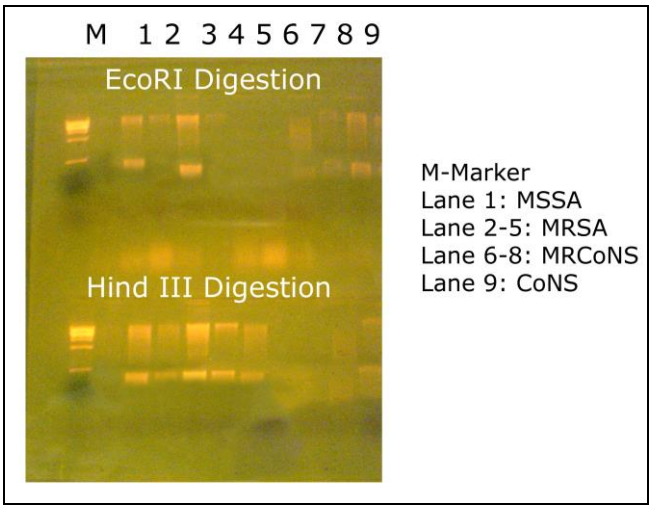

Fig 2: Restriction Digestion of Plasmid DNA from Staphylococcal isolates using EcoRI and HindIII Restriction Enzymes

4. Discussion

Methicillin resistant *Staphylococcus aureus* (MRSA) and Methicillin resistant coagulas negative *Staphylococci (*MRCoNS) was now problem in worldwide hospitals and was being incresingly recovered from nursing homes and other health care facilities. In this presend study, the 72 suspected sputum samples were collected from various hospitals.The result revealed that 4 isolates were MRSA, 3 were MRCoNS, 29 were MSSA, 21 were CoNS. The similar study have been reported by Chigbu**,** *et al.*, that a total of 35 ear and nasal swab samples were collecteted from the infected persons in the hospitals and examined for presence of Methicillin Resistant *Staphylococcus aureus* and Methicillin Resistant Coagulase Negative *Staphylococci* [9].

Pawa**,** *et al.*, focussed that the two distinct strains of Methicillin resistant *Staphylococcus aureus* (MRSA) isolated from patients in a dermatology ward were also resistant to mupiroxin. The mupirocin resitance plasmids from both strains were indistinguishable *Eco*RI and *Hind*III restriction digest analysis. Except for the presence of genes apparently mediating penicillinase production in some transconjugants conjugative transfer of the plasmid mediating mupirocin resistance from one of these strains to a recipient *S. aureus* was accompanied in some cases by co-transfer of plasmid mediating resistance to tetracycline or erythromycin; in some insistence the plasmid which possessed no apparent resitant markers was also transferred. The second strain demonstrated conjugative transfer of penicillin and mupirocin resistance as well as transfer of a plasmid mediating gentamicin resistance, but transfer of erthyromycin resitance was not apparently plasmid mediated [10].

5. Conclusion

In agreement with earlier work, in our present investication the restriction digestion was made on the isolate plasmids by two different restriction enzyme namely EcoRI and HindIII. The reliability of the fragment size (number of fragments) was obtained and recorded. Through this restriction study, it is clearly indicates that all the plasmid DNA obtained from various isolastes have simmilar restriction site to the above enzymes.

6. Acknowledgement

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