International Journal of Genetic Engineering and Biotechnology.
ISSN 0974-3073 Volume 2, Number 2 (2011), pp. 163-172

http://www.irphouse.com

Comparative Identification of Methicillin-Resistant *Staphylococcus aureus* (MRSA) based on amplification of *mec*A gene and Growth on MeReSa Agar and *Hi*Chrome MeReSa Agar

Arun Thangavel[1], Murugan Natarajan[2] and Rajasekara Pandiyan Moses[2]

[1]Centre for Biotechnology, Muthayammal College of Arts & Science, Rasipuram–637408, Tamilnadu, India
[2]Department of Zoology, Arignar Anna Govt. Arts College, Namakkal, Tamilnadu, India

Abstract

Detection of the *mec*A gene by polymerase chain reaction (PCR) is the pioneer method for identifying Methicillin-Resistant *Staphylococcus aureus* (MRSA). The aim of the study was to identify the presence of *mec*A gene in the clinical isolates of Methicillin-Resistant *Staphylococcus aureus* using Polymerase chain reaction with suitable forward and reverse oligonucleotide primer with product size of 527bp. The results were compared with the growth of Methicillin-Resistant *Staphylococcus aureus* in two new chromogenic media as MeReSa Agar medium and *Hi*Chrome MeReSa Agar medium. Ten of the 88 clinical isolates showed the presence of Methicillin-Resistant *Staphylococcus aureus* in the chromogenic media. But three strains should be amplified and confirmed the presence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) through Polymerase chain reaction.

Keywords: Methicillin-Resistant *Staphylococcus aureus* (MRSA), *mec*A gene, MeReSa Agar and *Hi*Chrome MeReSa Agar

Introduction

Increasing incidence of multiple resistances in human pathogenic microorganisms in the recent years, due to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases (Lorenzo Drago, *et al*., 2007). Resistance is a natural response to antimicrobial stress based on the selection which weakens the effect of chemotherapy. The introduction of large numbers of

chemotherapeutic agents into clinical practice has generated strains of microorganisms that survive and multiply *invivo* in the presence of high drug concentrations (Goretti Mallorqui, *et al*., 2004).

Staphylococcus aureus is a common, virulent pathogen associated with a variety of infections, ranging from moderate to severe, in the community as well as in the nosocomial setting. It has extensive genomic variability and easily acquires tools for resisting against antimicrobials, in particular against β-Lactam antibiotics (Goretti Mallorqui, *et al*., 2004). The first evidence of *Staphylococcus aureus* resistance to Penicillin appeared in 1941, only after 2 years since its introduction in clinical therapy. Penicillin resistance is plasmid mediated, so it spread out very quickly to several other strains. Unlikely, Methicillin-resistance is chromosome mediated, and therefore its diffusion is slower than the penicillin resistance.

Methicillin - Resistant *Staphylococcus aureus* (MRSA) was first detected in the early 1960's from European Countries. Methicillin, the first of the semi synthetic Penicillinase- resistant penicillin, was introduced in 1959 to target strains of Penicillinase- producing *Staphylococcus aureus.* Approximately 30 to 50 kb of additional chromosomal DNA in the *Staphylococci*, *mec* region is not found from the susceptible strains of *Staphylococci* but it is present in Methicillin-Resistant strains. Resistance in MRSA is related to a chromosomal *mec*A gene that specifies the production of an abnormal penicillin binding protein 2a (PBP2a) (Henry, 1997).

Over the last four decades, Methicillin-Resistant *Staphylococcus aureus* (MRSA) caused major problems in hospitals throughout the world and become highly endemic in many geographical areas (Kabir, *et al*., 2005; and Michelle Thouverez, *et al*., 2003). Classically, MRSA has been a nosocomial problem associated with long hospital stays, numerous or prolonged antibiotic courses, the presence of invasive devices and proximity to an already infected or colonized patients (Sara and Carol Moore, 2002). In addition to their resistance against all β-lactam antibiotics, MRSA strains may be resistant to several other classes of antibiotic, including the aminoglycosides, quinolones, clindamycin and erythromycin. Therefore, infections caused by these strains are serious and difficult to treat (Durmaz, *et al*., 1997).

Initially, MRSA nosocomial infections were mainly detected in large tertiary hospitals and in intensive care units, where colonized and infected patients as well as colonized health care workers were a significant source of cross-infection. Currently, MRSA is one of the most common pathogens in hospitals of all sizes worldwide (Marta Aires and Herminia, 2004). Methicillin - Resistant *Staphylococcus aureus* (MRSA) is an increasingly common cause of nosocomial infections, causing severe morbidity and mortality worldwide, and accounting in some hospitals for more than 50% of all *Staphylococcus aureus* diseases (Lorenzo Drago, *et al*., 2007).

Rapid detection of Methicillin-Resistant *Staphylococcus aureus* (MRSA) by standard clinical microbiological procedure is tedious and time consuming, since, it first requires the identification of isolated *Staphylococcus aureus* colonies within mixed flora samples before assessing their level of methicillin resistance. Detection of Methicillin-Resistant Staphylococcus aureus (MRSA) in clinical samples continues to be an important, since infections due to MRSA have high morbidity and mortality rates. Methods to detect MRSA in clinical samples ideally should have high sensitivity and a short time to reporting the results (Heiman Wertheim, *et al*., 2001).

Various techniques based on biochemical and molecular features have been applied to investigate MRSA spread including biotyping, antibiotic susceptibility patterns, immunoblotting, peptidoglycan analysis and bacteriophage typing. Recent approaches to typing MRSA exploit its molecular characteristics that include Polymerase chain reaction (PCR), plasmid analysis, restriction fragment length polymorphism (RFLP), ribotyping, and pulsed-field gel electrophoresis (PFGE) (Cotter, *et al*., 1997). The use of the PCR is a rapid and simple process for the amplification of target DNA sequences, which can be used to identify and test bacteria for antimicrobial resistant (Richard J. Jaffe, *et al.,* 2000).

Materials and Methods

Bacterial Isolates

A total of 126 clinical samples (Wound swab, Pus and Sputum) were collected using sterile cotton swabs and sterile containers (from Hi-Media, Mumbai, India) from different age groups of individuals including in-patients and out-patients from the private and Government hospitals in Namakkal District, Tamil Nadu, India. The collected samples were immediately transferred to the laboratory and processed using standard microbiological procedures.

Identification of *Staphylococcus aureus*

All the clinical isolates were identified as *Staphylococcus aureus* based on Gram staining, colony morphology in different media, namely Nutrient agar medium, Mannitol salt agar medium, Blood agar medium; positive catalase, positive coagulase and mannitol fermentation.

Preliminary Identification of MRSA

A Colony from Nutrient agar medium was streaked on the MeReSa Agar medium and *Hi*Crome MeReSa Agar (Special medium to isolate Methicillin - Resistant *Staphylococcus aureus* (MRSA) – *HiMedia*, Mumbai, India) medium and incubated for 18-24 hours at 37ºC.

Genomic DNA Isolation from MRSA, MSSA Clinical Isolates and MTCC - 96

The chromosomal DNA were isolated from Methicillin sensitive, resistant Staphylococcal isolates and MTCC-96 (*Staphylococcus aureus* – Sensitive Reference Strain) through Modified DNA extraction for rapid PCR detection of *Staphylococcus aureus* and MRS protocol (Aziz Japoni, *et al*., 2004) and confirmed through Agarose gel electrophoresis.

Genomic DNA Isolation Method

The Methicillin sensitive and resistance Staphylococcal isolates and MTCC-96 (*Staphylococcus aureus* – Sensitive Reference Strain) were grown in Luria Bertani Broth (LB) for 18 – 24 hours at 37ºC. The grown *Staphylococcus* sensitive and resistant isolates and MTCC-96 Staphylococcal strain were centrifuged at 8000 rpm for 10 minutes at 4ºC and the supernatant was discarded. The pellet was suspended

with 5ml Phosphate Buffer Saline, shacked for 30 minutes and then centrifuged at 8000rpm for 10minutes and the supernatant was discarded. Again the Pellet was suspended with 0.5ml of Phosphate Buffer Saline and the washed bacterial cells were transferred into 1.5ml microfuge tubes and 10µl of Lysozyme was added and incubated at 55˚C for 30 minutes. After the incubation, the tubes were added with 200µl of 10% Sodium Dodecyl Sulphate (SDS) and 5µl of (10mg/ml) Proteinase K and the tubes were incubated in water bath at 33˚C for 60 minutes. The tubes containing suspension was extracted twice with Phenol: Chloroform (1:1) and once with Chloroform (equal volume). Each step of the extraction was performed using a sterile microfuge tube and centrifuged at 12,000 rpm for 5 minutes. The Staphylococcal DNA containing fraction was recovered from supernatant and precipitated with Isopropanol for overnight at -20˚C freezer. The precipitated DNA was washed with 70% ethanol to separate the protein contamination and then resuspended with 40µl of TE buffer.

Amplification of *mec*A gene

The isolated DNA were amplified through the thermal cycler (*TECHNE*, UK) using suitable forward (5_GGGATCATAGCGTCATTATTC_3) and reverse (5_ AACGATTGTGACAGATAGCC_3) Oligonucleotide primers (*First Base*, Singapore), for the amplification of 527bp of *mec*A gene. The total volume of the PCR reaction mixture was 50µl and included as 33µl of sterile deionized water, 10µl of 5X buffer, 1.0µl of dNTP mix, 1.0µl of primer I, 1.0µl of primer II, 0.5µl of *Taq* DNA Polymerase, 1.5µl of $MgCl_2$ and 2µl 0f template DNA. The cyclic parameters were included as the initial denaturation for 2 minutes at 94°C, annealing for one minute at 49°C, extension for one minute at 72°C, repeat the step for 35 cycles, final extension for 7 minutes at 72°C and finally the tubes were stored at 4°C. The amplified gene was identified through Agarose gel electrophoresis with suitable 100bp ladder marker.

Results

Out of the 126 clinical samples, 50 samples (39.6%) yielded Methicillin-Sensitive *Staphylococcus aureus* (MSSA), 10 samples (7.9%) yielded Methicillin-Resistant *Staphylococcus aureus* (MRSA), 28 samples (22.2%) with Coagulase - Negative *Staphylococci* (CNS), and 38 samples (30.1%) were sterile (Table.1; Fig.1).

Table 1: Isolation of *Staphylococcus* species from Clinical Samples.

Clinical Samples	No. of Samples	Sex (Age Group Between 20 – 65)	No. of Isolates from Individuals	*Staphylococcal* Clinical Isolates			No Growth Identified
				MSSA	MRSA	CNS	
Wound	48 (38%)	Male	30 (23.8%)	12 (24%)	3 (30%)	9 (32.1%)	6 (15.7%)
		Female	18 (14.2%)	8 (16%)	2 (20%)	5 (17.8%)	3 (7.8%)

Pus	47 (37%)	Male	29 (23%)	15 (30%)	3 (30%)	7 (25%)	4 (10.5%)
		Female	18 (14.2%)	7 (14%)	1 (10%)	3 (10.7%)	7 (18.4%)
Sputum	31 (25%)	Male	18 (14.2%)	5 (10%)	1 (10%)	2 (7.1%)	10 (26.3%)
		Female	13 (10.3%)	3 (6%)	-	2 (7.1%)	8 (21%)
Total No. of Samples			126	50 (39.6%)	10 (7.9%)	28 (22.2%)	38 (30.1%)

MSSA – Methicillin Sensitive *Staphylococcus aureus*
MRSA – Methicillin Resistant *Staphylococcus aureus*
CNS – Coagulase Negative *Staphylococci*

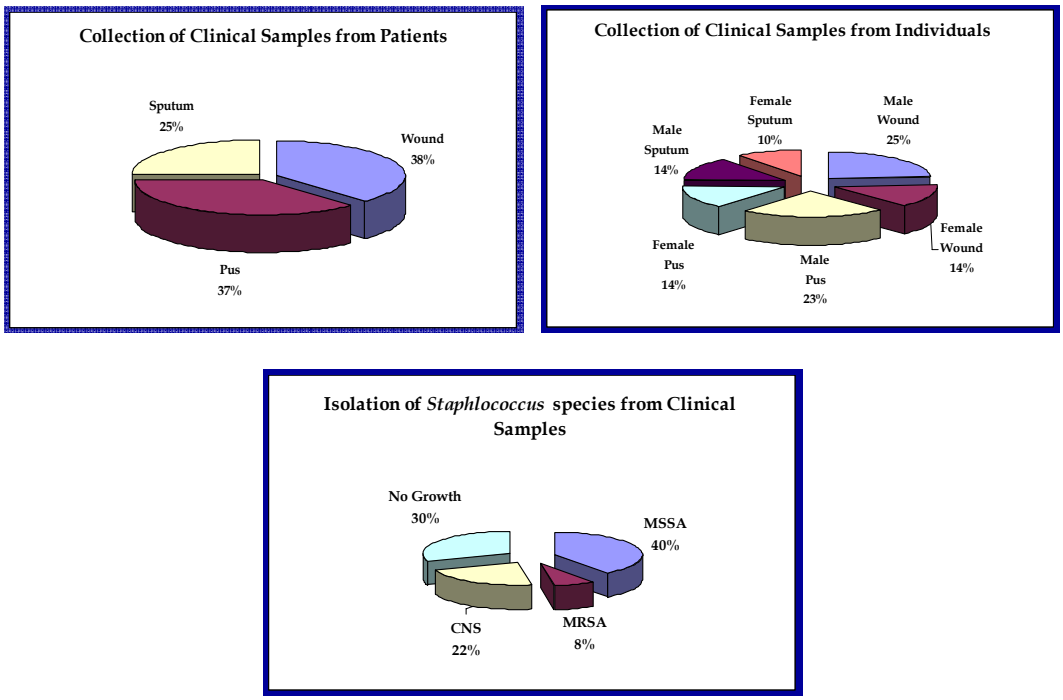

Figure 1: Collection of Wound, Pus and Sputum Clinical Samples and Isolation of Staphylococcus species from Individuals.

Identification of Methicillin-Resistant *Staphylococcus aureus*

The identification of *Staphylococcus aureus* and Methicillin - Resistant *Staphylococcus aureus* (MRSA) on the basis of Colony morphology with different media included as Nutrient agar medium, Mannitol Salt agar medium, MacConkey agar medium, Blood agar medium, DNAse agar medium, MeReSa Agar medium and *Hi*Crome MeReSa agar medium, gram staining reaction and the results of biochemical analysis also recorded and tabulated (Table.2).

Table 2: Preliminary Identification of *Staphylococcus aureus* & Methicillin Resistant *Staphylococcus aureus* (MRSA).

S.No	Tests	Results
1	Gram Stain	Gram Positive Cocci in Clusters.
2	Catalase Test	Bubble Formation indicates Positive Result
3	Coagulase Test - Slide Coagulase - Tube Coagulase	 Formation of Clumps indicates Positive result Coagulum Formation indicates Positive result
4	Mannitol fermentation	Acid/No Gas
5	Nutrient Agar	Formation of Golden Yellow colour colonies
6	Mannitol salt Agar	Formation of Yellow Colour Colonies
7	Blood Agar	Formation of β-Haemolysis
8	Growth on MeReSa Agar Medium	Formation of Greenish Blue Colour Colonies indicates the Presence of MRSA
9	Growth on *Hi*Crome MeReSa Medium	Formation of Bluish Green Colour Colonies indicates the Presence of MRSA

Amplification of *mec*A Gene

The Genomic DNA could be isolated from MTCC-96 (Sensitive Staphylococcal strain – *Staphylococcus aureus*), Methicillin Resistant and Sensitive clinical isolates. The isolated DNA could be confirmed through Agarose gel electrophoresis and visualized the DNA bands by UV-Transilluminator. The confirmed DNA from both the isolates and MTCC-96 could be amplified through thermal cycler (*TECHNE*, UK) using suitable forward and reverse Oligonucleotide primers. The amplified DNA (527-bp region of *mec*A gene) could be identified through Agarose gel electrophoresis with suitable 100bp ladder DNA marker. All the Methicillin sensitive isolates and MTCC-96 strain could not be amplified because the lack of *mec*A gene. But, few of the Methicillin-Resistant Staphylococcal isolates could be amplified and possessed the bands and confirmed the presence of *mec*A gene indicates the Methicillin resistance (Fig.3).

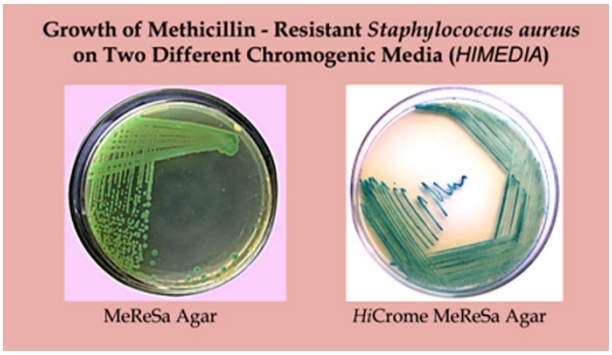

Figure 2

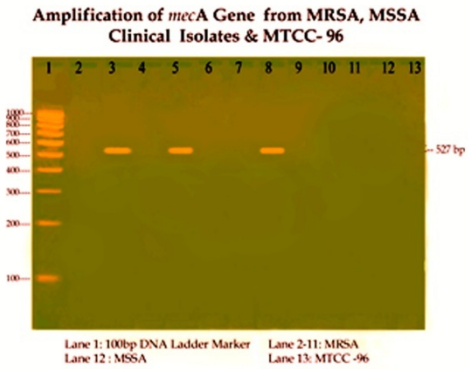

Figure 3

Comparative Analysis of MRSA Isolates

From the preliminary identification of MRSA clinical isolates, ten strains of MRSA have been grown in the Chromogenic media. But only three strains of MRSA have been amplified from the PCR reports. The identified PCR reports and comparison with the preliminary identification of Methicillin resistance through selective Chromogenic media could be recorded (Table.3).

Table 3: Identification and Comparison of Methicillin - Resistant *Staphylococcus aureus* (MRSA) by Two New Chromogenic Media with Polymerase Chain Reaction.

S.No	Clinical MRSA Isolates	Growth of MRSA on Two New Chromogenic Media		Amplification of *mec*A Gene Through PCR
		MeReSa Agar	*Hi*Crome MeReSa Agar	
1	$MRSA_{01}$	+	+	*mec*A$^-$
2	$MRSA_{02}$	+	+	*mec*A$^-$
3	$MRSA_{03}$	+	+	*mec*A$^+$
4	$MRSA_{04}$	+	+	*mec*A$^-$
5	$MRSA_{05}$	+	+	*mec*A$^+$
6	$MRSA_{06}$	+	+	*mec*A$^-$
7	$MRSA_{07}$	+	+	*mec*A$^-$
8	$MRSA_{08}$	+	+	*mec*A$^+$
9	$MRSA_{09}$	+	+	*mec*A$^-$
10	$MRSA_{10}$	+	+	*mec*A$^-$

Discussion

Multi drug resistance in Gram positive cocci have increased at an alarming rate in clinical settings. Drug resistance in microorganisms has predictable and perhaps inescapable response to the use of antimicrobial agent. This can arise from the

selection of resistant strains among naturally susceptible species or from the ingress of new strains of naturally resistant species. The extent of use of particular agents in a given environmental dictates, the rate at which resistance arises among microbial populations.

Our aim was to compare the identification of MRSA clinical isolates by Polymerase chain reaction (PCR) and with growth on chromogenic media. In the present research, 39.6% of Methicillin - Susceptible *Staphylococcus aureus* (MSSA), 7.9% of Methicillin - Resistant *Staphylococcus aureus* (MRSA), 22.2% of Coagulase-Negative *Staphylococci*, and 30.1% samples were sterile form out of 126 clinical samples. Similar reports have observed by Durmaz, *et al*., (1997) and identified the 73.8% of *Staphylococcus aureus*, 31.3% of MRSA, and 26.2% of CNS out of 513 Staphylococcal species. Sonal Saxena *et al*., (2003) have collected a total of 319 nasal swabs from healthy individuals and identified the *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* (MRSA) using Mannitol salt agar (MSA) medium and Oxacillin agar medium. In the present investigation, 48 wound swab samples were collected and identified the *S. aureus* using Mannitol Salt agar medium with yellow colour colonies and Methicillin-Resistant *Staphylococcus aureus* (MRSA) with MeReSa Agar medium and *Hi*Chrome MeReSa Agar medium with greenish blue and bluish green colonies respectively. Similar relevant observation made by Flayhart, *et al*., (2005) and used the CHROMagar MRSA agar medium with violet colour colonies.

Aziz Japoni, *et al*., (2004) have used the modified DNA extraction for rapid PCR detection of Methicillin-Resistant *Staphylococcus aureus* (MRSA) from the clinical samples. The PCR products were analyzed in 1.5% Agarose gel and visualized the amplicon size of 533bp with 100bp DNA ladder marker. In our present report, the above mentioned protocol used to isolate genomic DNA from clinical samples and the amplified product size of 527bp were visualized to confirm the molecular identification of methicillin resistance in *Staphylococcus aureus*. The present investigation shows that a total of 60 isolates of *Staphylococcus aureus* from different patients were isolated and identified that 10 isolates as MRSA. The identified strain could be further confirmed with PCR for the amplification of *mec*A gene responsible for the methicillin resistance. From this molecular observation with PCR, three strains were identified as *mec*A positive and remaining seven as *mec*A negative. Of all the Methicillin-Sensitive *Staphylococcus aureus* (MSSA) clinical isolates showed as *mec*A negative with the reference strains of MTCC-96 (Staphylococcal Sensitive Strain). Jureen, *et al*., (2001) have supported the present findings and isolated a total of 109 clinical isolates of *Staphylococcus aureus* from different patients. They have identified the 52 as *mec*A positive and 57 as *mec*A negative with the reference strains of *mec*A positive MRSA (CDC 2212) and *mec*A negative MSSA (ATCC 25923).

In the present study, the preliminary identification of Methicillin-Resistant *Staphylococcus aureus* (MRSA) from the various clinical samples could be further confirmed by the amplification of *mec*A gene through Polymerase Chain reaction (PCR). The confirmed MRSA clinical isolates through PCR could be compared with preliminary identification of MRSA using two new chromogenic media. From this observation, ten strains of MRSA could be identified through preliminary methods.

But, from the molecular identification, three strains only amplified and confirmed the presence of methicillin resistance in *Staphylococcus aureus*. Similar work has done by Kang-Ju Kim, *et al*., (2005) and reported the presence of MRSA isolates through the amplification of mecA gene and compared with the β-lactamase activity. All the thirteen strains of MRSA could be amplified except ATCC-25923 (Staphylococcal sensitive strain). But in the β-lactamase activity, only one strain showed the negative result along with the Staphylococcal sensitive strain.

Conclusion

From the above mentioned discussion, only three strains of Methicillin-Resistant *Staphylococcus aureus* (MRSA) could be confirmed by Polymerase Chain Reaction (PCR) from the preliminary identified ten MRSA clinical isolates using the two new chromogenic media. In the confirmation of antibiotic resistant bacteria from the clinical specimens, the use of chromogenic media may produce the false result. So, I have concluded that the PCR should be the basic method for their confirmation of antibiotic resistant bacteria.

Acknowledgment

Authors acknowledge the Muthayammal Educational & Charitable Trust, Rasipuram, Tamilnadu, India, provided the laboratory facilities.

References

[1] Aziz Japoni., Abdolvahhab Alborzi., Manoochehr Rasouli., and Bahman Pourabbas., 2004, "Modified DNA Extraction for Rapid PCR detection of MRS," Iranian Biomedical Journal., 8(3), pp.161-165.

[2] Cotter, L., Lynch, M., Cryan, B., Green, P., and S. Fanning. 1997. Investigation of a Methicillin-Resistant Staphylococcus aureus (MRSA) outbreak in an Irish hospital: triplex PCR and DNA amplification fingerprinting. Journal of Hospital Infection. 36: 37-47.

[3] Durmaz, B., Durmaz, R., and Sahin, K., 1997, "Methicillin-Resistance among Turkish isolates of Staphylococcus aureus strains from nosocomial and community infections and their resistance patterns using various antimicrobial agents," Journal of Hospital Infection., 37, pp.325-329.

[4] Flayhart, D., Walther, R., Lema, C., Snyder, H., and Carroll, K., 2005, "Enhanced detection of Methicillin-Resistant Staphylococcus aureus using Chromogenic MRSA media compared to traditional culture methods," Annual Association for Practitioners in Infection Control., pp.1-3.

[5] Goretti Mallorqui-Fernandez., Aniebrys Marrero., Sonia Garcia-Pique., Raquel Garcia-Castellanos., and Xavier Gomis-Ruth, F., 2004, "Staphylococcal Methicillin Resistance: fine focus on folds and functions," FEMS Microbiol Letters., 235, pp.1-8.

[6] Heiman Wertheim., Henri A. Verbrugh., Cindy Van Pelt., Peter De Man., Alex Van Belkum., and Margreet C. Vos., 2001, "Improved detection of Methicillin - Resistant Staphylococcus aureus using phenyl Mannitol broth containing Aztreonam and Ceftizoxime," J Clin Microbiol., 39(7), pp.2660-2662.

[7] Henry F. Chambers., 1997, "Methicillin Resistance in Staphylococci: Molecular and Biochemical basis and clinical implications," Clin Microbiol Rev., 10(4), pp.781-791.

[8] Jureen, R., Liland Bottolfsen, K., Grewal, H., and Digranes, A., 2001, "Comparative evaluation of a commercial test for rapid identification of Methicillin-Resistant Staphylococcus aureus," APMIS., 109, pp.787-790.

[9] Kabir O. Akinyemi., Olukayaode Oladapo., Chidi E. Okwara., Christopher C. Ibe., and Kehinde A. Fasure., 2005, "Screening of crude extracts of six medicinal plants used in South-West Nigerian Unorthodox medicine fro anti-Methicillin-Resistant Staphylococcus aureus activity," BMC Complementary and Alternative Medicine., 5(6), pp.1-7.

[10] Kang-Ju Kim., Hyeon-Hee Yu., Jung-Dan Cha., Se-Jeong Seo., Na-Young Choi., and Yong-Ouk You., 2005, "Antibacterial activity of Curcuma longa L. against Methicillin-Resistant Staphylococcus aureus," Phytotherapy Research., 19, pp.599-604.

[11] Lorenzo Drago., Elena De Vecchi., Lucia Nicola and Maria Rita Gismondo., 2007, "In vitro evaluation of antibiotics combinations for empirical therapy of suspected Methicillin-Resistant Staphylococcus aureus severe respiratory infections," BMC Infectious Diseases., 7(111), pp.1-7.

[12] Michelle Thouverez., Arno Muller., Didier Hocquet., Daniel Talon., and Xavier Bertrand., 2003, "Relationship between molecular epidemiology and antibiotic susceptibility of Methicillin-Resistant Staphylococcus aureus (MRSA) in a French teaching hospital," J Med Microbiol., 52, pp.801-806.

[13] Richard I. Jaffe., Janae D. Lane., Stephen V. Albury., and Debra M. Niemeyer., 2000, "Rapid Extraction from and direct identification in Clinical samples of MRS using the PCR," J Clin Microbiol., 38(9), pp.3407-3412.

[14] Sara I. Islam., and Carol Moore., 2002, "Prevalence of Methicillin-resistant Staphylococcus aureus and associated risk factors on admission to a specialist care eye hospital," Annals of Saudi Medicine., 22(3-4), pp.153-157.

[15] Sonal Saxena., Kavita Singh., and Vibha Talwar., 2003, "Methicillin-Resistant Staphylococcus aureus Prevalence in Community in the East Delhi Area," Jpn. J. Infect. Dis., 56, pp.54-56.