

Molecular Identification of Methicillin Resistant Staphylococcus (Mrsa) by Polymerase Chain Reaction (Pcr) Technology

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INTRODUCTION

Introduction of penicillin in the year 1940 had greatly improved the prognosis for patients with severe staphylococcal infections, but after a few years of clinical use, resistance appeared owing to production of β -lactamases. Methicillin was designed to resist β lactamase degradation, but Methicillin Resistant Staphylococcus aureus (MRSA) strains were soon identified. Since then, these staphylococcal strains have spread worldwide. Until recently, Methicillin Resistant Staphylococci were predominantly nosocomial pathogens causing hospital acquired infections but Methicillin Resistant Staphylococcal (MRS) strains are now being increasingly isolated from community acquired infections as well. β -lactam antibiotics such as methicillin inactivate Penicillin Binding Proteins PBP's 1, 2 and 3 in the bacterial cell wall which have an enzymatic role in the synthesis of Peptidoglycans. However Methicillin Resistant Staphylococcus Aureus soon acquired a new gene called *mecA* which encodes for a new PBP called PBP2a which has low affinity for β -lactam Antibiotics thus conferring resistance to these organisms. MRSA prevalence increased from 12% in 1992 to

80.83% in 1999. Indian literature shows that MRSA incidence was as low as 6.9% in 1988 and reached to 24% and 32.6% in Vellore and Lucknow in 1994 and was of the same order in Mumbai, Delhi and Bangalore in 1996 and in Rohtak and Mangalore in 1999. However, in some of the centres it was as high as 87%.

After the emergence of MRSA as a nosocomial pathogen in the early 1960s, there have been an increasing number of outbreaks of MRSA infections in hospitals reported from many countries. Life-threatening sepsis, endocarditis, and osteomyelitis caused by MRSA have also been reported[1]. Since resistance to multiple antibiotics among MRSA isolates is very common, there is a possibility of extensive outbreaks, which may be difficult to control. MRSA is now one of the commonest nosocomial pathogens, and asymptotically colonized healthcare workers are the major sources of MRSA in the hospital environment. Early detection of MRSA and formulation of effective antibiotic policy in tertiary care hospitals is of paramount importance from the epidemiological point. The higher price of

vancomycin, its unavailability in many parts of the country, and also the possibility of emergence of resistance to the drug should at least make the clinicians look into the alternatives. Therefore, regular surveillance of hospital-associated infections including antimicrobial susceptibility pattern of MRSA and formulation of a definite antibiotic policy may be helpful in reducing the burden of MRSA infections in the hospital.

Among all known bacteria, *Staphylococcus aureus* is possibly the greatest concern of all health-care-associated pathogens due to its ability to cause a wide variety of life-threatening infections (Lowy, 1998) (*Staphylococcus aureus* has the ability to rapidly adapt to different environmental conditions (Lowy, 1998). In 2003, *S. aureus* was reported to be the leading cause of health-care-associated infections globally (NNISS, 2004). Numerous anti-staphylococcal agents exist, including linezolid, daptomycin, tetracyclines and fluoroquinolones, but these are rapidly becoming of less value due to the ability of the bacterium to develop efficient mechanisms to neutralize these agents. The methicillin resistance mechanism is the most recognized in methicillin-resistant *S. aureus* (MRSA) strains. MRSA has been recognized as an important health-care-associated infection. Treatment as early as 1959 included semi-synthetic penicillin drugs such as methicillin for *S. aureus* infection. The rise of MRSA strains became apparent as early as 1960, approximately a year after methicillin introduction (Jevons, 1961). In the early 1980's MRSA strains were identified as a major cause of nosocomial infections due to the increase every decade. Since 1987, MRSA was increasingly found in the community and referred to as community-acquired MRSA (renamed community-associated MRSA) (CA-

MRSA). The development of methicillin resistance in *S. aureus* strains can be ascribed to the altered penicillin-binding protein (PBP2a), which has a reduced affinity to penicillin and β -lactam antibiotics. The *mecA* gene that encodes the altered protein (PBP2a) is not inactivated by methicillin during treatment. The *mecA* gene resides on a genomic island termed the staphylococcal cassette chromosome *mec* (SCC*mec*) MRSA strains that have been clinically identified as community associated have been shown to be more virulent with a high degree of severity of disease when compared with HA-MRSA.

This is due to the production of the Panton-Valentine leukocidin (PVL) toxin (Dufour et al., 2002). PVL is a toxin associated with deep skin infection, soft tissue infection and necrotizing pneumonia). PVL toxin stimulates pore formation in the leucocyte membrane resulting in the death of the cell, thus promoting tissue necrosis. Because, the PVL-associated genes of CA-MRSA are harboured by a bacteriophage, ϕ SLT, these toxin genes may be transmitted easily to other HA-MRSA strains. NPVL toxin has been identified as a genetic marker for CA-MRSA strains. In comparison with previous detection methods such as Southern blotting and pulsed-field gel electrophoresis (PFGE), PCR assays such as multiplex-PCR (M-PCR), real-time PCR, hypervariable region (HVR) and spa-typing techniques can provide a rapid amplification, detection and typing tool for MRSA strains. Healthcare providers should be aware of new technological approaches to the detection and confirmation of MRSA in clinical samples, and ensure that adequate health technology evaluation programmes are in place. *S. aureus* is a Gram-positive coccus where the round cells, approximately 1 μ m in diameter, form grape-like (Greek staphyle) clusters

indicative of the ability to divide in more than one plane. They are capable of both aerobic and anaerobic respiration and most strains ferment mannitol anaerobically. On blood agar they form characteristic golden (Latin aureum) or white colonies. They produce catalase, coagulase and an extracellular cell clumping factor, and some strains produce capsules.

Virtually all MRSA produce an additional penicillin-binding protein, PBP2a or PBP2' which confers resistance to all currently available β -lactam agents. PBP2a is encoded by the *mecA* gene.¹⁴ Matsuhashi M, Song MD, Ishino F et al. Molecular cloning of the gene of a penicillin-binding protein supposed to cause high resistance to β -lactam antibiotics in *Staphylococcus aureus*. Although methicillin is now not used in treatment, it was the first penicillinase-resistant penicillin to be used in the 1960s and was recognized at that time as the most reliable agent for routine susceptibility testing. Hence resistant strains were termed 'methicillin-resistant *S. aureus*' (MRSA). Later use of oxacillin as an alternative to methicillin in susceptibility tests resulted in the term 'oxacillin-resistant *S. aureus*' (ORSA). These designations are used interchangeably in the literature and are synonymous. Methicillin-resistant *Staphylococcus aureus* (MRSA) were first reported in 1961 and have since become a major nosocomial pathogen worldwide.^{2,3} In the UK, the mean incidence of MRSA bacteraemia is about 40% of *S. aureus* bacteraemia. An additional concern is the emergence of vancomycin-intermediate *S. aureus* (VISA) and more recently vancomycin-resistant *S. aureus* (VRSA).^{4,5} The reservoir of MRSA is infected and colonized patients,⁶ and the major mode of transmission from patient to patient is on the contaminated hands of healthcare workers.^{6,7} It is axiomatic that the

sooner an MRSA infection is diagnosed, and the susceptibility to antimicrobial agents established, the sooner appropriate therapy and control measures can be initiated. Laboratory diagnosis and susceptibility testing are crucial steps in treating, controlling and preventing MRSA infections.

Guidelines for the control of MRSA infections in the UK have been previously published by a joint Working Party of the British Society for Antimicrobial Chemotherapy (BSAC), and the Hospital Infection Society (HIS) in 1986, 1990 and together with the Infection Control Nurses Association (ICNA) in 1998.¹⁰ The Department of Health Special Advisory Committee on Antimicrobial Resistance (SACAR) asked the three Societies to revise the guidelines. Unlike the previous reports, which focussed on the prevention and control of MRSA infections, SACAR requested that guidelines should be extended to cover prophylaxis and therapy of MRSA infections and also the laboratory diagnosis and susceptibility testing of MRSA. Members of the Working Party were representatives of the BSAC, HIS and ICNA. This report deals with the laboratory diagnosis and susceptibility testing of MRSA in the UK (guidelines for the prophylaxis and therapy of MRSA infections are due to be published in JAC and guidelines for the control and prevention of MRSA in hospitals are due to be published in the Journal of Hospital Infection). The *mecA* gene is a gene found in bacterial cells. The *mecA* gene allows a bacterium to be resistant to antibiotics such as methicillin, penicillin and other penicillin-like antibiotics. The most commonly known carrier of the *mecA* gene is the bacterium known as MRSA. Apart from *Staphylococcus aureus* and other *Staphylococcus* species, it can also be found in *Streptococcus pneumoniae* strains

resistant to penicillin-like antibiotics. In *Staphylococcus* species, *mecA* is spread on the SCCmec genetic element. The *mecA* gene does not allow the ring like structure of penicillin-like antibiotics to bind to the enzymes that help form the cell wall of the bacterium (transpeptidases), and hence the bacteria is able to replicate as normal. The gene encodes the protein PBP2A (penicillin binding protein 2A). PBP2A has a low affinity for beta-lactam antibiotics such as methicillin and penicillin. This enables transpeptidase activity in the presence of beta-lactams, preventing them from inhibiting cell wall synthesis.

MATERIALS AND METHODS

SAMPLE COLLECTION

Discarded bandages from infected burn wounds were collected into polythene bags wearing hand gloves from Osmania General Hospital and immediately transferred to the Laboratory.

SERIAL DILUTIONS AND PLATING:

Materials required:

- Distilled water
- Sterile test tubes
- Sterile pipettes
- Samples
- Test tube stands

PROCEDURE :

The first step in making a serial dilution is to take a known volume (usually 1ml) of stock and place it into a known volume of distilled water (usually 9ml). This produces 10ml of dilute solution. The dilute solution has 1ml of extract /10ml. This is a 10-fold dilution. The concentration of stock in each ml of the diluted solution is 1ml. The technique used to make a single dilution is repeated sequentially

using a more and more dilute solution as the "stock" solution. At each step 1ml of the previous dilution is added to 9ml of distilled water. Each step results in a 10-fold change in the concentration from the previous concentration (10^{-1} to 10^{-8}). 0.5 ml from each dilution was inoculated by pour plate technique in nutrient agar medium and all the plates are incubated at 30 °C for 24 hrs.

MORPHOLOGICAL CHARACTERIZATION

Among 35 colonies obtained on 10⁻⁵ plate by serial dilution and plating, two isolated colonies with different colony morphology were selected and pure cultures of these isolates were obtained by repeated streaking on nutrient agar slants.

Finally, pure cultures were stored on nutrient agar slants at 4°C in a refrigerator. These cultures were first identified by preliminary morphological characterization as mentioned below:

GRAMS STAINING:

Materials required:

Set up a staining area, away from the Bunsen burner, with the following items:

- Crystal Violet stain.
- Gram's stain.
- Saffranin stain.
- Washing bottles with distilled water.
- 95% Ethanol solution in a washing bottle.

PROCEDURE:

- Smear and heat fix a clean microscope slide with bacterial culture.
- Put 10 to 15 drops of Crystal Violet stain on bacterial smear and leave on for one minute.
- Rinse the Crystal Violet stain off with water (from the washing bottle).

- Put the Grams' stain on the smear and leave it on for one minute, then rinse it off with water from the washing bottle.
- Add 10 to 15 drops of 95% ethanol and leave on for 10 to 15 seconds. Rinse off with water for the washing bottle.
- Place the Saffranin on the slide and leave on for 45 seconds. Rinse off with water from the washing bottle. Add a cover slip.
- Look under the microscope. Determine whether or not bacterial culture stained positive or negative.

OBSERVATION:

Presence of violet color indicates Gram Positive bacteria and presence of pink color indicates Gram Negative bacteria.

RESULT:

Violet color colonies were observed which indicates GRAM POSITIVE BACTERIA.

ENDOSPORE STAINING:

Materials required:

- Malachite green
- Saffranin

PROCEDURE:

- Prepare smears of organisms to be tested for endospores.
- Heat fix the smears.
- Cover the smears with a piece of absorbent paper cut to fit the slide and place the slide on a wire gauze on a ring stand.
- Saturate the paper with malachite green and holding the Bunsen burner in the hand heat the slide until steam can be seen rising from the surface.
- Remove the heat and reheat the slide as needed to keep the slide steaming for about three minutes. As the paper begins to dry add a drop

or two of malachite green to keep it moist, but don't add so much at one time that the temperature appreciably reduced. DO NOT OVERHEAT. The process is steaming and not baking.

- Remove the paper with tweezers and rinse the slide thoroughly with tap water.
- Drain the slide and counter stain 45 seconds with 0.5% Saffranin.
- Wash, blot, and examine.

OBSERVATION:

The appearance of vegetative cells in red color and spores in green color indicates positive test otherwise negative.

RESULT:

No spore formation.

CAPSULE STAINING:

Material Required:

- Indian ink/Nigrosin
- CuSO_4 (20% W/V)

PROCEDURE:

- Place one loopful of India ink at one end of the microscope slide.
- Mix 1 loopful of sterile saline with the Ink.
- Aseptically transfer and mix small amount of bacteria in the loopful Indian Ink.
- Take a second slide and hold at a 45 degree angle touch the end of the slide to the other slide and pull the slide to meet the drop.
- Without raising the slide push the top slide back to spread the stain.
- Dispose of the second slide.
- Do Not Heat Fix! Allow slide to thoroughly air dry.
- Flood smear with Ethylene blue for 3 minutes.
- Remove Ethylene blue and gently raise the slide with water.

- Do Not Heat Fix! Allow slide to thoroughly air dry.
- Add immersion oil to smear and observe.

OBSERVATION:

White capsule was observed in a black background .

RESULT:

The Organism Was Found To Contain A Capsule.

MOTILITY TEST: (Hanging drop method)

Materials Required:

- 12hours broth culture,
- cavity slide,
- petroleum jelly,
- cover slip etc.

PROCEDURE:

- Place a small drop of liquid bacterial culture in the center of a cover slip
- Place a small drop of water at each corner of the cover slip
- Invert a slide with a central depression over the cover slip
- The cover slip will stick to the slide and when the slide is inverted the drop of bacterial culture Will be suspended in the well
- Examine microscopically (X400) for motile organisms.

OBSERVATION:

If motility is seen on the disc slide the test is Positive otherwise the test is negative.

RESULT:

Non-Motility Of The Microorganisms Was Observed. Hence The Test Is Negative.

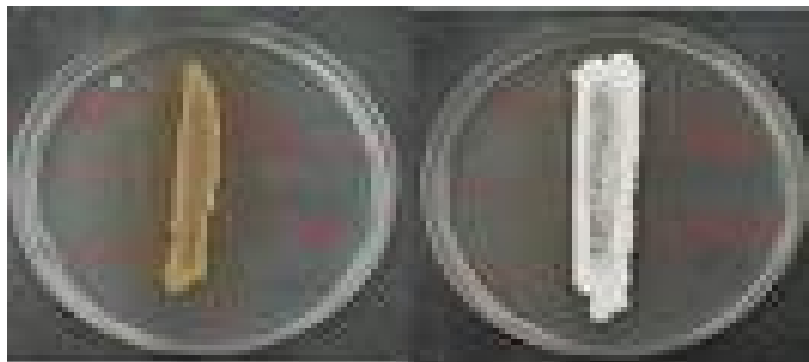


Figure 1: motility test. (Hanging drop method) non-motility of the microorganisms was observed. Hence the test is negative.

S.NO	TEST	RESULT
1	GRAM'S STAINING	POSITIVE
2	ENDOSPORE STAINING	NEGATIVE

3	CAPSULE STAINING	POSITIVE
4	MOTILITY	NEGATIVE

TABLE 1 : SUMMARY OF THE RESULTS ON MORPHOLOGICAL TESTS

BIOCHEMICAL TESTS

IMVIC TEST:

The IMViC tests are a group of individual tests used in microbiology lab testing to identify an organism in the coliform group. A coliform is a gram negative, aerobic or facultative anaerobic rod which produces gas from lactose within 48 hours. The presence of some coliforms indicate fecal contamination.

INDOLE PRODUCTION TEST

Medium : Tryptone broth media
 Peptone : 0.5 grams
 Glucose : 0.5 grams
 Nacl : 0.25 grams
 Yeast Extract : 0.5 grams
 Tryptone : 0.5 grams
 Distilled water : 50 ml

pH : 7.4

REAGENT : Kovac's Reagent

Procedure :

- Inoculate tryptone broth tubes with the bacterial culture and incubate for 24 to 48 hrs at 37⁰C.
- After incubation add 10 drops of Kovac's reagent directly to the culture tube and shake. Allow standing for few minutes and observed the test tubes, recording the result.

OBSERVATION:

Appearance of red color ring near the surface indicates Positive test and yellow color ring indicates Negative test .

RESULT:

Red color ring was not observed
 Hence the test is NEGATIVE

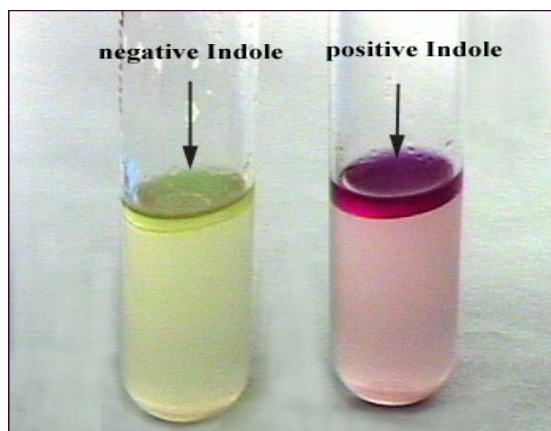


Figure 2 : Indole Production Test . Red Color Ring Was Not Observed Hence The Test Is Negative

METHYL RED TEST :

Medium: MRVP broth

Composition:

Peptone	:	0.7 gram
Glucose	:	0.5 grams
KH ₂ PO ₄	:	0.5 grams
Distilled water	:	100 ml
PH	:	6.9
Reagent	:	Methyl Red

Procedure :

- Inoculate tubes of Methyl red-Voges Proskauer broth with the test culture.
- Incubate the inoculate test tubes at 37⁰C for 24-48 hrs.
- After incubation, aseptically add a few drops of methyl red solution to the culture.
- Shake the vigorously for 30 seconds. Observed the test tubes and record the results.

OBSERVATION:

Appearance Of Red Color Indicates Positive Test And Yellow Color Indicates Negative Test .

RESULT:

Red color is observed. Hence the test is POSITIVE.

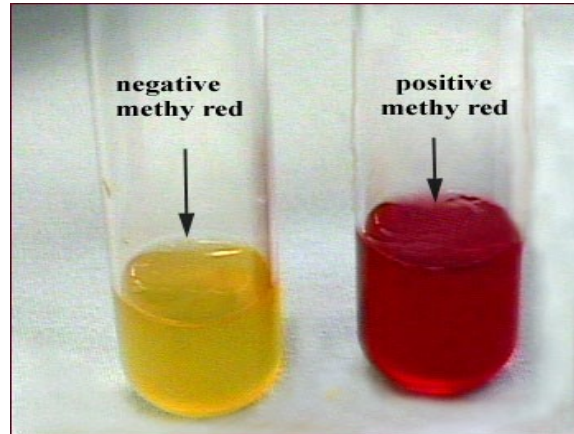


Figure 3 : Methyl Red Test. Red Color Is Observed. Hence The Test Is Positive

VOGES-PROSKAUER TEST:

Medium	: MRVP broth
Peptone	: 0.7 gram
Glucose	: 0.5 grams
KH ₂ PO ₄	: 0.5 grams
Distilled water	: 100 ml
PH	: 6.9

REAGENT:

VP I reagent (5% alpha-naphthol dissolved in absolute alcohol)

VP II reagent (40% KOH)

Procedure :

- Inoculate tubes of Methyl Red-Voges proskauer broth with each of the test culture and label the tubes.
- Incubate the inoculated test tubes at 37⁰C for 24-48 hours.
- After incubation, aseptically prepare 1 ml of culture to a clean test tube. To the 1 ml of culture add 0.5 ml of Barrett's reagent A & 0.5 ml of Barrett's reagent B. Shake vigorously 30 seconds.
- Observe the test tubes and record the results

OBSERVATION:

Color change from pink to crimson indicates Positive test and colorless indicates Negative test .

RESULT :

No Color Was Observed. Hence The Test Is NEGATIVE.

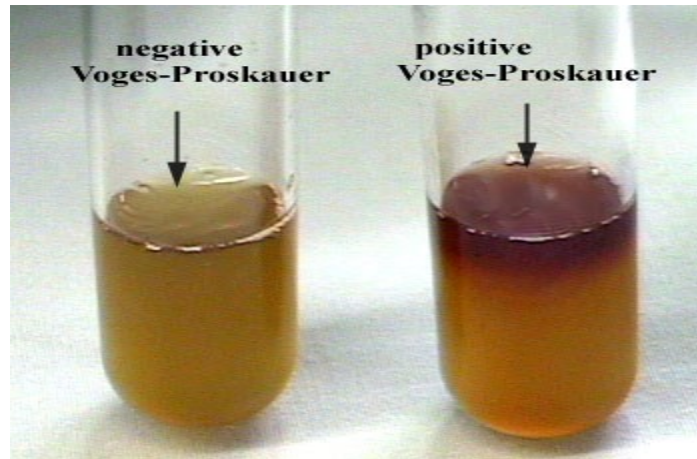


Figure 4: voges-proskauer test. no color was observed. Hence the test is negative.

CITRATE UTILIZATION TEST:

Medium : Simon's Citrate Agar

- Agar : 0.5 grams
- Nacl : 0.5 grams
- Ammonium-di hydrogen phosphate : 0.1 gram
- Di potassium hydrogen-phosphate : 0.1 gram
- Sodium Citrate : 0.2 grams
- Bromothymol blue : 0.08 grams
- Distilled Water : 50 ml
- pH : 6.9

PROCEDURE:

- Inoculate agar slant of Simmon's citrate medium with the test microorganism.
- Incubate the tubes at 37⁰C for 24 hours.
- After incubation observe the color of the test tubes and record the results.

OBSERVATION:

Change of color to blue indicates Positive test and no color change indicates Negative test.

RESULT:

The color did not change from green to blue. hence the test is NEGATIVE.

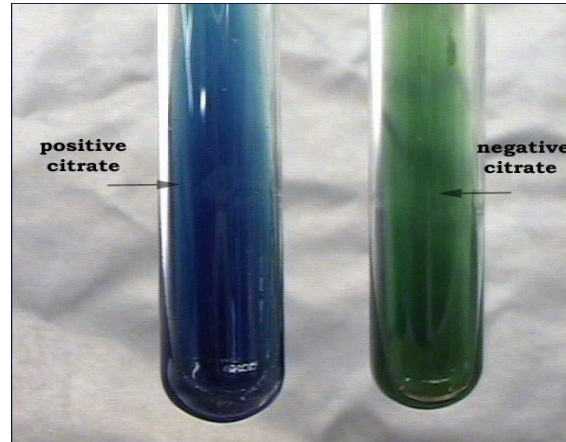


Figure 5: citrate utilization test. the color did not change from green to blue. hence the test is negative.

HYDROGEN SULFIDE (H₂S) PRODUCTION TEST:

Medium : Triple sugar iron agar (TSI Agar)
Beef Extract : 0.3 grams
East Extract : 0.3 grams
Peptone : 0.2 grams
Nacl : 0.5 grams
Glucose : 0.1 gram
Lactose : 1.0 gram
Sucrose : 1.0 gram

Na₂S₂O₃ : 2.5 gram
Ferrous ammonium-sulfate : 0.2 gram
Phenol red : 2.5 grams
Distilled Water : 100 ml
pH : 7.4

PROCEDURE:

- Agar butt with the inoculating needle to inoculate the subsurface regions of the agar.
- Incubate the test tube at 37⁰C for 24-48 hrs.
- After incubation observed with the inoculating needle, aseptically transfer culture to a tube of triple sugar iron agar medium. Stab the test tube and record the results.

OBSERVATION:

Appearance of black color indicates Positive test and colorless indicates Negative test.

RESULT:

Black color was NOT observed. Hence the test is NEGATIVE.

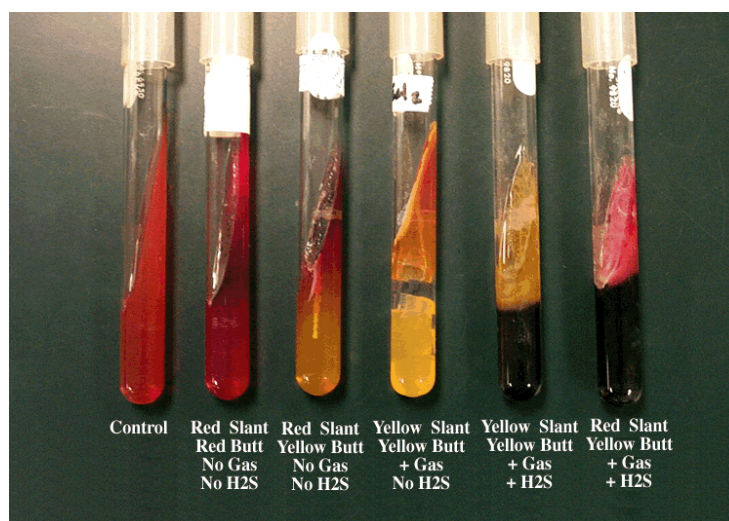


FIG 6 : HYDROGEN SULFIDE (H₂S) PRODUCTION. TEST BLACK COLOR WAS NOT OBSERVED. HENCE THE TEST IS NEGATIVE

CARBOHYDRATE FERMENTATION:

Medium :

Sugar : 0.25g
Yeast extract : 0.025g
Mgso₄ : 0.01g
K₂HPO₄ : 0.05g
NaCl : 0.25g
(NH₄) H₂PO₄ : 0.05g

Reagent : Phenol Red/Methyl Red

PROCEDURE:

- Take medium into test tubes.
- Invert Durham tubes into each test tube.
- Inoculate each test tube with culture.
- Incubate at 37°C for 24 hours.
- Examine for the result.

OBSERVATION:

Appearance of air bubbles and yellow color indicates both acid and gas production (Positive test). And absence of air bubbles and appearance of purple color indicates no fermentation and no gas production (Negative test).

RESULT:

Air bubbles were observed which indicates gas production and yellow color was observed which indicates acid production. Hence the test is POSITIVE

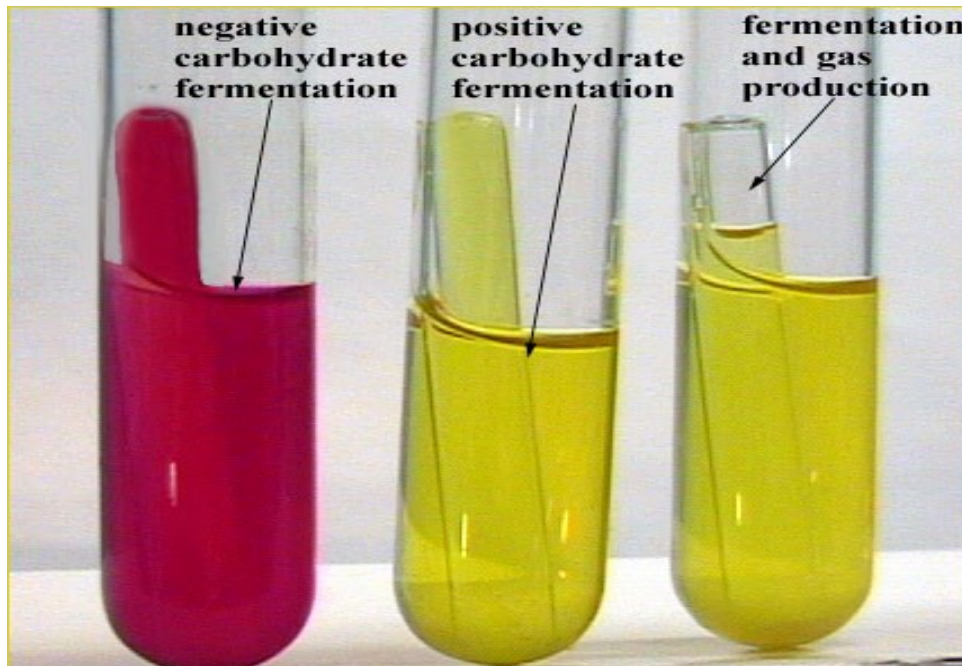


FIG 7: CARBOHYDRATE FERMENTATION. BUBBLES WERE OBSERVED WHICH INDICATES GAS PRODUCTION AND YELLOW COLOR THE TEST IS POSITIVE

NITRATE REDUCTION TEST:

- Medium : Nitrate broth
- Beef extract : 0.15 gm
 - Peptone : 0.25 gm
 - Potassium Nitrate : 0.05 gm
 - Distilled Water : 50 ml

Reagents:

Solution A : Sulfanilic acid

Solution B : beta-alpha-naphthalamine

Procedure :

- Inoculate the Nitrate broth with test organism from pure culture and incubate at 37°C for 24 hours.
- After incubation add 2,3 drops of reagent A & B to each test tubes.
- Observed the test tubes and record the result.

OBSERVATION:

Presence of Red Color indicates Positive test and absence of color indicates Negative test.

RESULT: Red color was observed. Hence the test is POSITIVE.

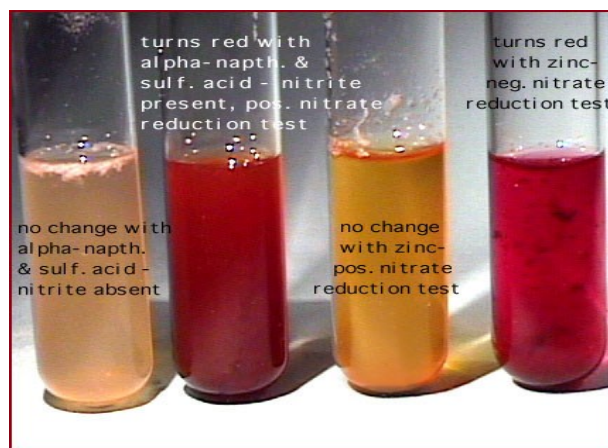


Figure 8: nitrate reduction test. Red color was observed. hence the test is positive

CATALASE TEST:

Medium : Trypticase soy agar

- Trypticase : 1.5gm
- Phytane : 0.5gm
- Nacl : 0.5 gm
- Agar : 2,0 gm
- Distilled Water : 100 ml

Reagent : 3% Hydrogen peroxide

Procedure :

- A bacterial colony was picked up with a toothpick or a platinum loop and mixed
- with a drop of hydrogen peroxide (10% v/v in water) taken on a glass slide.
- The effervescence indicates presence of the catalase enzyme..
- After incubation observe tube and record the result.

OBSERVATION:

Presence of air bubbles indicates Positive test and absence of air bubbles Indicates Negative test

RESULT: Air bubbles were observed with in 20 seconds. Hence test is POSITIVE

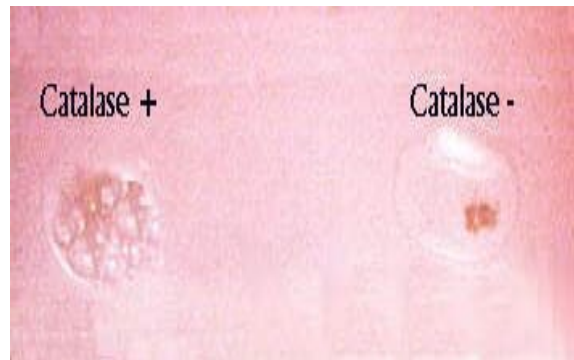


Figure 9: catalase test. air bubbles were observed with in 20 seconds. hence test is positiv

UREASE TEST:

Medium : Urea broth

Composition :

- Urea : 1.0 gm
- Distilled Water : 100 ml

Reagent : Phenol Red

PROCEDURE:

- Inoculate the surface of the Urea broth with test organism.
- Incubated at 37 C for 48 hours.
- After incubation observe tube and record the result.

OBSERVATION:

Appearance of pink color indicates Positive test and pale yellow color indicates Negative test.

RESULT: Pink color was not observed. Hence test is NEGATIVE.

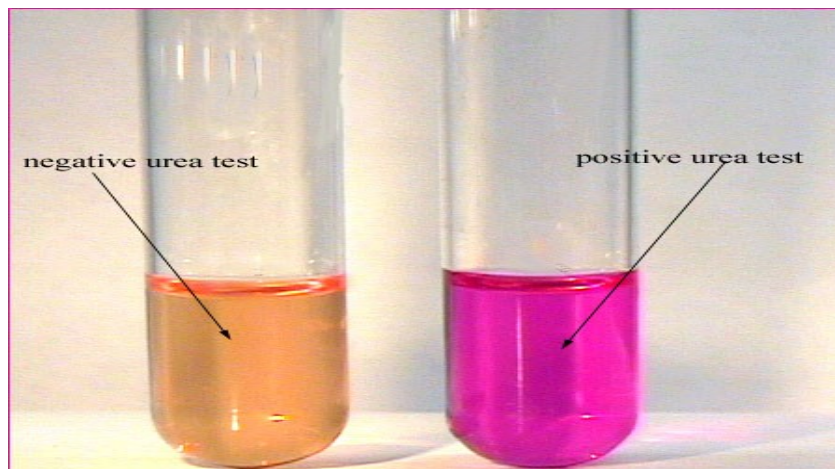


Figure 10: catalase test . pink color was not observed. hence test is negative

S.NO	TEST	RESULT
1	INDOLE PRODUCTION TEST	NEGATIVE
2	METHYL RED TEST	POSITIVE
3	VOGES-PROSKAUE	NEGATIVE
4	CITRATE UTILIZATION TEST	NEGATIVE
5	H ₂ S PRODUCTION	NEGATIVE
6	CHO FERMENTATION	POSITIVE
7	NITRATE REDUCTION	POSITIVE
8	CATALASE TEST	POSITIVE
9	UREASE TEST	POSITIVE

TABEL 2 : SUMMARY OF THE RESULTS ON BIOCHEMICAL TESTS

RESULT

THE ORGANISM ISOLATED FROM BURN WOUNDS WAS IDENTIFIED AS STAPHYLOCOCCU AUREUS FROM MORPHOLOGICAL AND BIOCHEMICAL TESTS ACCORDING TO BERGEY'S MANUAL

DETECTION OF METHICILLIN RESISTANCE

OXACILLIN AND CEFOXITIN DISC DIFFUSION METHOD:

The test organism was subcultured into nutrient broth & incubated for 2-4hrs at 37°C. The turbidity was matched with 0.5 Mcfarland .

A sterile cotton swab was dipped into the inoculum, it was rotated firmly against the inner wall of the test tube to remove excess fluid.

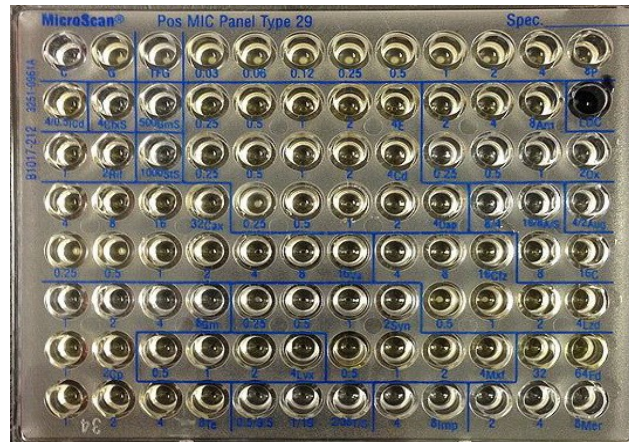
The test strain was swabbed over Mueller Hinton agar (MHA) with 4 % NaCl for oxacillin and MHA without NaCl for cefoxitin. After 3-5 mins, 1 µg oxacillin and 30µg cefoxitin discs were applied on the corresponding plates. The plates were incubated at 35°C for 18-24 hrs. Zone diameters were measured with a scale. Interpretation was done as per CLSI guidelines.

OXACILLIN DISC DIFFUSION TEST:

Staphylococcus aureus was considered as sensitive if zone diameter was ≥ 13 mm. Diameter of 11-12 mm was considered intermediate.

The strain was considered as resistant if the zone diameter was ≤ 10 mm.

MRSA NCTC 12493 was used as control. The Clinical and Laboratory Standards Institute (CLSI) set the oxacillin susceptibility breakpoint for *S aureus* at a minimum inhibitory concentration (MIC) ≤ 2 µg/mL and resistance at an MIC ≥ 4 µg/mL using the broth microdilution method, as was done for the above result. Although methicillin is no longer commercially available and was replaced by oxacillin in the 1990s to test for MRSA, the term "MRSA" has remained in use. The term "ORSA" (oxacillin-resistant *S aureus*) can be used interchangeably with MRSA. Resistance to oxacillin implies resistance to beta-lactam antibiotics, including penicillins, carbapenems, and cephalosporins (excluding ceftaroline, which is a new cephalosporin drug with activity against MRSA).



(B). MRSA GROWTH IS UNINHIBITED BY CEFOXITIN. THE ZONE OF GROWTH INHIBITION (≥ 22 MM) AROUND THE CEFOXITIN DISK INDICATES THAT THE ISOLATE IS MSSA.

GENOTYPIC IDENTIFICATION AND CONFORMATION OF MRSA

Our **GenoType MRSA** test system permits molecular genetic identification of *S. aureus* and *S. epidermidis* from cultures. Differentiation of the clinically most significant pathogens causing staphylococcal infections is thus assured. Since *S. aureus* exhibits particularly high pathogenicity, its reliable detection is crucial for the introduction of targeted treatment and hygiene measures. In addition, the *mecA* and *mecC* genes that impart methicillin resistance as well as a specific fragment of the PVL gene are simultaneously detected with the **GenoType MRSA**. The presence of PVL gives an indication of CA-MRSA strains acquired in the environment. In contrast to nosocomial MRSA, CA-MRSA have a higher pathogenicity, but do not demonstrate any multiresistance to antibiotics. Distinguishing CA-MRSA from nosocomial MRSA therefore makes sense from a therapeutic and epidemiological viewpoint.

PROCEDURE:

PLASMID ISOLATION:

L.B (Luria Bertani) medium was inoculated with one test colony and incubated for 16 hours before carrying out the plasmid isolation. PCR was run to amplify the *mec A* gene followed by gel electrophoresis to confirm the presence of *mec A* gene

MOLECULAR IDENTIFICATION OF MRSA STRAIN

Acquisition of SCC*mec* in methicillin-sensitive staphylococcus aureus (*MSSA*) gives rise to a number of genetically different MRSA lineages. These genetic variations within

different MRSA strains possibly explain the variability in virulence and associated MRSA infections. The first MRSA strain, ST250 MRSA-1 originated from SCC*mec* and ST250-MSSA integration. Historically, major MRSA clones: ST2470-MRSA-I, ST239-MRSA-III, ST5-MRSA-II, and ST5-MRSA-IV were responsible for causing hospital-acquired MRSA (HA-MRSA) infections. ST239-MRSA-III.

DETECTION OF *MECA* GENE BY PCR

In order to identify methicillin-resistant staphylococci from clinical sources with ease and reliability, enzymatic detection of polymerase chain reaction (ED-PCR) was applied. ED-PCR is based on the capture of amplified products via biotin-streptavidin affinity and the detection of an incorporated hapten in amplified products with an enzyme-linked antibody. In order to identify methicillin-resistant staphylococci of all species, a 150-bp fragment of the *mecA* gene was targeted for ED-PCR. After PCR was performed with a pair of biotin and dinitrophenol 5'-labeled primers, the reaction mixture was applied to a microtiter well precoated with streptavidin. Thereafter, bound PCR products were detected colorimetrically with alkaline phosphatase-conjugated anti-dinitrophenol antibody. The extraction of DNA from staphylococcal cells for PCR was simplified so that it could be performed within one tube. The total assay, including PCR, took less than 3 h.

The sensitivity of *mecA* gene detection ranged from greater than 5×10^2 CFU per tube for

Staphylococcus aureus to greater than 5×10^3 CFU per tube for Staphylococcus epidermidis. Genotyping results obtained by ED-PCR of 161 tested strains from the colonies (97 strains of S. aureus and 64 strains of coagulase-negative staphylococci) were compared with the phenotypic susceptibilities of the strains to oxacillin. The results of ED-PCR showed excellent agreement with the MICs of oxacillin with very few exceptions; only one strain of S. aureus and two strains of coagulase-negative staphylococci were found to possess the mecA gene, which was discrepant with their phenotypes. Fifty-five blood culture samples were also tested by ED-PCR. For staphylococcal isolates in 33 of the cultures, oxacillin MICs were >4 microgram/ml; 31 of the 33 staphylococcal isolates were determined by ED-PCR to be mecA gene positive. These results suggest that ED-PCR can be used with reasonable confidence in the clinical microbiological laboratory

GENOMIC DNA ISOLATION PROCEDURE

Harju-Buffer preparation

MATERIAL:

- 2% Triton X-100
- 1% SDS,
- 100 mM NaCl

POLYMERASE CHAIN REACTION:

MATERIAL:

- Water: 9.8 μ l
- Buffer: 2.0 μ l
- $MgCl_2$: 1.2 μ l
- dNTPs: 0.5 μ l
- Primer (F): 1.0 μ l
- Primer (R): 1.0 μ l
- Taq polymerase: 0.5 μ l
- DNA: 4.0 μ l

- 10 mM Tris-HCl, pH 8.0,
- 1 mM EDTA

PROCEDURE:

1. 1.5 ml of liquid culture grown for 20 - 24 h at 30°C is transferred into a micro centrifuge tube. Pellet cells by centrifugation at 10,000 rpm for 5 minutes.
2. 200 μ l of Harju- buffer is added .
3. the tubes are placed in a ice- box for 2 minutes,
4. Tubes are Transferred to a 95°C water bath for 1 minute.
5. last two steps are repeated
6. Vortex for 30 seconds done .
7. 200 μ l of chloroform is added and 2 minutes vortex is done .
8. Centrifugation done for 5 minutes at room temperature, 10,000 rpm.
9. Transferring of the upper aqueous phase to a micro centrifuge tube containing 400 μ l ice-cold 100% ethanol done . Mixing by inversion or gentle vortexing done.
10. Incubation at room temperature, 5 minutes. Alternatively, precipitation of DNA at -20°C to increase yield.
11. Centrifugation for 5 minutes at room temperature, 10,000 rpm.
12. Removal of the supernatant and Resuspension of pellet in 25- 50 μ l TE (pH 8.0)] or water was done.

- Final volume should be 20 μ l

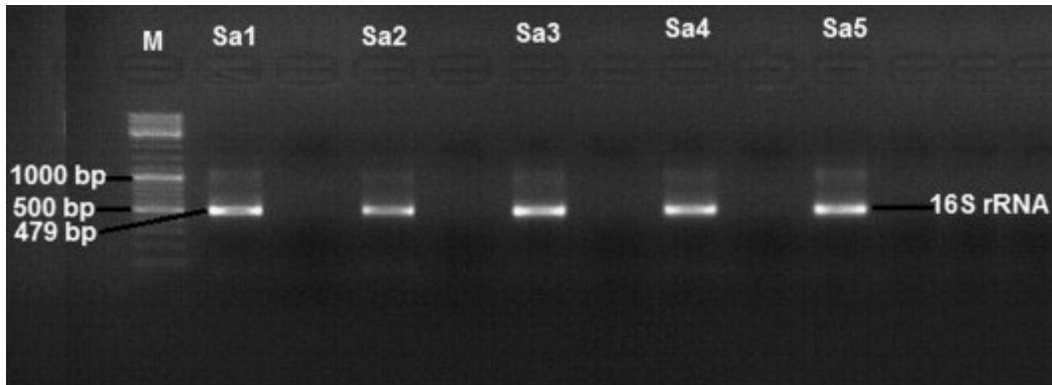


FIG 13 :GEL ELECTROPHORESIS SHOWS THE 16S RRNA FRAGMENTS OF *S. AUREUS*,
M: DNA MARKER, SA *S. AUREUS*.

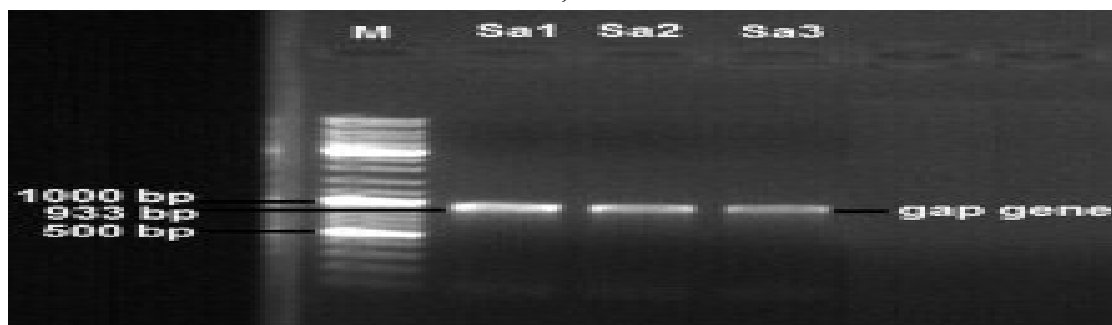


FIG 14. GEL ELECTROPHORESIS SHOWS THE GAP GENE FRAGMENTS OF *S. AUREUS*,
M: DNA MARKER, SA *S. AUREUS*.

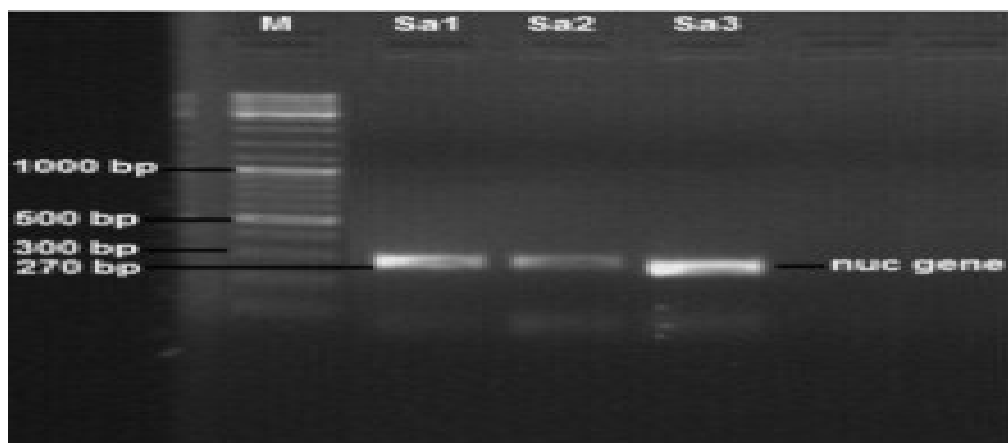


FIG 1 5. GEL ELECTROPHORESIS SHOWS THE NUC GENE FRAGMENTS OF *S. AUREUS*, M: DNA MARKER, SA *S. AUREUS*.

BIOINFORMATICS (INSILICO) STUDIES

METHODOLOGY

PRIMER DESIGN:

For designing the primer, DNA template sequence is required that can be taken from any of the available sequence databases, e.g., RefSeq database. The in silico validation can be carried out using BLAST tool and Gene Runner software, which check their efficiency and specificity. Thereafter, the primers designed in silico can be validated in the wet lab. After that, these validated primers can be synthesized for use in the amplification of concerned gene/DNA fragment. Two of the primers used for the detection of the *mecA* gene (named P2 and P3) have already been published and one more sense primer was designed by us (P1). The primer sequences were:

P15(911)GGTCCCATTA ACTCTGAAG(929)-3'; P2, 5'-

(130)ATCCTCCTTATATAAGACTAC(150)-3'; antisense SA19, 5'-
 (277)CATATCGTGAGCAATGAACTG(257)-3'

(1427)ATCGATGGTAAAGGTTGGC(1445)-3'; and P3, 5'-
 (1956)AGTTCTGCAGTACCGGATTTGC(1935)-3'

(Genset, Paris, France), according to the sequence numbers as described by Matsuhashi *et al.* The two sense primers (P1, P2) and one antisense (P3) have been applied in two reactions, and gave rise to PCR products of 1046 and of 530 bp, respectively. For the detection of the regulatory genes *mecR1-mecI*, five pairs of eight primers were used in combination, as follows: for the detection of the 5' end of the *mecR1* gene designated as membrane spanning (MS), we used the primers: sense SA18,

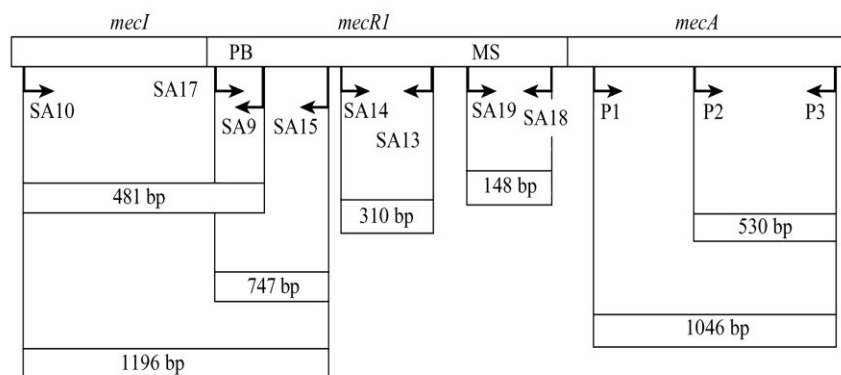


FIG 16 : SCHEMATIC REPRESENTATION OF THE GENOMIC ORGANIZATION OF THE *MEC* REGION. THE ARROWS INDICATE THE POSITIONS OF THE PRIMERS USED. THE SIZES OF THE PCR PRODUCTS ARE INCLUDED. PB, PENICILLIN-BINDING DOMAIN; MS, MEMBRANE SPANNING PART OF *MECRI*

RETRIEVAL OF TARGET SEQUENCE:

The protein sequence of the protein PBP2a had been retrieved from UniProt, and saved in FASTA format that gives the specific information regarding the number of amino acids in the sequence and other sequence related information

FASTA FORMAT OF MECA GENE STRUCTURE

>gij88193823:907598-908317 Staphylococcus aureus subsp. aureus NCTC 8325 chromosome, complete genome

```
ATGAGAATAGAACGAGTAGATGATACAACGTGAAAATTGTTTATAACATATAGCGATATCG
AGGCCCGTG
GATTTAGTCGTGAAGATTTATGGACAAATCGCAAACGTGGCGAAGAATTCTTTTGGTCAATG
ATGGATGA
AATTAACGAAGAAGAAGATTTTGTGTGTAAGGTCCATTATGGATTCAAGTACATGCCTTTG
AAAAAGGT
GTCGAAGTCACAATTTCTAAATCTAAAATGAAGATATGATGAATATGTCTGATGATGATGC
AACTGATC
AATTTGATGAACAAGTTCAAGAATTGTTAGCTCAAACATTAGAAGGTGAAGATCAATTAGA
AGAATTATT
CGAGCAACGAACAAAAGAAAAGAAGCTCAAGGTTCTAAACGTCAAAGTCTTCAGCACGT
AAAAATACA
AGAACAATCATTGTGAAATTTAACGATTTAGAAGATGTTATTAATTATGCATATCATAGCAA
TCCAATAA
CTACAGAGTTTGAAGATTTGTTATATATGGTTGATGGTACTTATTATTATGCTGTATATTTTG
ATAGTCA
TGTTGATCAAGAAGTCATTAATGATAGTTACAGTCAATTGCTTGAATTTGCTTATCCAACAG
ACAGAACA
GAAGTTTATTTAAATGACTATGCTAAAATAATTATGAGTCATAACGTAACAGCTCAAGTTTCG
ACGTTATT
```

PERFORMING TEMPLATE SEARCH

The protein sequence of the protein PBP2a had been retrieved from UniProt and the search for the template had been done using *BLAST* algorithm. Homology Modeling

Homology modeling, also known as comparative modeling of protein refers to constructing an atomic-resolution model of the "target" protein from its amino acid sequence and an experimental three-dimensional structure of a related homologous protein. Homology modeling relies on the identification of one or more known protein structures likely to resemble the structure of the query sequence, and on the production of an alignment that maps residues in the query sequence to residues in the template sequence.

The sequence alignment and template structure are then used to produce a structural model of the target. Because protein structures are more conserved than DNA sequences, detectable levels of sequence similarity usually imply significant structural similarity. The quality of the homology model is dependent on the quality of the sequence alignment and template structure. The approach can be complicated by the presence of alignment gaps (commonly called indels) that indicate a structural region present in the target but not in the template, and by structure gaps in the template that arise from poor resolution in the experimental procedure (usually X-ray crystallography) used to solve the structure. Homology modeling can produce high-quality structural models when the target and template are closely related, which has inspired the formation of a structural genomics.

MODEL ASSESSMENT:-

Assessment of homology models without reference to the true target structure is usually performed with two methods: statistical potentials or physics-based energy calculations. Both methods produce an estimate of the energy (or an energy-like analog) for the model or models being assessed; independent criteria are needed to determine acceptable cutoffs. Neither of the two methods correlates exceptionally well with true structural accuracy, especially on protein types underrepresented in the PDB, such as membrane proteins. Physics-based energy calculations aim to capture the interatomic interactions that are physically responsible for protein stability in solution, especially van der Waals and electrostatic interactions. These calculations are performed using a molecular mechanics force field; proteins are normally too large even for semi-empirical quantum mechanics-based calculations.

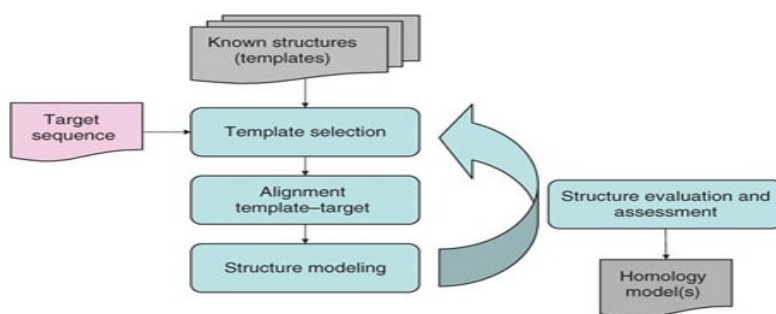


FIG 17 : FLOW CHART OF HOMOMOLOGY MODELING

VALIDATION OF MODELED PROTEIN USING RAPPER:

As the loop of the structure is built up and the terminals are removed, the modeled structure is now given for validation through RAPPER. The structure is given in .pdb form to it to analyze the structure and to generate the RAMACHANDRAN plot of the structure to find the validity of the structure. This is again also carried out after minimization of the structure is done

POCKET DETERMINATION USING CASTP:

Now that we have the final modeled structure the active pockets of the protein are found by uploading the .pdb file of modeled protein to castP. The residues in the pockets are noted.

MOLECULAR MODELLING:

Molecular modelling is a collective term that refers to theoretical methods and computational techniques to model or mimic the behaviour of molecules. The techniques are used in the fields of computational chemistry, computational biology and materials science for studying molecular systems ranging from small chemical systems to large biological molecules and material assemblies. The simplest calculations can be performed by hand, but inevitably computers are required to perform molecular modelling of any reasonably sized system. The common feature of molecular modelling techniques is the atomistic level description of the molecular systems; the lowest level of information is individual atoms (or a small group of atoms). This is in contrast to quantum chemistry (also known as electronic structure calculations) where electrons are considered explicitly. The benefit of molecular modelling is that it reduces the complexity of the system, allowing many more particles (atoms) to be considered during simulations.

LIGANDS FOR DOCKING STUDIES:

Antibiotics shorten the course of the disease and reduce the severity of the symptoms; however, oral rehydration therapy remains the

principal treatment. Tetracycline is typically used as the primary antibiotic, although some strains of *V. cholerae* have shown resistance. Other antibiotics that have been proven effective against *V. cholerae* include cotrimoxazole, erythromycin, doxycycline, chloramphenicol, and furazolidone, Fluoroquinolones such as norfloxacin also may be used. Ligands are extracted from various sources such as PUBCHEM, DRUGBANK,

DESIGNING OF METHICILLIN:

Methicillin Antibiotic structure was designed for docking studies. The structure of this antibiotic was constructed and optimized using “Chemsketch” software.

ACTIVE SITE IDENTIFICATION:

Active site of PBP2a was identified using CASTp server. A new program, CASTp, for automatically locating and measuring protein pockets and cavities, is based on precise computational geometry methods, including alpha shape and discrete flow theory. CASTp identifies and measures pockets and pocket mouth openings, as well as cavities. The program specifies the atoms lining pockets, pocket openings, and buried cavities; the volume and area of pockets and cavities; and the area and circumference of mouth openings.

DOCKING METHOD:

Molecular docking was performed using the Gold version 3.0.1 (Genetic Optimization of Ligand Docking) software which is based on genetic algorithm (GA), to study the binding orientation of compounds into

the PBP2a structure. This method allows partial flexibility of protein and full flexibility of compounds. The designed Methicillin was docked to the active site of the PBP2a. The binding site identification of PBP2a structure was carried out using CastP server. CastP identifies and measures pockets and pocket mouth openings, as well as cavities. The program specifies the atoms lining pockets, pocket openings, and buried cavities; the volume and area of pockets and cavities; and the area and circumference of mouth openings. The interaction of methicillin with the active site residues are studied using molecular mechanics calculations. The parameters used for GA were population size (100), selection pressure (1.1), number of operations (10,000), number of island (1) and niche size (2). Operator parameters for crossover, mutation and migration were set to 100, 100 and 10 respectively. Default cutoff values of 3.0 Å° (dH-X) for hydrogen bonds and 6.0 Å° for vanderwaals were employed. During docking, the default algorithm speed was selected and the binding site in the PBP2a was defined within a 10 Å° radius with the centroid

as CE atom of GLN207. The number of poses for each inhibitor was set 100, and early termination was allowed if the top three bound conformations of a compound were within 1.5Å° RMSD. After docking, the individual binding poses of each compound were observed and their interactions with the protein were studied. The best and most energetically favorable conformation of each compound was selected.

RESULTS AND DISCUSSION

PRIMER DESIGN:

The primers for mec A gene were identified using Primer3 software. The staphylococcus aureus genome was submitted to primer3 and the forward and reverse primers which amplify the mecA gene were predicted. The primers were 20 nucleotide in length with high GC content. These primers were synthesized and used to amplify the mecA gene of MRSA.

OLIGO	start	len	tm	gc%	any	3' seq
LEFT PRIMER	4591	20	60.02	45.00	4.00	2.00 AAGAAGCGTTGTCGCTGAAT
RIGHT PRIMER	4820	20	60.04	50.00	7.00	2.00 GGACAAAGCCGTTGTACGTT

HOMOLOGY MODELLING OF HISTAMINE (PBP2a):

All molecular simulations were performed on AMD 64 bits dual processing hi-end Linux desktop. The amino acid sequence of PBP2a from MRSA was obtained from the protein sequence databank in the Swiss-prot or Uniprot_KB at the site www.expasy.org.

Primary sequence of PBP2a Protein in MRSA

```
>tr|Q7DHH4|Q7DHH4_STAAU MecA OS=Staphylococcus aureus GN=mecA PE=4 SV=1
MKKIKIVPLILIVVVVGFYFYASKDKEINNTIDAIEDKNFKQVYKDSYISKSDNGEV
EMTERPIKIYNSLGVKDINIQRKIKKVSKNKRVDAQYKIKTNYGNIDRNVQFNFKED
GMWKLDDWHSVIIPGMQKQDSIHENLKSERKILDRNNVELANTGTAYEIGIVPKNVSK
KDYKAIKELSISEDIKQQMDQNWVQDDTFVPLKTVKMKMDEYLSDFAKKFHLTTNETES
```

RNYPLGKATSHLLGYVGPINSEELKQKEYKGYKDDAVIGKKGLEKLYDKKLQHEDGYRVT
 IVDDNSNTIAHTLIEKKKKDKGKDIQLTIDAKVQKSIYNNMKNDYSGGTAIHPQTGELLAL
 VSTPSYDVYPFMYGMSNEEYNKLTEDKKEPLLKFKQITTSFGSTQKILTAMIGLNNKTLTLD
 DKTSYKIDGKGWQKDKSWGGYNVTRYEVVNGNIDLKQAISSDNIFARVALELGSKKFE
 KGMKCLGVGEDIPSDYPFYNAQISKNKLNLDNEILLADSGYGQGEILINPVQILSIYSALEN
 NGNINAPHLLKDTKNKVVKKNIISKENINLLTDGMQQVVNKTHKEDIYRSYANLIGKSGT
 AELKMKQGETGRQIGWFISYDKDNPNNMMMAINVKDVQDKGMASYNAKISGKVYDELYENG
 NKKYDIDE

PRIMRAY SEQUENCE ANALYSIS OF MRSA PBP2a PROTEIN:

Amino Acid	Number	Mol%
Ala A	13	6.40
Cys C	4	1.97
Asp D	15	7.39
Glu E	13	6.40
Phe F	11	5.42
Gly G	6	2.96
His H	5	2.46
Ile I	10	4.93
Lys K	12	5.91
Leu L	23	11.33
Met M	9	4.43
Asn N	11	5.42
Pro P	4	1.97
Gln Q	8	3.94
Arg R	13	6.40
Ser S	10	4.93
Thr T	10	4.93
Val V	16	7.88
Trp W	4	1.97
Tyr Y	6	2.96

FIG 18 : REPRESENTS THE AMINO ACID COMPOSITION AND MOLECULAR WEIGHT OF THE PBP2a PROTEIN, IN OUR RESULTS SHOWS LEU IS HIGHLY PRESENT COMPARED TO OTHER RESIDUES.

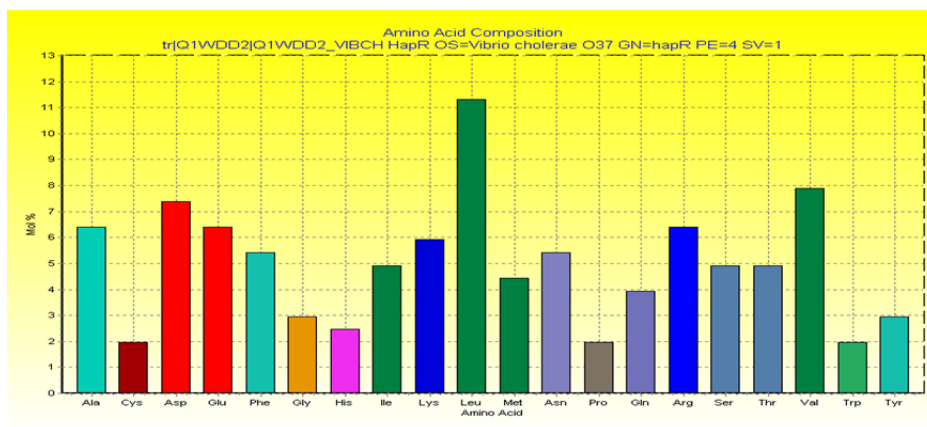


FIG 19 : GRAPHICAL REPRESENTATION OF AMINO ACID COMPOSITION

HYDROPHOBIC PROFILE OF PBP2a PROTEIN SEQUENCE:

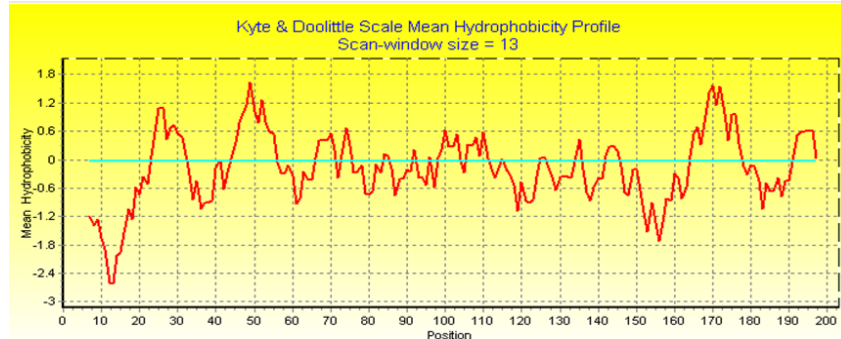


FIG 20 : DIAGRAM SHOWS HYDROPHOBICITY PROFILE OF PBP2A ,WHICH WAS DEVELOPED BY KYTE &DOOLITE PROGRAM ,IT WAS REPRESENTING HYDROPHOBIC REGIONS 22-32,42-58,162-180

PARKER HPLC –HYDOPHILICITY PROFILE:

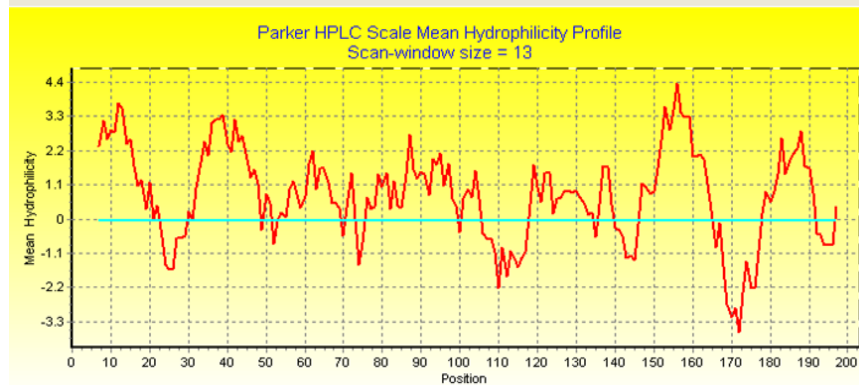


FIG 21 : DIAGRAM SHOWS HYDROPHILICITY PROFILE OF PBP2A ,WHICH WAS DEVELOPED BY PARKER HPLC PROGRAM ,IT WAS REPRESENTING HYDROPHILIC REGIONS 31-50,148-165 ETC

HELICAL-WHEEL DIAGRAM

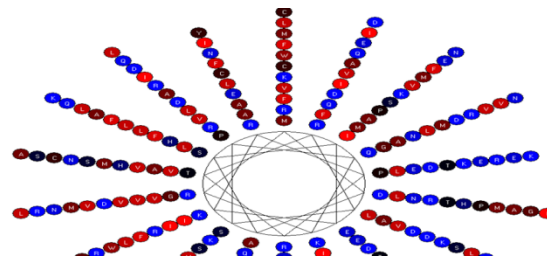


FIG 22 : SHOWS PATTERN OF AMINO ACID REPRESENTATION BY HELICAL WHEEL DIAGRAM ,CENTRAL REGION IS THE HYDROPHOBIC AND SIMILAR COLOR RESIDUES REPRESENT THEY ARE BELONGS TO SAME BRANCH OF AMINO ACIDS
SECONDARY STRUCTURE ANALYSIS OF PBP2a PROTEIN:

SOPMA result for : UNK_98030

[Abstract](#) Geoujon, C. & Deléage, G., SOPMA: Significant improvement in protein secondary structure prediction by consensus prediction from multiple alignments., *Cabios* (1995) 11, 681-684

View SOPMA in [\[AnTheProt \(PC\)\]](#), [\[Download..\]](#) [\[HELP\]](#)

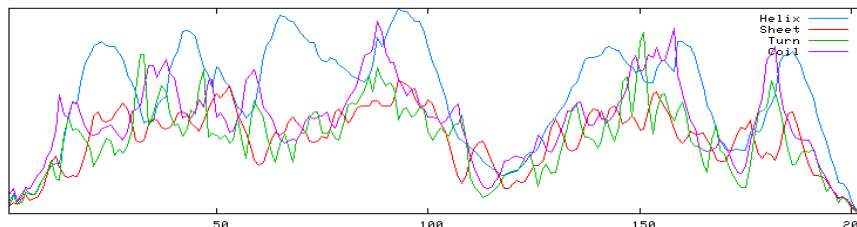
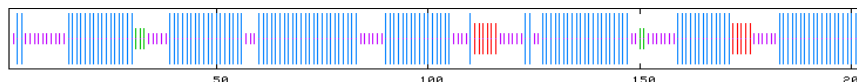
```

      10      20      30      40      50      60      70
MDASIEKRPRTLSFQKRKIQIMEIALEVFVAKRGIKRGDADIAEIAQVSVATVFNFFPTREDLVDDVLN
ccchcccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccc
FVVRQYSNFLTDHLDLQVTKNLQTLCKEMVKLAMDCHWLKRVWFWSASTRDEVWFLVSTNRINQLL
hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh
IRNMFKAMERGLCEKHDVDMNASLFGHGFYSIFLQVNRLEGEAVYKLDASYLNMLCIYKN
hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh
    
```

Sequence length : 203

SOPMA :

Alpha helix (Hh) :	133 is	65.52%
₃ 10 helix (Gg) :	0 is	0.00%
Pi helix (Ii) :	0 is	0.00%
Beta bridge (Bb) :	0 is	0.00%
Extended strand (Ee) :	11 is	5.42%
Beta turn (Tt) :	5 is	2.46%
Bend region (Ss) :	0 is	0.00%
Random coil (Cc) :	54 is	26.60%
Ambiguous states (?) :	0 is	0.00%
Other states :	0 is	0.00%



Parameters :

Window width	: 17
Similarity threshold	: 8
Number of states	: 4

FIG 23 : SHOWS SECONDARY STRUCTURE ANALYSIS OF PBP2A PROTEIN SEQUENCE

TEMPLATE SELECTION:

Is a process of identifying a suitable protein which shares nearly the same structure of the query protein which doesn't possess the 3D structure. Template selection is very important in comparative protein modeling. Templates can be chosen by various tools such as BLAST, FASTA, Swiss-model, etc.

In the case of Blast and Fasta the sequence of protein in fasta format can be uploaded and the templates can be manually selected by considering the score value and the E value. In the case of Swiss-Model server, it automatically chooses the template and models the protein structure.

BLAST SEARCH:

A high level of sequence identity should guarantee more accurate alignment between the target sequence and template structure. In the results of BLAST search against PDB, only one-reference protein 2PBX has a high level of sequence identity and the identity of the reference protein with the Tau-protein domain are 80%.

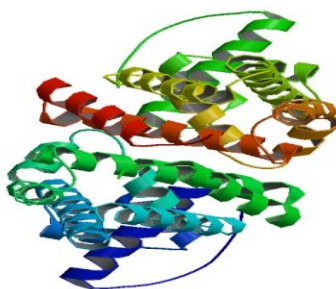


FIG 24 : TEMPLATE STRUCTURE: 2PBX

SEQUENCE ALIGNMENT:

In the following study, we have chosen 2PBX as a reference structure for modeling PBP2a domain. Coordinates from the reference protein (2PBX) to the SCRs, structurally variable regions (SVRs), N-termini and C-termini were assigned to the target sequence based on the satisfaction of spatial restraints. Sequence of the reference structures were extracted from the respective structure files and aligned with the target sequence using the default parameters in ClustalW.

THEOREYICAL MODEL OF PBP2a PROTEIN:

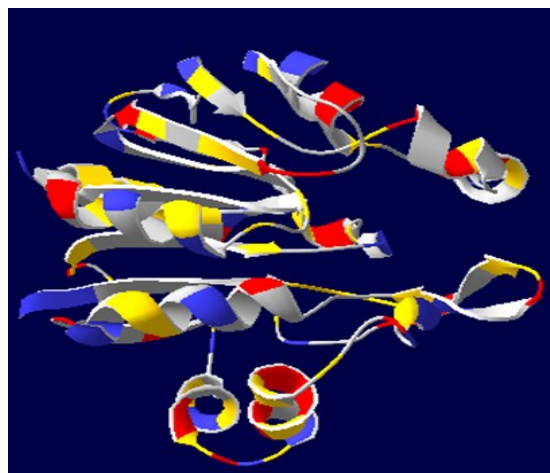


FIG 25 : PBP2A STRUCTURE

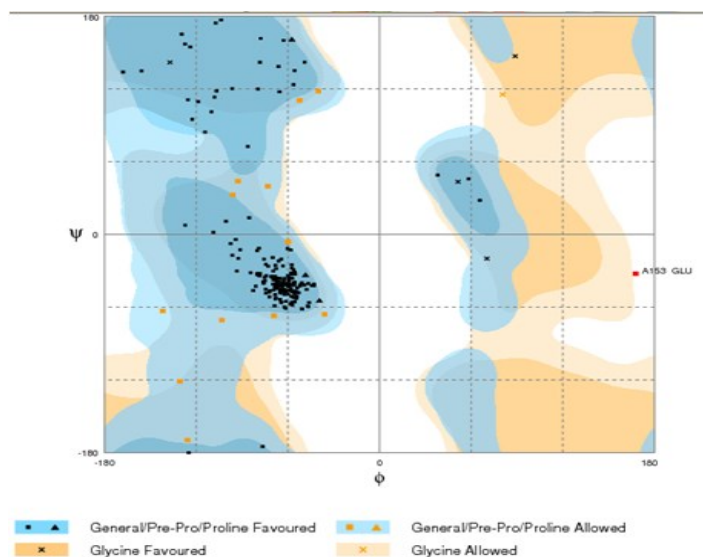


FIG 26 : MODEL VERIFICATION BY RAPPER

Number of residues in favoured region (~98.0% expected) : 182 (92.4%)
 Number of residues in allowed region (~2.0% expected) : 14 (7.1%)
 Number of residues in outlier region : 1 (0.5%)

ACTIVE SITE IDENTIFICATIN:

The selected docked conformations of the PBP2a binding site are shown in Figure 28 .The docked conformations revealed that methicillin located in the hydrophobic binding pocket surrounding

the binuclear copper active site. In this study, docked methicillin was found to have some interaction between an oxygen atom of the methicillin and PBP2A. Moreover, these docked conformations also formed an H-bonding interaction with in the active site. In the binding pocket, common H-bonding interactions were formed between all docked compounds and GLY 135, GLN 137, GLN 140, HIS 143, GLU 145, GLN 145, GLN 207, ASP 209, HIS 232, THR 300, and HIS 311.

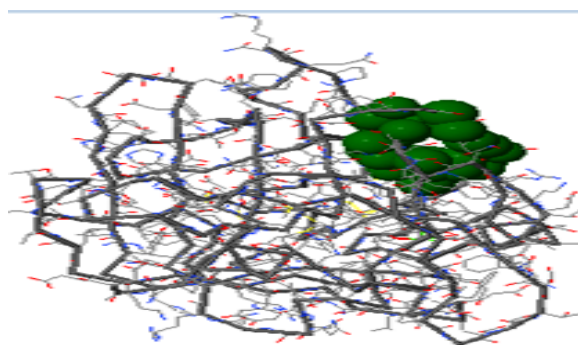


FIG 27 : ACTIVE SITE OF PBP2A

MECHANISM OF BINDING METHICILLIN TO PBP2a:

After constructing the methicillin and optimizing them using “Chemsketch” software, the crystal model and the possible binding sites of PBP2a were searched with CASTP server. From the binding site analysis of PBP2a we identified that, the binding pockets are identical in all chains and the largest binding pocket was taken for further docking studies. The crystal structures of PBP2a are similar and we have therefore taken 1VQQ (chain A) as representative structure for docking studies. The docking of compounds into the active site of PBP2a was performed using the GOLD software and the docking evaluations were made on the basis of GoldScore fitness functions. We preferred Gold fitness score than Chemscore fitness as Gold fitness score is marginally better than Chemscore fitness function. In order to explain the binding of methicillin, the H-bonding interactions with the other surrounding residues in the hydrophobic binding pocket were also investigated. The docking results showed that methicillin has affinity towards PBP2a.

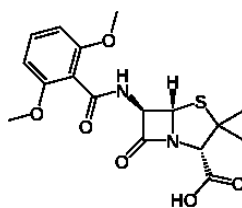


FIG 28 : METHICILLIN STRUCTURE

DOCKING OF THE METHICILLIN:

Docking of the methicillin with PBP2a was performed using GOLD 3.0.1, which is based on genetic algorithm. This program generates an ensemble of different rigid body orientations (poses) for

each compound conformer within the binding pocket and then passes each molecule against a negative image of the binding site. Poses clashing with this ‘bump map’ are eliminated. Poses surviving the bump test are then scored and ranked with a Gaussian shape function. We defined the binding pocket using the ligand-free protein structure and a box enclosing the binding site. This box was defined by extending the size of a cocrystallized ligand by 4Å. This dimension was considered here appropriate to allow, for instance, compounds larger than the cocrystallized ones to fit into the binding site. One unique pose for each of the best-scored compounds was saved for the subsequent steps. The compounds used for docking was converted in 3D with SILVER. To this set, the substrate corresponding to the modeled protein were added. Docking of best inhibitor with the active site of protein showed the activity of the molecule on protein function.

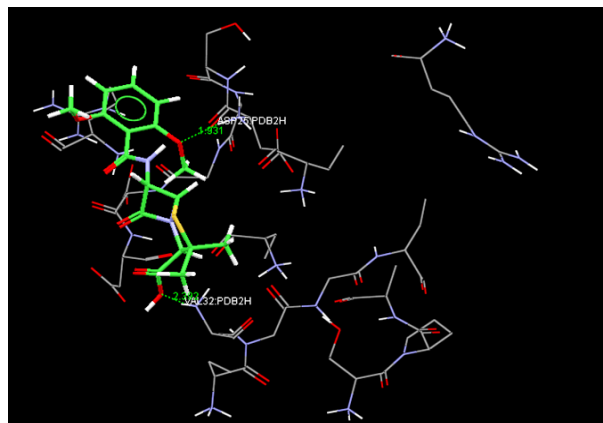


FIG 29 : DOCKING STUDIES OF METHICILLIN WITH PBP2A

Fitness	S(hb_ext)	S(vdw_ext)	S(hb_int)	S(int)	Compound
31.62	0.00	40.06	0.00	-23.45	Methicillin

TABLE 3 : DOCKING METHICILLIN SCORE

BETA-LACTAMASE RESISTANCE OF PBP2a MECHAISM:

The mechanism was identified using KEGG database for PBP2a of MRSA. The PBP2a binds to the Methicillin with its active site and prevents inhibition of cell membrane proteins.

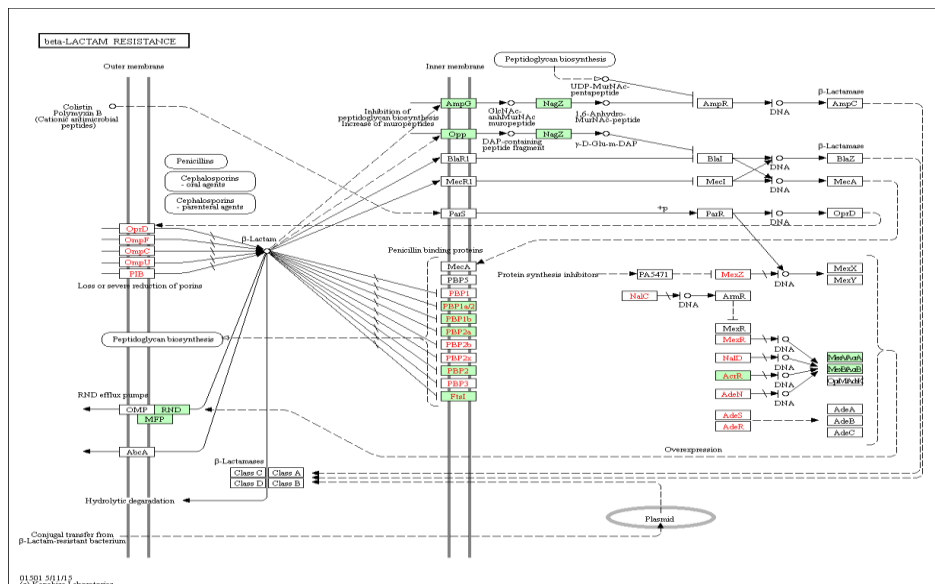


Figure 30: Mechanism Of Pbp2a Binding To Methicillin

CONCLUSIONS

Molecular Diagnostic Techniques are expected to play a significant role in clinical and diagnostic bacteriology. Although their adoption may never replace the conventional methods their efficiency, quality, quickness and their role in the detection of slow growing organisms cannot be overlooked. The docking results showed that Methicillin showed docking fitness and this revealed the binding orientation in the PBP2a binding pocket surrounding the active site, which resulted that PBP2A has a affinity for beta-lactam antibiotics such as methicillin. This enables transpeptidase activity in the presence of beta-lactams, preventing them from inhibiting cell wall synthesis. From the results we can conclude that PBP2a is competitor protein to Methicillin to bind to other proteins.

REFERENCES:

1. A Hartman BJ, Tomasz A. Low-affinity penicillin-binding protein associated with β -lactam resistance in *Staphylococcus aureus*. *J Bacteriol* 1984; 158: 513–6. which confers resistance to all currently available β -lactam agents.
2. Acar JF, Courvalin P, Chabbert YA. Methicillin-resistant staphylococemia: bacteriological failure of treatment with cephalosporins. *Antimicrob Agents Chemother* 1970; 10: 280–5.
3. Andrews JM. BSAC standardized disc susceptibility testing method (version 3). *J Antimicrob Chemother* 2004; 53: 713–28.

4. Annear DI. The effect of temperature on resistance of *Staphylococcus aureus* to methicillin and some other antibiotics. *Med J Aust* 1968; 1: 444–6.
5. Arakawa Y, Ike Y, Nagasawa M. Where has vancomycin-heterogeneously resistant *Staphylococcus aureus* gone? *Lancet* 2004; 363: 1401.
6. Arbique J, Forward K, Haldane D et al. Comparison of the Velogene Rapid MRSA Identification Assay, Denka MRSA-Screen Assay, and BBL Crystal MRSA ID System for rapid identification of methicillin-resistant *Staphylococcus aureus*. *Diagn Microbiol Infect Dis* 2001; 40: 5–10.
7. Archer GL, Pennell E. Detection of methicillin resistance in staphylococci by using a DNA probe. *Antimicrob Agents Chemother* 1990; 34: 1720–4.
8. Archer GL, Pennell E. Detection of methicillin resistance in staphylococci by using a DNA probe. *Antimicrob Agents Chemother* 1990; 34: 1720–4.
9. Aucken HM, Warner M, Ganner M et al. Twenty months of screening for glycopeptide-intermediate *Staphylococcus aureus*. *J*
10. Avison MB, Bennett PM, Howe RA et al. Preliminary analysis of the genetic basis for vancomycin resistance in *Staphylococcus aureus* strain Mu50. *J Antimicrob Chemother* 2002; 49: 255–60.
11. Baba T (2002) Genomic and virulence determinants of high virulence community-acquired MRSA. *Lancet* 359: 1819–1827.
12. Bannerman TL. *Staphylococci, Micrococcus and other catalase-positive cocci that grow aerobically*. In: Murray PR, Baron EJ, Jorgensen JM et al. eds. *Manual of Clinical Microbiology*, 8th edn. Washington: American Society for Microbiology Press, 2003; 384–404.
13. Barber M. Naturally occurring methicillin-resistant staphylococci. *J Gen Microbiol* 1964; 35: 183–90.