MATERIALS & METHODS

3. MATERIALS AND METHODS

The present study was conducted in the Department of Microbiology, Jawaharlal Nehru Medical College, A.M.U., Aligarh and the Department of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar, Bareilly during June 2003 to March 2006. The study aimed for evaluation of phenotypic and genotypic characters of *Staphylococcus aureus* isolates obtained from clinical specimens and from foods of animal origin.

3.1. Collection of Samples:

3.1.1. Clinical Specimens:

A total of 3514 human clinical specimens including Pus, Urine, CSF, Throat swab, Ear swab, Conjuctival swab, Cervical swab, Semen, CSF, Aspirated Fluid, Stool and Vomitus were collected from the patients admitted in various wards or from outdoor patients of Jawaharlal Nehru Medical College and hospital, A.M.U., Aligarh. A total of two hundred thirteen raw milk samples were collected as animal origin clinical specimens from Indian Veterinary Research Institute, Izatnagar, Bareilly. The samples were collected in sterile containers / swabs according to standard techniques (Mackie and McCartney. 2000) for the isolation of *Staphylococcus aureus*.

3.1.2. Food Samples:

A total of 1006 food samples were collected according to the methods described by Agarwal *et al.* (2003). The details of the food samples are provided below:

3.1.2.1. Milk products: About 2 gm each of Khoa (Milk concentrate used for the preparation of sweets), Paneer (cottage cheese), Chamcham and Sweets were collected with aseptic precautions in sterile glass containers.

3.1.2.2. Raw meat: Meat sample used in this study were collected aseptically and quickly transported to laboratory under chilled conditions.

3.1.2.3. Cooked meat Products: About 2 gm each of Kabab (a meat cutlet) and Salami were collected in the sterile polybags aseptically.

3.2. Laboratory procedures:

3.2.1. Direct Microscopic examination: Smears were prepared from all the clinical samples on clean sterile glass slides for Gram's staining. The smears were allowed to dry and then fixed by passing through flame and stained by Gram's technique. The smears were examined under oil immersion to look for Gram positive cocci in clusters.

3.2.2. Processing of food samples:

(a) Processing of milk products: The samples were homogenized at room temperature in 10 ml of NS in sterile beakers. The homogenization was done by electrically operated food homogenizer and then used for bacterial isolation.

(b) Processing of raw meat samples: The surface of the collected meat samples were trimmed off with sterile scissors and forceps. From each sample, a small portion was taken and cut into small pieces and then ground in 10 ml NS by electrically operated food homogenizer.

(c) Processing of cooked meat products: The collected meat products were homogenized in 10 ml NS using a homogenizer and them subjected to culture.

3.2.3. Bacterial culture:

All clinical and food samples, were cultured according to standard procedure appropriate to the type of specimen.

Samples were streaked on 5% Sheep blood agar, nutrient agar and Baird-Parker agar medium and incubated for 24-72 hours at 37^0C. The isolates were stocked in agar stabs and stored at 4^0C for further characterization.

3.3. Phenotypic tests for identification / characterization:

Staphylococci were identified as per standard methods (Mackie and McCartney, 2000).

3.3.1. Colony Morphology: On nutrient agar and sheep blood agar:

The circular, smooth, 1-3 mm, low convex, glistening and opaque colonies, which were easily emulsifiable, butyrous in consistency golden yellow/creamy colour, surrounded by zone of β-hemolysis on blood agar were identified as the colonies of *S. aureus*. On Baird-Parker medium distinct black colored colonies were found.

3.3.2. Microscopic Morphology: Smears were prepared from the colonies for Gram's staining to look for the characteristic morphology of Staphylococci.

3.3.3. Other tests:

The Staphylococcal isolates were further characterized on the basis of:

3.3.3.1. Enzymatic Tests:

3.3.3.1.1. Catalase Test:

Requirement: 3% (V/V) Hydrogen peroxide.

Method: A drop of H_2O_2 was placed on a clean glass slide. Using a clean glass rod, small amount of colony to be tested was picked up from nutrient agar plate and immersed into H_2O_2 drop. Production of gas bubbles immediately indicates positive reaction.

Controls:

Catalase positive control: *S. aureus* oxford strain number 6571.

Catalase negative control: Streptococcus species

3.3.3.1.2. Coagulase Test:

Requirements:

Rabbit plasma

Normal Saline (0.85%) or Nutrient broth

Controls:

Coagulase- positive strain (*S. aureus* oxford strain number 6571).

Coagulase- negative Staphylococci (*S. epidermidis*)

a. Slide coagulase test: (William and Harper, 1946): To detect 'bound coagulase' (Clumping factor):

Method: A drop of physiological saline solution was placed on a clean glass slide and with minimum spreading one or two colonies of culture under test was emulsified in it. A control suspension from a known coagulase-positive and negative culture was also made to confirm the reactivity of the plasma. With inoculating wire, a drop of plasma was added at room temperature and mixed gently. The wire was flamed and the procedure was repeated for control suspensions. The appearance of coarse clumps visible to the naked eye within 10 seconds indicates positive reaction. The absence of clumping or any reaction taking more than 10 seconds to develop was considered as negative.

b. Tube Coagulase Test (Modified from Gillespie 1943): To detect 'free coagulase':

Method:

A 1:10 dilution of rabbit plasma was prepared in saline (0.85% NaCl) solution. 0.5ml of the diluted plasma was placed in a small sterile tube. Few colonies of Staphylococci under test were emulsified in nutrient broth to give a dense suspension. Subsequently 0.1 ml of this suspension (about 10^9 cocci) was added to the diluted plasma tube. Similarly control test with known coagulase–positive and coagulase-negative cultures were set up. A tube of unseeded diluted plasma was also included to confirm that it does not coagulate spontaneously. The tubes were incubated at 37^0C in a water-bath and examined for clot formation by tilting the tube through 90^0 at 1, 2 and

4 hrs. and again if still negative, after standing overnight at room temperature.

Test was read positive when the plasma had been converted into a stiff gel when the tube was tilted or inverted. Test in which the plasma remained wholly liquid or showed only a flocculent or ropy precipitate or free flowing was read as negative.

3.3.3.1.3. DNAse Test (Lachica *et al.*, 1971):

Requirements: DNA agar (Oxoid DNAse Agar) with 0.1% toluidine blue obtained from Hi Media.

Method:

Petriplates were prepared by pouring toluidine blue DNA agar. After solidification, 2 mm diameter wells (10-12 wells per plate) were made and agar plugs were removed by aspiration, 0.01 ml each heated sample (15 min. in boiling water bath) of BHI broth culture was added into wells on prepared plates, and incubated at 35^{0}C. Positive reaction was the formation of a bright pink halo extending at least 1 mm from the periphery of well. The results were noted after 4 hrs. of incubation and observed up to 18 hrs.

3.3.3.1.4. Phosphatase Test:

Requirements: Phenolphthalein diphosphate agar (Hi Media), liquor ammonia solution (SG 0.88).

Method: Test strain was streaked on the phenolphthalein diphosphate agar (PPA) plates and incubated for 18–20 hours at 37^{0}C. After incubation 01–02 ml ammonia solution was placed in the lid of petridish

and culture plate was inverted above it. Bright pink colonies were considered as positive. The test was performed using the method of Agarwal *et al.*, 2003.

3.3.3.1.5. β- Lactamase Production:

All the isolates of *Staphylococcus aureus* were tested for β-lactamase production using iodometric method.

Iodometric Method

Penicillinase hydrolyses penicillin to penicilloic acid, which reduce iodine and reverse the formation of the blue colour when latter complexes with starch. The presence of penicillinase in a test system is shown by decolorization of Starch-iodine complex The test was performed using the method of Sykes (1978).

Requirements:

Starch solution: 1% soluble starch solution was prepared by dissolving starch at 100^0C.

Iodine Reagent:

Iodine	2.03gm
Potassium Iodine	5.32gm
Distilled Water	100ml

Method:

A heavy suspension (about 10^9 colony units / ml) was prepared in wells of a microtitre plate from an overnight culture of test organism in

0.1 ml solution of benzylpencillin (6 mg/ ml in 0.1 mol/liter phosphate buffer, pH 7.3). The control tests with penicillinase negative and positive cultures were also set up. The microtitre plate was incubated for one hour at 37^{0}C, and then two drops of freshly prepared 1% solution of soluble starch was added to each well. In positive test, the blue colour was lost rapidly (within 10 seconds). In negative test blue colour persisted for at least 10 minutes.

3.3.3.2. Biochemical Tests:

All the isolates were subjected to various biochemical tests:

a. Production of acid from Sugars:

Each isolate was tested for production of acid from glucose and mannitol (both aerobically and anaerobically) and from trehalose (aerobically).

Method: Carbohydrate test solution were inoculated by test culture from nutrient agar plate and incubated overnight at 37^{0}C. Reddish pink colour of the medium indicates positive reaction.

b. Hugh and Leifson's O/F test:

Hugh and Leifson's O/F test was performed to see the ability of test strain to produce acid from glucose aerobically and / or anaerobically.

Method: Using a sterile straight wire test organism was stab inoculated throughout the length of sugar tube in duplicate. After inoculation the surface of one tube was covered with a 1-2 inch layer of sterile liquid paraffin. The tubes were then incubated at 37^{0}C for 5 days. Development of yellow colour indicated acid production. Acid production in the open

tube only indicated oxidative utilization of carbohydrate. Acid production both in open (with paraffin) and sealed tube indicated fermentation of sugar (1953).

c. Acetoin Production : Voges-Proskauer test:

Requirements:

Glucose Phosphate Peptone water

40% Potassium Hydroxide (KOH)

5% α-naphthol in absolute alcohol

Method: A large drop of broth culture of the strain under test was inoculated into 2 ml glucose phosphate peptone water tube and incubated at 30^0C for 18-48 hours.

The presence of acetoin production was detected by Barritt's method (1936):

1 ml of 40% Potassium hydroxide and 3 ml of 5% solution of α-naphtol in ethanol was added and shaken vigorously for at least 30 seconds. Positive reaction was indicated by the development of pink colour with in 5-10 minutes, becoming rose red colour in 30 minutes. The test was performed using the method of Mackie and McCartney, 2000.

d. Bacitracin Sensitivity:

Bacitracin sensitivity was done to differentiate between Micrococci and Staphylococci. *S. aureus* were resistant to bacitracin.

Resistance to bacitracin was tested on Mueller-Hinton agar medium (Hi Media) with 0.04 units of bacitracin disc (Hi Media). Resistance to bacitracin was reported when zone of inhibition of growth

was found less than 10 mm.

e. Novobiocin Sensitivity:

Resistance to Novobiocin distinguishes *S. saprophyticus* from other Staphylococci. Sensitivity to novobiocin was tested on Mueller-Hinton agar medium (Hi Media) with paper disc (7mm diameter) containing 5 µg of novobiocin. Sensitivity to novobiocin was reported when zone of inhibition was ≥15 mm in diameter.

3.3.3.3. Antimicrobial drug sensitivity test:

(a) Antibiotic sensitivity test was done by the disc diffusion method as proposed by Kirby Bauer (1961).

Requirements:

(i) Media:

Mueller-Hinton Agar (Hi Media)

Mueller-Hinton agar supplemented with an additional 5% NaCl (for Methicillin sensitivity)

(ii) Antibiotic discs (Hi-Media): Commercially available antibiotic discs obtained from (Hi Media) were used for antibiotic sensitivity testing. The antibiotics and their contents were:

Antibiotics	Abbreviation	Disc potency
Penicillin G	P	10 units
Amikacin	Ak	30 µg
Amoxycillin	Ac	30 µg

Chloramphenicol	C	30 µg
Ciprofloxacin	Cf	5 µg
Co–Trimoxazole	Co	25 µg
Cefoclor	Cj	30 µg
Ceftriaxone	Ci	30 µg
Cefepime	Cpm	30 µg
Ceftazidime	Ca	30 µg
Erythromycin	E	30 µg
Tetracycline	T	30 µg
Gentamycin	G	10 µg
Oxacillin	Ox	1 unit
Vancomycin	Va	30 µg
Teicoplanin	Te	30 µg

All antibiotic discs were stored in refrigerator. On removal from the refrigerator for use, the vials were left at room temperature for about an hour to allow the temperature to equilibrate, thus preventing the amount of condensation that occurs immediately after. Before use each lot of antibiotic disc was tested with standard strain of *S. aureus* 6571.

The results were interpreted according to the standard table provided by the supplier.

(iii) Standard control Strains:

Oxford *S. aureus* 6571

S. aureus (MRSA) in-house control

(iv) Test strains (Bacterial inoculum)

(v) Opacity Standard (0.5 McFarland)

(vi) Sterile Nontoxic cotton swabs.

Inoculum:

Test strains of *Staphylococcus aureus* and standard oxford *S. aureus* 6571 were isolated on nutrient agar. 4-5 identical colonies were picked up from both strains (standard and test strains) and inoculated in 5ml of nutrient broth separately and incubated at 37^0C for 4-6 hours. The density of the suspension was compared with the opacity standard tube i.e. 0.5 McFarland standard.

Method:

Drug sensitivity test for all antibiotics was carried out on Mueller-Hinton agar plate (Hi Media). While, for methicillin resistance oxacillin discs were used on Mueller-Hinton agar supplemented with 5% NaCl and incubated for 18-24 hour at 35°C.

The test strain was applied on the surface of the Mueller-Hinton agar plate using sterile swabs and allowed to dry for 10 minutes at room temperature. The antibiotic discs having the standard strength were lightly pressed on the surface of agar so as to ensure firm even contact of the disc with the seeded agar. Care was also taken to make sure that the placed discs straddle the gap uniformly at both sides and are about 1 cm

away from the rim of the plate. The plates were then incubated for overnight at 37^0C.

(b) Oxacillin agar screening (6µg/ml)

Inoculum: 2-5 colonies from overnight growth on a non-selective agar medium were suspended in broth or saline to a turbidity that matched with 0.5 Mc Farland standard. The suspension was diluted 1: 10 times and was adjusted to 10^7 cfu/ml.

Method: Plates were inoculated within 30 minutes after preparation of the inoculum since longer delay may lead to changes in inoculum size. By using a pipette, 0.001 to 0.002 ml of suspension of 10^7 cfu/ml was delivered to agar surface resulting in the final desired inoculum of approximately 10^4 to 10^5 cfu per spot. After inoculation plates were incubated for full 24 hrs. at 35^0C.

Interpretation: The presence of more than one colony or a haze of growth was considered as resistanct.

3.3.3.4. Bacteriophage typing:

Phage typing was done by the standard method described by Blair and Williams (1961) at National Staphylococcal Phage Center, Department of Microbiology Maulana Azad Medical College, New Delhi. For the propagation of the phages, testing of phage filtrate and typing the strains of *Staphylococcus aureus* isolates the propagating strain was first sub cultured onto a blood agar plate. A single colony was picked up and the phage pattern was checked using the 23 phages of the basic set at routine test dilution (1 RTD) and 100x RTD.

Propagation of the phages:

The phages were propagated on their homologous propagating strains. The aim of propagation was to produce a phage suspension of adequate dilution.

Propagation methods:

(a) Propagation in liquid medium:

The following phages were propagated in liquid medium: 55, 71, 53, 95 and 96.

(b) Propagation in soft agar:

The following phages namely 29, 52, 52A, 79, 80. 3A, 3C, 6, 42E, 47, 54, 75, 77, 83A, 84, 85, 81, and 91 were propagated in soft agar.

Determination of RTD: The phage preparation was diluted in ten-fold step. One drop of each dilution was then applied to a nutrient agar plate spread with the homologues propagating strain. The plate was incubated at 30°C overnight. The RTD (routine test dilution) was taken as the highest dilution of a phage that gave just less than confluent lysis.

Determination of 100 x RTD: The phage preparation was diluted in 100-fold steps. One drop of each dilution was then applied to a nutrient agar plate spread with the homologues propagating strain. The plate was incubated at 30°C overnight. The 100x RTD (routine test dilution) was taken as the highest dilution of a phage that gave just less than confluent lysis.

Performance of phage typing:

The basic set of phages, consisting of 23 phages was used for typing the strains of *Staphylococcus aureus*.

A Single colony of *Staphylococcus aureus* was inoculated into 5ml nutrient broth and incubated at 37° C for 4 to 6 hours. This culture was used to inoculate 4-inch peteridishes containing nutrient agar supplemented with 0.04% fused calcium chloride. The plates used for typing were freshly made and dried for 45 minutes. The broth culture of the test strain was added on the surface of the plate with a Pasteur pipette and the excess broth was pipetted off. These were then left for drying with lids open for one hour at room temperature.

The phages were loaded in a block containing 27 wells, using sterile Pasteur pipettes. Phages were delivered from the loading chamber to agar plate with the help of mechanical multi-loop/ phage applicator.

After each application, the prongs of the applicator were dipped in spirit and were sterilized by flaming. After the charged phages had dried, the plates were incubated at 30°C for 18 hrs. The readings were taken on the following day.

Interpretation of the lytic pattern:

The plates were examined by indirectly transmitted light against a dark background and read semi-quantitatively.

Strains that were non-typeable at 1 RTD were typed at 100xRTD. Non-typeablity was recorded when the strains was non-typeable at 100x RTD.

The phage patterns were recorded as follows:

++, CL = Confluent lysis (Strong reaction)

\+ =Moderate lysis

± = Weak lysis

o = inhibition reaction

NT = Not typeable

Conventional Phages

Lytic Group of Phages	Designation of Phages
I	29,52,52A,79,80
II	3A, 3C, 55, 71
III	6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85
Non – allocated	81, 94, 95, 96

TYPING OF METHICILLIN-RESISTANT *Staphylococcus aureus* BY MRSA PHAGES

All methicillin resistant staphylococcal stains were phage typed using 9 supplementary MRSA phages.

The 9 phages used were:

M3, M5, M12, M8, MR25, 622, C30, C33, C38

3.4. Tests for genotypic characterization:

Genotypic characterization was done using polymerase chain reaction for thermostable nuclease gene, coagulase gene, enterotoxin genes and methicillin gene.

3.4.1. Polymerase Chain Reaction (PCR):

Standardization of PCR:

Standardization of Polymerase Chain Reaction (PCR) was done using the standard strains of *S. aureus* obtained from IVRI, Izatnagar.

3.4.1.1. Template DNA Preparation / Extraction:

Various methods of cell lysis and release / extraction of DNA as described below were compared for their use in PCR assay.

3.4.1.1.1. Genomic DNA Extraction:

By Phenol Chloroform Extraction:

All isolates under study were cultured in Tryptone Soya Broth (TSB, oxide Ltd. England) at 37°C for 18h. Bacteria were pelleted @ 8000 rpm for 10 mins. Pellet was resuspended in 2ml TE buffer. 40 µl of lysozyme (50 mg / ml) was added, vortexed to mix properly and incubated at 37°C for 1h. Then 400 µl SDS (10%) and 30 ml Proteinase-K (20mg / ml) was added and incubated at 37°C for 2h. After incubation, 800 µl of NaCl (5M) and 800 µl CTAB (10%, Preheated at 65°C), were added and incubated in water bath at 65°C for 30 minutes. The aqueous phase was extracted with equal amount of Phenol + Chloroform (1:1). The phenol-chloroform extraction step was repeated as to the extracted aqueous phase, 0.6 volume of isopropanol and 0.1 volume of 3M sodium

acetate (pH 5.2) were added. The components were mixed properly, and kept at -20°C for 8-10h. Centrifugation at 20,000 rpm for 10 minute was performed to collect DNA pellet. The pellet was washed twice with 70% ethanol and air-dried. Finally the pellet was dissolved the pellet in 400 µl of TE buffer containing 20 µg RNase / ml (MBI Fermentas) and kept at 65 °C for 1 hr. Subsequently cooled and stored at -20°C till further use.

3.4.1.1.2. By Using Bacterial Cell Lysate:

(a) By Triton X – 100:

As per the method described by Wang *et. al*, (1992), cells were pelleted and suspended in 50 -100 µl of 1% Triton X-100. It was then heated in a boiling water bath for 5 minutes and snaps chilled.

(b) By SDS:

The bacterial cells were pelleted and suspended in about 20 µl of 0.05% SDS and heated in a boiling water bath for 5 minutes and snap chilled.

(c) By Sonication:

About 100-200 µl of BHI broth culture was subjected to sonication and was used in PCR.

(d) By Boiling and Chilling:

In this method about 0.5-1.0 ml of BHI broth culture was subjected to vigorous heating in a boiling water bath for 10 minutes and then snap chilled. From this about 5 µl was used as a template in PCR.

For standardization with DNA extraction, initially all the methods described above were applied on the standard strains. As all these

methods showed comparable results, we subsequently followed the boiling and chilling method for the DNA extraction from the test strains.

3.4.2. Specificity of the PCR:

About 0.5µl of overnight BHI cultures was subjected to heating in a boiling water bath and then snap chilled in ice. The PCR was performed as described below and agarose gel electrophoresis as described in section 3.5, and observed for any product generated from other bacteria.

3.4.3. PCR amplification of thermostable nuclease gene (Nuc):

PCR amplification was performed by using the primers as used by Brakstad *et al.,* (1992) with slight modifications. The thermostable nuclease gene (nuc) was amplified by standard PCR protocol. The template was prepared as described in 3.4.1.1.2 (d). Sequences of the primers used were

nuc 1- G C G A T T G A T G G T G A T A C G G T T

nuc 2 - A G C C A A G C C T T G A C C A A C T A A A G C

Size of the amplified product – 447 bp

Reactions were carried out on 50µl as follows.

DNA	-	5µl
10x PCR buffer	-	5 µl
$MgCl_2$ (1.5mM)	-	1.5 µl
dNTP's (2mM)	-	5 µl
Primers (10 pmol/ µl)	-	N1– 2.0 µl
		N2– 2.0 µl
Taq DNA polymerase (1u/µl Labware, USA)	-	0.4 µl
Milli Q water to make volume 50 µl.		

The PCR tube containing the reaction mixture was flash spun in a micro centrifuge to get the reactants at the bottom. The reactions were performed in a master cycler Gradient thermocycler (Eppendrof, Germany) with a preheated lid.

The PCR programme included denaturation at 94°C for 5 minutes followed by 34 cycles of denaturation (94°C for 1 minute), annealing (55°C for 1 minute) and extension (72°C for 1 minute). Final extension was carried out at 72°C for 10 minutes After the reaction, tubes with PCR products were held at 4°C until further analysis / confirmation by agarose gel electrophoresis (1.5% agarose) described as in 3.5. gene Ruler™ 100 bp DNA ladder was used as molecular weight marker.

3.4.4. PCR and nested PCR amplification of coagulase gene (*coa*):

PCR and nested PCR were performed for amplification of coagulase gene.

3.4.4.1. PCR amplification of coagulase gene (*coa*):

By using the primers Goh *et al.*, (1992) with slight modification the coagulase gene (*coa*) was amplified by standard PCR protocol. Template was prepared as described in 3.4.1.1.2 (d). Sequences of the primers used were

Coa 1- ATACTCAACCGACGGACACCG

Coa 2- GATTTTGGATGAAGCGGATT

Reactions were carried out on 50 µl as follows

DNA	-	4µl
10x PCR buffer	-	5µl
$MgCl_2$ (1.5mM)	-	1.5 µl
dNTP's (2mM)	-	5µl
Primers (10 pmol /µl)	-	coa 1 – 2.00 µl
		coa 2 – 2.00 µl
Taq DNA polymerase	-	0.4 µl

(1unit / reaction, Lab ware, USA)

Milli Q water to make volume 50 µl.

The PCR tube containing the reaction mixture was flash spun in a micro centrifuge to get the reactants at the bottom. The reactions were performed in a master cycler Gradient thermocycler (Eppendrof, Germany) with a preheated lid.

The PCR programme included denaturation at 94°C for 5 minutes followed by 34 cycles of denaturation (94 °C for 1 minute), annealing (56 °C for 1 minute) and extension (72°C for 1 minute). Final extension was carried out at 72 °C for 10 minutes. After the reaction, tubes with PCR products were held at 4°C until further analysis / confirmation by agarosegel electrophoresis (2% agarose) described as in section 3.5. Gene Ruler™ 100 bp DNA ladder plus was used as molecular weight marker.

3.4.4.2. Nested PCR amplification of coagulase gene (*coa*):

Nested PCR was standardized as per method described by Goh *et al.* (1992) with slight modifications. All isolates were tested in duplicates. Depending on the intensity of the primary amplicon generated by primer set no. 1, 2 microlitre of undiluted to 1:10 diluted primary

amplicon was taken for nested PCR. The internal primer set no. 2 was used to generate nested PCR products. Sequences of the primers used were

coa N – 1 CGAGACCAAGA[illegible]TCAACAAG

coa N – 2 AAAGAAAACCA[illegible]TCACATCA

Reactions were carried out on 50 µl as follows.

Size of the amplified products - Ranged between 440 bp to 915 bp

Ist PCR product	-	2.0µl
10x PCR buffer	-	5µl
$MgCl_2$ (1.5mM)	-	1.5 µl
dNTP's (2mM)	-	5µl
Primers (10 pmol / µl)	-	coa 1- 2.0µl
		coa 2- 2.0µl
Taq DNA polymerase	-	0.4 µl

(1u/ µl, Lab ware, USA)

Milli Q water to make volume 50 µl.

The PCR tube containing the reaction mixture was flash spun in a micro centrifuge to get the reactants at the bottom. In this study the previous PCR product used as template. The reactions were performed in a master cycler Gradient thermocycler (Eppendrof, Germany) with a preheated lid.

The PCR program and other procedures were same as described earlier for the first amplification of *coa* gene.

3.4.4.3. PCR restriction analysis:

Restriction enzyme digestion:

For the confirmation of the PCR product generated by primer set no. 1, restriction digestion with *Alu 1* was done. To about 10µl of nested PCR product, 6 unit (0.6 µl) of *Alu 1* was added (Bangalore, Genie) with 2µl of 10x buffer and the volume was made up to 20 µl with autoclaved milli Q water and incubated at 37°c for overnight. The enzyme activity was stopped by adding 4µl of 6x loading dye. Then the digested product was subjected to agarose gel electrophoresis (2% agarose) and observed for the products of desired molecular weight. All isolates were again tested in duplicate.

3.4.5. PCR amplification of enterotoxin gene (SEA, SEB and SEC):

A total of 202 isolates (102 human clinical isolates and 100 animal-origin isolates) were tested for production of enterotoxin A, enterotoxin B and enterotoxin C. PCR amplification for enterotoxin genes were performed by using the methods described by Johnson *et al.* (1991) (for SEA and SEB) while the method of Chen *et al* (2000) was used for SEC with slight modification. Templates were prepared as described in 3.4.1.1.2 (d). Sequences of the primers used were

For SEA SEA – 1 TTGGAAACGCTTAAAACGAA

SEA – 2 GAACCTTCCC ATCAAAAACA

Size of the amplified product – 120 bp

For SEB SEB – 1 TCGCATCA AACTGACAAACG

SEB – 2 GCAGGTAC TCTA AAGTGCC

Size of the amplified product – 478 bp

For SEC SEC – 1 ACATTAGTGATAAAAAACTGAAA

SEC – 2 TTGTAAGTTCCCATTATCAAAGTG

Size of the amplified product – 234 bp

Reactions were carried out on 50µl as follows

DNA	-	4µl
10x PCR buffer	-	5µ
$MgCl_2$ (1.5mM)	-	1.5µl
d NTP'S (2mM)	-	5 µl
Primers (10 pmol / µl)	-	forward – 2.0 µl
		reversed – 2.0 µl
Taq DNA polymerase	-	0.4 µl
(1u / µl, Labware, USA)		

Milli Q water to make volume 50 µl.

The PCR tube containing the reaction mixture was flash spun on a micro centrifuge to get the reactants at the bottom. The reactions were performed in a master cycler Gradient thermocycler (Eppendrof, Germany) with a preheated lid.

The PCR programme included denaturation at 94°C for 5 minutes followed by 34 cycles of denaturation (94 °C for 30 seconds), annealing (55 °C for 30 seconds) and extension (72°C for 20 seconds). Final extension was carried out at 72°C for 10 minutes. The PCR products were characterized by gel electrophoresis on 2.5% agarose gel (Q. Biogene, USA) as described in 3.5.

Gene Ruler™ 100 bp DNA ladder was used as molecular weight marker.

3.4.6. PCR amplification of methicillin resistance gene (*mec A*):

By using the primers as used by Prasad *et al.* (2000), with slight modification, the methicillin resistant gene (*mec A*) was amplified by the standard PCR protocol. Template was prepared as described in 3.4.1.1.2 (d). Sequences of the primers used were

mec A 1 – A G T T G T A G T T C T C G G G T T T G

mec A 2 – A G T G G A A C G A A G G T A T C A T C

Size of the amplified product – 604 bp

Reactions were carried out on 50 µl as follows.

DNA	-	10µl
10x PCR buffer	-	5µl
$MgCl_2$ (1.5mM)	-	1.5 µl
dNTP's (2mM)	-	5µl
Primers (20 pmol / µl)	-	mec A1 - 2.0µl
		mec A2 - 2.0µl
Taq DNA polymerase (1unit/reaction, Lab ware, USA)	-	0.4µl

Milli Q water to make volume 50 µl.

The PCR tube containing the reaction mixture was flash spun in a micro centrifuge to get the reactants at the bottom. The reactions were performed in a master cycler Gradient thermocycler (Eppendrof, Germany) with a preheated lid.

The PCR programme includes denaturation at 94°C for 5 minutes followed by 34 cycles of denaturation (94°C for 1 minute), annealing

(54°C for 1.5 minutes) and extension (72°C for 1 minute). Final extension was carried out at 72°C for 10 minutes. After the reaction, tubes with PCR products were held at 4°C until further analysis / confirmation by agarose gel electrophoresis (2% agarose) described as in 3.5. Gene Ruler™ 100 bp DNA ladder was used as molecular weight marker.

3.5. Agarose gel electrophoresis:

Agarose gel was prepared by boiling molecular biology grade agarose (Bangalore, Genie) in 0.5-x TBE buffer to dissolve it completely. After cooling it to about 50°C, ethidium bromide (SRL) was added to the agarose solution to a final concentration of 0.5 µg / ml. Before dissolving the agarose, the gel-casting platform was placed on a leveled surface and the open sites were sealed with adhesive tape. The gel comb was then placed across the gel-casting platform, so that the teeth of the comb remained 1mm above the base of the platform. The molten agarose was then poured on to the gel-casting platform and it was kept undisturbed for about an hour to solidify the gel. After the gel solidified the comb was taken out and adhesive tape was removed The set gel with the gel-casting platform was then submerged in the elect ophoresis tank with the wells at the cathode end of the tank with sufficient quantity (about 1 mm level) of electrophoresis buffer (TBE, 0.5x) above the surface of the gel.

About 10 µl of PCR products was mixed with 2 µl of bromophenol blue (6x) loading dye and loaded into the well. Electrophoresis was performed at 6 V/cm and the progress of mobility was monitored by the migration of the dye. At the end of electrophoresis the gel was visualized under UV transilluminator / gel documentation system for the bands of desired molecular weight.