CERTAIN PLASMID - MEDIATED CHARACTERS OF STAPHYLOCOCCI ISOLATED FROM BOVINE MASTITIS

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"CERTAIN PLASMID-MEDIATED CHARACTERS OF STAPHYLOCOCCI ISOLATED FROM BOVINE MASTITIS"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled **"CERTAIN PLASMID-MEDIATED CHARACTERS OF STAPHYLOCOCCI ISOLATED FROM BOVINE MASTITIS"** is a record of research work done independently by **Sri. M. Anil Kumar**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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To My Beloved Parents

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ABBREVIATIONS

Ac	-	Acriflavin
AGE	-	Agarose gel electrophoresis
Am	-	Ampicillin
ANTI	-	Antibiogram typing
Ao	-	Ammonium molybdate
At	-	Antimony trichloride
Ax	-	Amoxycillin
B	-	Barium chloride
BIO	-	Biotyping
Bo	-	Boric acid
BSA	-	Bovine Serum Albumin
C	-	Chloramphenicol
CCC	-	Covalently clossed circular
Cd	-	Cadmium chloride
Cm^r	-	Chloramphenicol resistance
CNS	-	Coagulase negative staphylococci
CPS	-	Coagulase positive staphylococci
Cs	-	Copper sulphate
Ct	-	Cetrimide
Cv	-	Crystal violet
Cx	-	Cloxacillin
°C	-	degree Centigrade
DNA	-	Deoxyribo Nucleic Acid
DNase	-	Deoxyribo Nucelase
D Value	-	Numerical Index of Discrimination
E	-	Erythromycin
EEO	-	Electro Endosmosis
EDTA	-	Ethylene Diamine Tetra Aceticacid
F	-	Flumequine
Fs	-	Ferrous sulphate
Fus^r	-	Fusidic acid resistance

G	-	Gentamicin
HBM	-	Hypertonic Buffer Medium
Hly	-	Haemolysin
I	-	Iodine
IU	-	International Unit
K	-	Kanamycin
kb	-	kilobase
kbp	-	kilo base pair
L	-	Lead oxide
LB	-	Luria Bertani
M	-	Methicillin (antibiotic resistance)
M	-	Molar (concentration of reagents)
mA	-	milli Ampere
Mc	-	Mercuric chloride
mcg	-	microgram
mcm	-	micrometer
mcl	-	microliter
MDa	-	Mega Dalton
MDR	-	Multiple Drug Resistance
mM	-	milli Molar
MRSA	-	Methicillin resistant *S. aureus*
MSSA	-	Methicillin sensitive *S. aureus*
Na	-	Nalidixic acid
Ni	-	Nitrofurantoin
No	-	Novobiocin
Pb	-	Polymixin-B
PEG	-	Polyethylene Glycol
%	-	per cent
Pf	-	Pefloxacin
Pg	-	Penicillin-G
pH	-	Hydrogen ion concentration
PLAS	-	Plasmid profiling
Pp	-	Potassium permanganate
Pt	-	Potassium tellurite
QAC	-	Quarternary Ammonium Compound

R	-	Rifampicin
RESI	-	Resistogram typing
R-factor	-	Resistance factor
Rh	-	Relative humidity
Rif^r	-	Refampicin resistance
R medium	-	Regeneration medium
R-plasmid	-	Resistance plasmid
Sa	-	Sodium arsenate
SDS	-	Sodium Dodecyl Sulphate
SEC	-	Staphylococcal Ent◊erotoxin-C
Sf	-	Sulfurazole
SMTB	-	Sucrose Magnesium Tris Buffer
Sn	-	Silver nitrate
SLR	-	Single Lense Reflex
St	-	Streptomycin
T	-	Tetracycline
TBE	-	Tris Borate Electrophoresis Buffer
TE	-	Tris EDTA Buffer
TEG	-	Tris EDTA Glucose Buffer
TEM	-	Tellurite Egg Medium
UV	-	Ultra Violet
V	-	Vancomycin (antibiotic resistance)
V	-	Voltage (electrophoresis)
VP test	-	Voges Proskauer test

Introduction

INTRODUCTION

There has been a spurt in the incidence of staphylococcal infections both in man and animals in recent years. Mastitis is an important economic problem confronting dairy farmers all over the world and staphylococci is the most important single causative agent of bovine mastitis (Tuteja *et al.*, 1993). The mastitis caused by staphylococci may be either clinical or subclinical. According to Bramley (1985) the staphylococci causing mastitis are seen in the udder and in the surroundings of the cow. The milk and dairy products from animals suffering from staphylococcal mastitis may cause food-borne infections, and antibiotic residues in milk of treated animals can cause health problems in man.

Strains of staphylococci isolated prior to 1942 were found sensitive to most of the drugs, while strains isolated since then were found resistant to most of them (Parker, 1983).

Staphylococci are identified by the morphological characters including staining reaction, cultural characters and biochemical characters. Mathew (1986) and Paul (1992) have grouped staphylococci isolated from cases of bovine mastitis in Kerala and Tamil Nadu respectively, based on

mannitol fermentation, coagulase production, haemolysin production, resistance to heavy metals, antibiogram, lysostaphin sensitivity and phage typing. Paul (1992) has demonstrated the plasmid profile of 50 strains of *S. aureus* isolated from bovine mastitis. Baumgartner *et al.* (1984) and Wegener and Schwarz (1993) have reported that plasmid profile analysis can be used successfully to trace strains of staphylococci in animal infections.

Mastitis is produced by both coagulase positive staphylococci (CPS) and coagulase negative staphylococci (CNS), but CNS are currently the most prevalent organism causing mastitis (Honkanen-Buzalski *et al.*, 1994).

The genes responsible for antibiotic resistance and heavy metal resistance in staphylococci are located either in the chromosomal DNA (Altenbern, 1968) or in the plasmid DNA (Novick and Roth, 1968). But genes for a number of phenotypic characters of this organism like haemolysin production, coagulase production, pigment production and enterotoxin production are found to be plasmid-borne (Brock and Madigan, 1988).

A number of plasmid-borne characters in staphylococci were found to be transferred inter or intragenerically (Muhammad *et al.*, 1993). The R-plasmids play an important role in the infectious transfer of drug-resistance in

staphylococci either by conjugation, transformation, transduction or protoplast fusion.

This project was undertaken to suggest most suitable antibiotic/chemotherapeutic agent for treating staphylococcal mastitis and to compare the plasmid profiles of the organisms vis-a-vis the plasmid-mediated characters and to establish the relationship, if any, between these characters and the plasmid profile of the organism.

In the present investigation following parameters are studied, viz:

a. Isolation of staphylococci from clinical and subclinical cases of bovine mastitis.

b. Characterization of the organism based on morphological, cultural and biochemical characters.

c. Biotyping of the isolates based on haemolysin production, coagulase production, pigment production, urease production and hydrolysis of Tween-80.

d. Study the antibiogram and the resistogram of the isolates.

e. Study the plasmid profile of the isolates.

f. Transfer the various plasmid-borne characters to other bacteria by various gene transfer methods like conjugation/transformation/protoplast fusion.

Review of Literature

REVIEW OF LITERATURE

Staphylococci are a group of organisms causing various diseases in man and animals. The organism was first demonstrated by Bollinger in 1869 from botryomycosis in horses and later cultivated by Pasteur in 1880. The occurrence of staphylococcal mastitis in sheep was recorded by D'Arboval in 1823. The first thorough study of this organism was made by Rosenbach in 1884 (Smith, 1959).

Barber (1914) reported the occurrence of food poisoning in man due to staphylococci, isolated from cow suffering from mastitis in Philippines.

Staphylococci are spherical or ovoid cells usually arranged in grape-like clusters, growing aerobically or as facultative anaerobe, liquifying gelatin and fermenting a number of sugars (Cowan, 1974). At present, the staphylococci are classified in the family Micrococcaceae, under the genus Staphylococcus along with Micrococcus, Stomatococcus and Planococcus (Schleifer, 1986).

The two important species under the genus Staphylococcus are *Staphylococcus aureus* and *Staphylococcus epidermidis*, which can be distinguished by coagulase test and anaerobic fermentation of mannitol (Baird-Parker, 1965).

Eventhough *S. aureus* represented the pathogenic group of this genus, strains of *S. epidermidis* were also found to cause bovine mastitis (Forbes and Hebert, 1968).

2.1 Isolation of staphylococci from mastitis cases

Bovine mastitis is caused by different infective agents like bacteria, yeast and other fungi and among them bacteria play the most important role (Blood and Rodostits, 1989). Members of the genus Staphylococcus were reported to be the most important bacterial etiology of bovine mastitis (Watts and Owens, 1989; Tuteja *et al.*, 1993).

Evans (1916) isolated the bacterial flora from 192 milk samples and recovered 'micrococci' from 58.8 per cent of the samples. Based on haemolysin production, mannitol fermentation and pigment production, 50 per cent of these isolates were considered to be *S. aureus* and opined that pathogenic staphylococci could also be isolated from milk drawn from normal udders.

Jhala (1976) and Suribabu *et al.* (1979) reported the occurrence of *S. aureus* in cases of mastitis to the tune of 45.38 and 55 per cent respectively.

Sudharma *et al.* (1985) obtained 31.67 per cent of staphylococci out of the 240 bacterial organisms isolated

from 483 milk samples in Kerala. But, Mathew (1986) could obtain only 14.44 per cent of staphylococci from 360 bovine mastitis cases.

Watts and Owens (1989) have isolated 521 (84%) staphylococcal organisms out of 620 bacterial isolates from bovine mastitis cases in four dairy herds in Lousiana and as per their observation *S. aureus*, *S. hyicus* (Staphylococcus) and *S. epidermidis* were the predominant organisms.

Jarp (1991) reported that 66 (44.6%) strains of *S. simulans* could be identified from 148 strains of CNS isolated from clinical and subclinical mastitis in cows in Norway and *S. simulans* was the most frequently isolated species.

Paul (1992) isolated 50 strains of *S. aureus* from 354 bovine mastitis cases in Tamil Nadu and out of this 50 strains, 21 (42%) were from subclinical cases and 29 (58%) from clinical cases of mastitis. Birgersson and co-workers (1992) isolated 203 strains of CNS from bovine milk samples in Sweden and reported that *S. simulans*, *S. chromogenes*, (Staphylococcus) *S. epidermidis* and *S. xylosus* were the most common species in both abnormal and normal milk.

Matsunaga *et al.* (1993) reported the isolation of 58 strains of *S. aureus* from bovine mastitis cases from 27

dairy farms in Japan and out of this 58 strains, four were from per acute cases, 18 from acute cases and 36 from chronic cases. Tuteja *et al.* (1993) studied the bacterial flora of udder of 178 apparently healthy cows in an organized herd and found that staphylococci were predominant (44.3%) among 307 bacterial isolates.

Tuteja and Kapur (1995) reported the occurrence of 12 different species of staphylococci in quarter milk samples of 178 apparently healthy cows. A total of 143 isolates were obtained and *S. hyicus*, *S. intermedius*, *S. haemolyticus* and *S. anreus* were the frequent isolates.

2.2 Characterization of staphylococci

Characterization of staphylococci is usually carried out by morphological character including staining reaction, cultural character and various biochemical reactions (Cowan, 1974; Cruickshank, 1975). The use of catalase test in the characterization of staphylococci isolated from bovine mastitis cases was reported by various workers (McDonald and Anderson, 1981; Hodges *et al.*, 1984).

2.3 Biotyping of staphylococci

Several tests have been developed to biotype and to determine the pathogenic significance of staphylococci

isolated from mastitis. Wilson *et al.* (1966) determined coagulase production, alpha and beta-haemolysin production, fermentation of mannitol and growth on tellurite glycine agar and opined that the organisms which were positive to three out of the four tests could be considered as potential pathogens.

The most common criteria to determine the virulence of staphylococcal isolates were coagulase production and haemolysin production. These organisms also produced enterotoxin, leucocidin, necrotoxin, lethal toxin, lysozyme, hyaluronidase, TSST-I (Toxic Shock Syndrome Toxin-I) and lipase, which were used to biotype and to characterize staphylococcal isolates (Koiranen, 1969; Hajek and Balusek, 1988).

Coia *et al.* (1990) conducted biotyping of methicillin-resistant *S. aureus* of human origin based on urease production, hydrolysis of Tween-80 and pigmentation on Tween-80 agar.

Paul (1992) grouped 50 isolates of *S. aureus* of bovine mastitis origin into 4 biotypes namely A, B, C and D based on the method described by Coia *et al.* (1990).

2.3.1 Coagulase production

Loeb (1903) discovered that *S. pyognes* could coagulate goose plasma *in vitro*. Smith (1947) opined that the production of coagulase was the only absolute criterion of pathogenicity among isolates of staphylococci from mastitis.

The staphylococci produced two types of coagulase: free coagulase and 'bound ' coagulase/clumping factor (Minor and Marth, 1976).

Muth (1971), while studying the cultural characters and coagulase production of *S. aureus* opined that the genes for coagulase production and unique colonial morphology were linked and extra-chromosomal.

The coagulase test for staphylococci is generally done with rabbit or human plasma. But it was reported that the ability to coagulate bovine plasma within a relatively short period of time was primarily a property of bovine strains (Hajek and Marselek, 1969; Meyer, 1966).

Coagulase positive staphylococci (CPS) were only considered to be pathogenic, but this was not found to be true as far as the animal strains were concerned. Jayappa and co-workers (1977) detected 27 (41%) CPS and 39 (59%) CNS

out of 66 staphylococci isolated from bovine mastitis. Kapur *et al.* (1979) obtained 103 (33.88%) CPS and 201 (66.12%) CNS out of 304 staphylococcal isolates from bovine udders.

Mathew (1986) obtained 32.69 per cent CPS and 67.31 per cent CNS from bovine mastitis. Hogan *et al.* (1986) detected 203 (67%) CPS and 100 (33%) CNS out of 303 staphylococci from bovine mastitis.

Honkanen-Buzalski *et al.* (1994) reported from Finland that clinical mastitis due to CNS was common in cows and *S. hyicus*, *S. simulans* and *S. epidermidis* were the most frequently isolated organisms.

2.3.2 Haemolysin production

The production of haemolysins, viz. alpha, beta, gamma and delta, as a parameter in the characterization of staphylococci had been reported by various workers (Slanetz and Bartley, 1953; Loken and Harvey, 1962; Poutrel, 1984).

Elek and Levy (1950) reported that CPS could produce one or a combination of several haemolysins, but it appeared that beta-haemolysin was produced more frequently by strains of animal origin. Joshi and Dale (1963) found that

beta-haemolytic strains were predominant among haemolytic staphylococci isolated from milk samples of mastitis.

Zemelman and Longeri (1965) reported that of the 404 CPS isolated from bovine raw milk, 77 per cent produced beta-haemolysin, where as none of 371 CNS produced beta-lysin.

Mathew (1986) reported that out of 52 strains of staphylococci isolated from bovine mastitis, 21 (40.38%) strains produced haemolysins.

De Centorbi and co-workers (1988) reported from Argentina, that out of 36 strains of *S. aureus* isolated from young goats, 29 (80.6%) strains produced alpha-lysin and 15 (41.7%) strains produced beta-lysin. Paul (1992) reported that 46 (92%) out of 50 strains of *S. aureus* isolated from cases of bovine mastitis produced beta-haemolysin.

Matsunaga *et al.* (1993) reported that out of 58 *S. aureus* strains isolated from bovine mastitis in Japan, 74.1 per cent produced alpha-haemolysin, 65.5 per cent produced beta-haemolysin and 12.1 per cent produced delta-haemolysin. They have also observed that all the *S. aureus* isolates from peracute mastitis produced TSST-I, Staphylococcal Enterotoxin-C (SEC), alpha-haemolysin and beta-haemolysin.

Calvinho and Dodd (1994) reported that out of the 79 *S. aureus* isolates obtained from bovine mastitis, the majority (92.4%) produced alpha, beta and delta-haemolysins in combination and those producing only one or two haemolysins were rarely seen.

Some workers were of the opinion that haemolysin and coagulase production by staphylococci are plasmid-mediated characters (Falkow, 1975; Brock and Madigan, 1988).

2.3.3 Pigment production

Evans and Niven (1950) reported that 22 strains of enterotoxigenic *S. aureus* isolated from food-borne infections produced either orange or cream pigment on agar medium with 7.5 per cent sodium chloride. Reid and Wilson (1959) reported that all strains isolated from acute bovine staphylococcal mastitis produced orange or yellow pigment. Zemelman and Longeri (1965) studied 404 CPS isolated from bovine raw milk and reported that 96 per cent of these strains produced a deep orange pigment.

Many staphylococci produce a pigment that ranges in colour from deep gold to orange, cream or white (Minor and Marth, 1976). They are water-insoluble in nature (Merchant and Packer, 1983).

Brock and Madigan (1988) proposed that pigment production by staphylococci is a plasmid-borne character. Hajek and Balusek (1988) used pigment production to characterize 86 *S. aureus* and 25 *S. intermedius* strains isolated from rooks and gulls and found that 78 (90.7%) *S. aureus* strains produced either cream or orange pigment and none of the *S. intermedius* strains produced pigment.

De Centorbi *et al.* (1988) reported that all 36 strains of *S. aureus* isolated from young goats produced pigment and they have used pigment production character for biotyping these isolates. Jarp (1991) used production of pigment by 148 CNS from bovine mastitis on Tryptic Soy Agar as a method to characterize staphylococci and used Api-Staph system to confirm the diagnosis of strains. Paul (1992) used pigment production on Tween-80 agar as a criterion for biotyping 50 strains of *S. aureus* isolated from bovine mastitis and found that these strains can be grouped into 4 biotype groups.

2.3.4 Urease production, Tween-80 hydrolysis and Casein hydrolysis

Urease production, Tween-80 hydrolysis and casein hydrolysis were used for classifying different strains of staphylococci (Minor and Marth, 1976).

Jarp (1991) classified 148 strains of CNS isolated from bovine mastitis into different species and used Tween-80 hydrolysis, urease production and caseinase production by these strains for classification. Paul (1992) used Tween-80 hydrolysis and urease production for biotyping 50 strains of *S. aureus* isolated from bovine mastitis and grouped them into 4 biotype groups, with a reliability of 8 per cent in differentiating *S. aureus* isolates.

Aarestrup *et al.* (1995b) have biotyped 105 *S. aureus* isolates from bovine milk samples in Denmark based on production of fibrinolysin and beta-haemolysin, coagulation of bovine plasma and crystal violet reaction.

2.4 Antibiogram of staphylococci

For successful chemotherapy of mastitis, selection of proper antimicrobial agent is highly essential. Because of this reason antibiotic sensitivity test, which is a very useful tool in assessing the effectiveness of the antibiotic against specific microorganisms, has assumed great importance. The term resistance and susceptibility were used in antibiotic sensitivity tests to express the ability or lack of ability of an organism to multiply in the presence of a given concentration of an antibiotic under defined conditions. Many antibacterial substances when

tested in critical concentrations and in very fine steps could be capable of distinguishing between strains of different bacteria.

One of the first suspicions that drug resistance might have its foundation in a mechanism other than simple mutation and selection, came with the observation by Barber (1949), that staphylococci could occasionally lose their resistance to penicillin. Later on it was demonstrated that the penicilliase production in *S. aureus* was mediated by plasmid. Such plasmids are not restricted only to penicillin resistance, but also coded for resistance to erythromycin, tetracycline, chloramphenicol and kanamycin as well as to toxic cations, arsenate and arsenite (Falkow, 1975).

Mechanism of emergence of drug resistant staphylococci has not been understood clearly. Of the three identified modes, R-plasmid mediated resistance has been reported to be the most important in Enterobacteriaceae and staphylococci (Rush *et al.*, 1969; Novick, 1980; Cohen *et al.*, 1982). The other two mechanisms reported are chromosome-mediated and transposon - mediated resistance (Chopra and Howe, 1978). The resistance genes located in R-plasmids, chromosome or transponsons of one strain of staphylococci may be transferred to another strain by conjugation, transduction or transformation (Mathew, 1986).

Antibiogram of bacteria is detected by using various antibiotics in critical concentrations by the agar diffusion test (Bauer *et al.*, 1966). The various strains of bacteria could be distinguished by detecting the antibiogram of the isolates. Novick and Roth (1968) reported the existence of resistance genes against penicillin and erythromycin in the penicillinase plasmids of *S. aureus* along with resistance genes for a series of inorganic ions like cadmium, arsenite, arsenate, lead, bismuth etc.

Van and Devriese (1971) tested 132 strains of *S. aureus* isolated from cases of bovine mastitis and found that 38 per cent were resistant to penicillin, 26 per cent to sterptomycin, 21 per cent to tetracycline, 3.5 per cent to chloramphenicol and 3 per cent possessed multiple drug resistance to all the four antibiotics. But all these strains were sensitive to neomycin, virginiamycin and rifamycin.

Jenetschke and Rizk (1973) reported from Syria that *S. aureus* was the most common organism isolated from bovine mastitis and it was observed that 78 per cent of the isolates were penicillin-resistant, 29 per cent to streptomycin, 6.6 per cent to chloramphenicol. Jhala (1976) reported that neomycin and chloramphenicol gave the best results in inhibiting the majority of organisms isolated

from mastitis cases, while streptomycin and terramycin showed variable sensitivity.

Frost and O'Boyle (1981) reported that studies on 1657 isolates of *S. aureus* from bovine mastitis in Australia during 1974-1979, revealed the commonest resistance was to penicillin and streptomycin. All strains were sensitive to methicillin and MDR was rare. Resistance to penicillin declined from 35 per cent in 1974-75 to 7 per cent in 1979.

Ramachandra *et al.* (1984) studied the antibiotic sensitivity pattern of 136 strains of staphylococci isolated from bovine udder infections in Karnataka and reported that they were highly sensitive to neomycin (64.7%), chloramphenicol (41.9%), furadantin (33.8%), polymixin-B (22.2%), kanamycin (25.7%) and majority of them were resistant to ampicillin, penicillin, streptomycin and erythromycin.

Kapur and co-workers (1984) in a study of *S. aureus* isolated from bovine mastitis observed sensitivity to various chemotherapeutic agents in the following order: furadantin (98.18%), cloxacillin (97.56%), chloraphenicol (90.2%), oleandomycin (80.77%), neomycin (79.25%), streptomycin (71.82%), oxytetracycline (40.54%), polymixin-B (49.5%), ampicillin (33.33%) and penicillin (36.36%).

Sudharma *et al.* (1985) studied the antibiogram of staphylococci isolated from bovine mastitis in Kerala by agar diffusion method and found that 84 per cent of the organisms were sensitive to gentamicin, 78.48 per cent to chloramphenicol, 62.5 per cent to neomycin, 60.96 per cent to tetracycline, 55.56 per cent to furadantin, 50 per cent to ampicillin and 43.17 per cent to penicillin. None of these isolates were resistant to all the antibiotics tested.

Mathew (1986) studied the antibiogram of 52 strains of staphylococci isolated from bovine mastitis by both agar diffusion and agar dilution methods and reported that by agar diffusion method all the isolates were found sensitive to bacitracin, while the percentage of isolates resistant to streptomycin, sulphamethoxazole, amoxycillin, ampicillin, tetracycline, penicillin, erythromycin, cloxacillin, nitrofurantoin, neomycin, methicillin and gentamicin varied from 64.70 per cent to 5.88 per cent. All the 35 CNS strains were sensitive to cloxacillin, gentamicin, neomycin, methicillin, and nitrofurantoin. By agar dilution method, all the *S. aureus* strains were found sensitive to gentamicin, erythromycin and rifampicin, while all the CNS strains were sensitive to ampicillin, gentamicin, chloramphenicol and rifampicin.

Mackie and co-workers (1988) studied the antibiogram of 848 strains of *S. aureus* isolated from milk samples of cows suffering from clinical and subclinical mastitis in England from 1984-87. All the 214 strains of *S. aureus* isolated during this period from clinical mastitis were sensitive to cephaloridine and cloxacillin. A very high percentage (>93%) of the isolates was sensitive to novobiocin in 1984 and 1985. Erythromycin, neomycin and tetracycline were effective against most of the isolates with little variation over the four years. Ampicillin and penicillin-G inhibited the growth of 62 and 55 per cent respectively, of the isolates in 1984, but in 1986 the figures dropped to 19 and 33 per cent respectively, and then returned to the 1984 figures in 1987. The antibiogram of *S. aureus* from clinical as well as subclinical cases was similar during the period 1984-87.

Trinidad *et al.* (1990) studied the antibiotic sensitivity of staphylococcal isolates from the mammary secretions and teat canal of dairy heifers in Lousiana. More than 92 per cent of the 311 isolates were sensitive to all the 12 antimicrobial agents tested, viz. ampicillin, penicillin, streptomycin, sulphamethoxazole-trimethoprim, ampicillin-clavulanic acid, cephalothin, erythromycin, gentamicin, novobiocin, oxacillin, tetracycline, vancomycin. Staphylococci other than *S. aureus* demonstrated an over all

susceptibility of 98.3 per cent to all the antibiotics and *S. aureus* demonstrated a 97 per cent susceptibility.

Paul (1992) studied the antibiotic sensitivity pattern of 50 isolates of *S. aureus* from cases of bovine mastitis and found that all the isolates were resistant to metronidazole and ampicillin, 96 per cent of strains were resistant to methicillin. The resistance against different antibacterial agents were as follows: framycetin (94%), nalidixic acid (86%), bacitracin (78%), penicillin (62%) and rifampicin (58%).

Dasgupta *et al.* (1993) studied the drug sensitivity patterns of organisms isolated from clinical mastitis cases of goats in West Bengal and found that the staphylococci constituted the main causative organism (37.5%) and 80 per cent of the staphylococcal isolates were sensitive to norfloxacin and pefloxacin, 73.33 per cent to neomycin, 60 per cent to nitrofurantoin, 23.33 per cent to chloramphenicol, 20 per cent to ampicillin, lincomycin and flumequine, 13.33 per cent to oxytetracycline and 10 per cent to trimethoprim.

Saini and co-workers (1994) studied the etiological agents causing subclinical mastitis in cows and buffaloes in Punjab and found that *S. aureus* (48.57%) and CNS (17.14%) were the most frequent agents among the 70 isolates and

found that more than 90 per cent of the staphylococcal isolates were sensitive to gentamicin, chloramphenicol, cotrimazole, nitrofurantoin and neomycin.

Bagherwal (1994) reported that pefloxacin was highly effective in treating acute mastitis due to different organisms including staphylococci, in cattle and buffaloes.

2.5 Resistogram of staphylococci

Staphylococci are often encountered in complex environments, which contain biological, physical and chemical agents that are potentially deliterious to their growth and survival (Minor and Marth, 1976).

Novick and Roth (1968) reported that penicillinase plasmids of *S. aureus* were found to carry determinants of resistance to a series of inorganic ions as well as resistance to penicillin and in some cases erythromycin. They have observed that there were separate genetic loci for resistance to arsenate, arsenite, lead, cadmium, mercuric and bismuth ions. Resistance to antimony and zinc were also found, but were not separated genetically from resistance to arsenite and cadmium, respectively.

Altenbern (1968) reported that the genes for resistance to vancomycin, novobiocin, acriflavin and nitrofurazone were

located on the chromosome of *S. aureus* and used this knowledge for chromosome mapping of *S. aureus*. Koiranen (1969) analysed the occurrence of strains resistant to penicillin, streptomycin and both penicillin and streptomycin among mercury-resistant and mercury-sensitive strains of staphylococci isolated from bovine milk samples in Finland and observed that, of the mercury-resistant strains, 32.9 per cent were penicillin-resistant, 22.8 per cent were resistant to streptomycin and 13.9 per cent were resistant to both penicillin and streptomycin. The occurrence of strains belonging to all three groups was relatively higher among mercury-resistant strains than among mercury-sensitive strains.

Kondo *et al.* (1974) reported that the penicillinase plasmids of *S. aureus* carry resistance genes for mercury, arsenate, lead, cadmium and bismath ions and found that they were linked. Weiss *et al.* (1977) reported that penicillinase plasmids of *S. aureus* often contained genes conferring resistance to inorganic mercury (Hg^{2+}) and the organomercurial phenyl acetate. In one group, 39 per cent of penicillin-resistant strains were resistant to phenyl mercury also. *S. aureus* exhibited higher frequency of resistance to cadmium to the tune of 70 per cent.

Jayappa and co-workers (1977) reported that a high incidence of penicillin - resistant staphylococci from cases of bovine mastitis in Banglore and observed that penicillin resistant isolates varied widely in their resistance to other antibiotics and also metal ions such as mercury, cadmium, cobalt and arsenate. Co-existence of penicillin and mercury resistance was observed in 73 per cent of the isolates.

There appeared to be a strong correlation between antibiotic resistance and resistance to heavy metal ions like mercury, cadmium, lead and chemical agents like cetrimide, acriflavin etc. (Nakahara *et al.*, 1977a,b).

Benson (1984) reported that tellurite egg medium (TEM) containing 0.02 per cent tellurite can be used as a selective medium for staphylococci and a concentration of 1 in 5,00,000 crystal violet inhibited staphylococci, but not streptococci. It was also reported that some strains of staphylococci were resistant to a concentration of 1 in 27,500 mercuric chloride and such strains were found to be resistant to penicillin and tetracyclines.

Brumfitt *et al.* (1985) observed that Methicillin Resistant *S. aureus* (MRSA) strains were four, nine and three times as resistant as Methicillin Sensitive *S. aureus*

(MSSA) strains to chlorhexidine, propamidine and QAC and cetrimide respectively.

Mathew (1986) reported that all the 52 strains of staphylococci isolated from bovine mastitis, were sensitive to mercuric chloride at a concentration of 1 in 27,500 by agar dilution method.

Al-Masaudi and co-workers (1991) studied the effects of various biocides like chlorhexidine diacetate, dibromo propamidine, isothionate, cetyl pyridium chloride, phenyl mercuric nitrate and sodium dodecyl sulpahate on *S. aureus*. Most of them were considered to be membrane - active agents and all the tested agents decreased the transfer of plasmids.

Paul (1992) conducted resistogram typing of 50 strains of *S. aureus* from cases of bovine mastitis using phenyl mercury acetate, basic fuchsin, acriflavin, potassium metabisulphite, acetyl trimethyl ammonium bromide, boric acid, cadmium acetate, lead nitrate, 3-amino phenol, sodium arsenate and found that there were 35 different patterns with reliability of 70 per cent in differentiation of strains. High rate of resistance was noticed against phenyl mercury acetate and basic fuchsin (86% each). It was observed that resistogram typing in conjunction with plasmid

profile analysis could differentiate and identify the isolates.

2.6 Plasmid profile analysis

Plasmids are extra-chromosomal, autonomous, circular, double-stranded DNA molecules found in many species of bacterial cells. They are endosymbionts of bacteria and they are not essential for the maintenance of bacteria. They are replicons, i.e., DNA molecules that can replicate independently. The term 'plasmid' was coined by Lederberg (1952) as a generic name for extra-chromosomal genetic element. They are covalently closed circular (CCC) DNA molecules and range in size from few kilo-bases (kb) to several hundred kilo-bases. Some genes carried by plasmids were responsible for drug resistance, heavy metal resistance, production of toxins, virulence factors like capsule and fimbria, bacteriocin production, degradation of organic compounds etc. (Brock and Madigan, 1988).

The plasmid profile analysis is a sensitive, rapid, reproducible, relatively inexpensive and suitable for examination of large number of bacterial cultures, and it also detects the interference of chromosomal DNA fragments. The plasmid profiles of several bacterial species were elucidated by agarose gel electrophoresis (AGE) method

(Meyers *et al.*, 1976; Crosa *et al.*, 1977). Willshaw *et al.* (1979) applied the technique for analysing the strains with more number (5-6) of plasmids of different types and sizes. The plasmid profile analysis was found to be useful for identifying and characterizing the organisms for the epidemiological studies.

2.6.1 Plasmid profile analysis techniques

Sambrook *et al.* (1989) described a technique used for isolation of plasmid DNA by lysing bacterial cells so that the plasmid DNA is preserved intact and could be physically separated from the more massive chromosomal DNA.

2.6.1.1 Isolation of plasmid DNA

The techniques employed for the isolation of plasmid DNA were based on their supercoiled, covalently closed circular (CCC) configuration within the host bacterial cell. The 'Clear lysate method' with the incorporation of Brij, a detergent, was widely used (Clewell and Helsinki, 1969; Marcina *et al.*, 1978).

Guerry *et al.* (1973) reported that the plasmid DNA could be isolated from chromosomal DNA by the preferential precipitation of the higher molecular weight chromosomal DNA

in the presence of Sodium lauryl sulphate and a high concentration of Sodium chloride (5 M).

Hansen and Olsen (1978) isolated large plasmids from *E. coli* and other organisms with alkaline denaturation and subsequent neutralization and removal of membrane-chromosome complexes with Sodium dodecyl sulphate (SDS) and Sodium chloride (NaCl).

Brinboim and Doly (1979) applied the principle of selective alkaline denaturation of high molecular weight chromosomal DNA wherein, a narrow range of pH (12.0-12.5) was used to denature the linear DNA but not CCC DNA and that this property could be used for purifying CCC DNA.

Kado and Liu (1981) reported a rapid procedure for the detection and isolation of plasmids of various sizes (2.6 to 350 MDa) harboured in bacteria like Escherichia, Erwinia, Salmonella, Pseudomonas etc., by lysing bacterial cells using alkaline Sodium dodecyl sulphate (pH-12.6) at elevated temperatures of 55°C to 65°C depending upon the bacterial species.

Blackall (1988) applied the alkaline extraction technique and alkaline detergent lysis technique for the isolation of plasmid from enterotoxigenic isolates of *E. coli* and *Haemophilus paragallinarum*.

Basha and Palanivelu (1994) reported a modified alkaline lysis procedure, in which they have used sodium chloride solutions instead of sugar solutions in the initial buffer and this modified method was effective for both small scale and large scale isolation of plasmids from *E. coli*.

Holmes and Quigley (1981) evolved the boiling method of plasmid isolation. This method begins with lysis of cell with detergent and lysozyme followed by boiling. This method could be efficiently used for rapid small scale isolation as well as large scale isolation of plasmid DNA.

Dunkle and Sippel (1984) reported a rapid microprocedure for extraction of plasmid DNA from *S. aureus* by boiling method.

Gomez-Marquez *et al.* (1987) modified the boiling method by Holmes and Quigley (1981), for large scale purification of plasmid DNA. The recovered plasmids were free from RNA and chromosomal DNA and were supercoiled and suitable for restriction enzyme analysis.

Barness (1977) developed a procedure of rapid disruption of bacterial colonies to test the size of plasmid DNA. Newland *et al.* (1984) developed 'in the well lysis technique'. While Bennet *et al.* (1986) developed 'an ultra

- rapid isolation technique'. Likewise a 'rapid isolation technique' was developed by Roberts *et al.* (1986).

2.6.1.2 Lysostaphin

Lysostaphin is a mucolytic protein produced by *Staphylococcus simulans* and it is enzymatically degrading the polyglycine crosslinks of the cell wall of *S. aureus* (Oldham and Daley, 1991). It is an enzyme, with a molecular weight of about 30,000 and lyses staphylococci, but not micrococci. It is highly active against *S. aureus*, but has little or no effect on *S. epidermidis* or other bacterial genera (Eickhoff, 1972).

Lysostaphin is highly essential for plasmid isolation from staphylococcal cells both by alkaline lysis and boiling methods. Schwarz and Blobel (1990) used lysostaphin at the rate of 40 mcg/ml for plasmid extraction from 32 *S. hyicus* strains isolated from pigs with exudative epidermitis and Paul (1992) used lysostaphin at the rate of 100 mcg/ml of the bacterial pellet, for plasmid extraction from 50 isolates of *S. aureus* from bovine mastitis, by modified alkaline lysis method.

Matthews *et al.* (1992) reported the use of lysostaphin at the rate of 200 mcg/ml to extract plasmids from 95 isolates of staphylococci from bovine mammary secretions and

Wegener and Schwarz (1993) used lysostaphin at the rate of 70 mcg/ml to establish plasmid profile of 100 strains of *S. hyicus* isolated from pigs with exudative epidermitis and from healthy pigs, by boiling method.

2.6.1.3 Agarose Gel Electrophoresis (AGE) of plasmid DNA

The utility of agarose gel electrophoresis in the analysis of plasmid DNA molecules has been well established (Southern, 1975; Willshaw *et al.*, 1979; Sambrook *et al.*, 1989).

Meyers *et al.* (1976) reported that agarose gel electrophoresis (AGE) could detect and characterize plasmid DNA present in Gram-negative microorganisms. The method was very sensitive, and useful for the estimation of plasmid mass, from the extent of DNA migration in gels. The method was proved to be a useful tool for survey work and the epidemiological investigation of plasmid dissemination, as well as an important adjunct to the genetic analysis of plasmids. Hansen and Olsen (1978) reported that a variety of large plasmids ranging from 70-300 MDa were readily visualised with AGE.

Willshaw *et al.* (1979) applied AGE to study the migration of open circular and linear plasmid DNA, since these forms caused difficulty in the interpretation of the

plasmid content of uncharacterized strains. Various agarose concentrations were employed to resolve clearly plasmid DNA from chromosomal DNA fragment in the crude preparations. It was noticed that the AGE provided information on the distribution of particular plasmids.

2.6.1.4 Molecular size estimation of plasmid DNA

Plasmid size may be estimated conveniently by AGE of either purified plasmid DNA or plasmid containing cell lysates that have been cleared of chromosomal DNA by high speed centrifugation, along with plasmid molecules of known molecular size in the same electrophoretic run.

Aaij and Borst (1972) reported that the migration rates of purified bactriophage and mitochondrial CCC DNAs ranging from 3.4×10^6 to 10×10^6 daltons were inversely related to the logarithm of their masses in 0.6 per cent agarose gels. However the migration properties of higher molecular weight CCC DNA were overlooked. Meyers *et al.* (1976) reported that there was a linear relationship between the logarithm of the relative migration of CCC DNA molecules and the logarithm of the plasmid molecular size.

Electron microscopy of DNA was done to determine the contour length of plasmid DNA. Molecular weight of CCC DNA was calculated from the contour length by using the

conversion factor of 1 mcm = 2.07×10^6. Hansen and Olsen (1978) applied similar method for molecular weight estimation.

A strain of *E. coli* designated V 517 was analysed, which contained multiple plasmid species of eight distinct sizes 1.36×10^6 to 35.8×10^6 daltons (2.1, 2.7, 3.0, 3.9, 5.2, 5.6, 7.3 and 54 kb). It was noticed that the strain would serve as a single source of plasmid reference molecules (molecular weight markers) (Marcina *et al.*, 1978; Wegener and Schwarz, 1993).

Schwarz and Blobel (1990) reported the use of a marker DNA, generated by partial Eco RI digestion of λdv 21/8 DNA for calculating the molecular weight of plasmid DNA of *S. hyicus*.

Matthews *et al.* (1992) reported the use of Hind III digested λ DNA as molecular weight marker, for plasmid profile analysis of staphylococci isolated from bovine mammary secretions.

The molecular sizes of the plasmid DNA were estimated by comparison with the plasmids of *E. coli* V 517. Using the least square technique for fitting the regression line of the logaraithms of the molecular weights to the distance

migrated in cms, the molecular weights were computed for the measured distances of migration (David *et al.*, 1991).

2.6.1.5 Staphylococcal plasmids

Dornbusch (1971) reported that methicillin resistance, production of enterotoxin B and beta-haemolysin by staphylococcal isolates from clinical cases may be plasmid-linked. Some workers have suggested that the ability to produce pigment, coagulase, haemolytic toxin and fibrinolysin were plasmid-linked in some strains of staphylococci and there was a striking parallel between the R-factors and other plasmids of enteric bacteria and the plasmids of staphylococci (Falkow, 1975; Brock and Madigan, 1988).

Staphylococci are notorious for their drug-resistance and Novick and Roth (1968) reported that plasmids of *S. aureus* were coded with genes responsible for resistance to antibiotics and metal ions. The best studied were the plasmids specifying penicillin-resistance (penicillinase plasmids), which also possess a variety of marker patterns including erythromycin resistance and resistance to inorganic cations and anions. Some other plasmids also have been identified in some clinical isolates of staphylococci that appeared to be genetically homologous to 'penicillinase' plasmids, which lack penicillinase gene but

contained cadmium-resistance genes and either this gene or penicillin resistance gene was invariably present in this class of plasmids.

Bardowski *et al.* (1985) reported that six out of 22 *S. aureus* and five out of 12 *S. epidermidis* isolates from bovine, contained one to three plasmids. Three of the *S. aureus* strains carried a 4.4 kb tetracycline resistance plasmid, which seemed to be identical with pT 181 tetracycline resistance plasmid isolated from *S. aureus* strain 4250 of human origin.

Mathew (1986) studied the staphylococci isolated from bovine mastitis and reported that the plasmid-mediated resistance against streptomycin only could be transferred to *S. aureus* RN450 RF, when CNS were used as donors. But, Mathew and Punnoose (1988) reported that when isolates of *S. aureus* from bovine mastitis cases were used as donors, plasmid-mediated resistances against tetracycline, streptomycin and penicillin could be transferred to *S. aureus* RN450 RF recipient.

Thompson and Holding (1986) reported that plasmids were more common in strains of *S. aureus* associated with live poultry than with strains endemic in poultry plants and strains of human origin. They have reported the presence of 1.65, 17 and 18.2 kbp plasmids in *S. aureus* isolates. They

have opined that 18.2 kbp plasmid-coded genes for caseolysis. Both 1.65 and 18.2 kbp plasmids were responsible for production of acid from lactose.

Schwarz and Blobel (1990) conducted restriction endonuclease analysis of 4.55 kb tetracycline resistance plasmid of *S. hyicus* from exudative epidermitis of pigs and named this plasmid as p ST 1.

Cardoso and Schwarz (1992) reported the presence of chloramphenicol resistance plasmids of 4.6 kb in 11 out of 217 *S. aureus* isolates from bovine subclinical mastitis.

Noble and Allaker (1992) observed that many plasmids were present in the 168 isolates of staphylococci from pigs and their attempts to study the resistance plasmids were frustrating because of the presence of large number of plasmids even in strains, which were resistant to only one antibiotic.

Matthews *et al.* (1992) reported that the plasmid profiles of staphylococci isolated from bovine mammary secretions were heterogeneous and plasmids were identified in 19 (20.2%) of 94 staphylococcal isolates. Number of plasmids per isolate varied from 1 to 4 and size of plasmids ranged from 1.2 to 45 MDa. They have observed that the

ability to differentiate isolates was not enhanced when antibiograms were used in conjunction with plasmid profiles.

Paul (1992) studied the plasmid profile analysis of 50 isolates of *S. aureus* from bovine mastitis by alkaline lysis method followed by agarose gel electrophoresis, and found 32 profiles (64%) with plasmids ranging from 1 to 7 in numbers and in few isolates, no plasmids were present.

Wegener and Schwarz (1993) studied the antibiotic-resistance and plasmids in 100 strains of *S. hyicus* isolated from pigs. Plasmid profiles of all *S. hyicus* strains studied have revealed the plasmid bands corresponding to molecular weight ranging from 1.5 kb to 19.7 kb in size. Plasmids in numbers from zero to five were detected in different strains and on an average, 2.1 plasmids per strain was isolated from diseased piglets and 1.6 plasmid per strain was isolated from healthy piglets. Nine strains contained no plasmids.

Todhunter *et al.* (1993) reported the plasmid content of CNS from 86 different bovine intramammary infections and plasmids were isolated from 30.2 per cent of CNS. Number of plasmid bands ranged from 1 to 5. With the exception of tetracycline resistance, the presence of plasmids was not related to antibiotic resistance.

Schwarz (1994) found chloramphenicol resistance (Cm^r) in fourteen of the eighteen *S. lentus* strains, but in none of the *S. intermedius* and *S. xylosus* strains used in the study. This was shown to be mediated by small plasmids of 3.6 to 4.6 kb and four different types of Cm^r plasmids, designated as p SC S14-17 could be obtained.

Schwarz and Noble (1994) reported the isolation of 47 tetracycline-resistant staphylococci from skin of pigs and they have observed the presence of six different plasmids in these isolates.

2.7 Plasmid transfer between staphylococci

Various plasmid transfer techniques like conjugation, transformation and protoplast fusion are reported in staphylococci.

2.7.1 Conjugation

It is a process whereby DNA is transferred from one bacterial cell to another by a mechanism requiring cell to cell contact. This process has invariably been found to be encoded by bacterial plasmids, and such plasmids are designated 'conjugative' (Willets and Skurray, 1980).

Conjugation was not considered as a mode of transfer of DNA between strains of staphylococci, while transduction and transformation were thought to be the only means of transfer of drug resistance between strains of staphylococci (Lacey, 1975).

Falkow (1975) opined that conjugation is a very rare event in staphylococci and therefore staphylococcal plasmids are not self-transmissible. According to Novick (1980) staphylococcal R-factors could not apparently promote their own transfer by conjugation.

Lacey (1980) reported the rarity of gene transfer between animal and human strains of *S. aureus in vitro*. Fawcett *et al.* (1981) reported the failure of CNS to transfer antibiotic resistance to *S. aureus* 1030 in mixed cultures. Forbes and Schaberg (1983) were the first investigators to report conjugation in staphylococci by filter-mating method, though they failed to transfer resistance markers in broth cultures. Mathew and Punnoose (1986) reported for the first time the conjugal transfer of streptomycin-resistant plasmid DNA from CNS to *S. aureus* RN450 RF in mixed cultures.

Mathew (1986) observed that drug-resistant *S. aureus* as well as CNS strains failed to transfer their resistances to *S. epidermidis* 131 S. They have also observed that only

streptomycin-resistance could be transferred from CNS to *S. aureus* RN450 RF.

Mathew and Punnoose (1988) reported that when *S. aureus* RN450 RF was used as recipient, six of the ten selected *S. aureus* isolates could transfer either one or more drug resistance markers (i.e. tetracycline, streptomycin and penicillin resistances) and the mode of transfer was suspected to be by conjugation.

Jones *et al.* (1987) reported the conjugative transfer of tetracycline resistance transposon-Tn 916 - by 2 conjugative plasmids of *Streptococcus faecalis* namely p AD 1:: Tn 916 and p AD 5:: Tn 916 to *S. aureus* by intergeneric membrane-filter matings.

Muhammad *et al.* (1993) reported the *in vitro* transferability study of penicillin, streptomycin, tetracycline and erythromycin resistances from CNS to *S. aureus*, and among CNS species of bovine mammary gland origin. They have conducted bacterial mating studies on filters and mixed-culture matings in broth and in skim milk. They have suggested that conjugation appeared to be the mode of streptomycin-resistance transfer and transfer of resistance between staphylococci of bovine mammary gland origin appeared to be fairly uncommon.

2.7.2 Protoplast fusion

The availability of literature regarding the protoplast fusion is scanty and this is only an artificial method of transfer of plasmids in bacteria.

Gotz *et al.* (1981) reported the protoplast fusion experiments and used the methods and media for preparation of protoplasts were mainly those described for *Bacillus subtilis* by different scientists (Wyrick and Rogers, 1973; Chang and Cohen, 1979). The authors have described the experimental conditions for plasmid transfer and genetic recombination in *S. aureus* and some CNS by protoplast fusion with the help of polyethylene glycol 6000 (PEG).

The protoplast fusion is unique mode of genetic exchange in prokaryotes, because the transfer of genetic information is bidirectional and entire chromosomes are combined in the same cytoplasm at high frequencies (Stahl and Pattee, 1983). They have also reported that the interest in protoplast fusion with *S. aureus* centered primarily on its potential as a supplementary technique for chromosome mapping.

Jones *et al.* (1987) reported the transfer of tetracycline resistance transposon, Tn 916 by two conjugative

plasmids p AD 1::Tn 916 and p AD 5::Tn 916 of *Streptococcus faecalis* to *S. aureus* by intergeneric protoplast fusions.

2.8 Numerical Index of Discrimination of typing methods

The ability of typing methods used alone, or in combination, to discriminate bacterial strains can be calculated by the Numerical Index of Discrimination (D). Gaston and Hunter (1989) have formed an equation to find out the Numerical Index of Discrimination of different typing methods.

$$D = 1 - \frac{1}{(N\ (N-1))} \sum_{j=1}^{s} nj\ (nj-1)$$

D = Numerical Index of Discrimination

s = Total number of types

nj = Number of strains belonging to the j^{th} type

N = Number of strains tested

Aarestrup *et al.* (1995a) have used the original formula of Gaston and Hunter (1989), in order to compare coagulase genetyping, ribotyping and their combination, used to type 187 *S. aureus* isolates of bovine mastitis origin.

Aarestrup *et al.* (1995b) have used a modified form of this formula to find the Numerical Index of Discrimination

of different typing methods i.e., biotyping, antibiogram typing, plasmid profiling, phage typing, ribotyping and their 26 combinations used for the typing of 105 *S. aureus* isolates from bovine mastitis in Denmark.

Materials and Methods

MATERIALS AND METHODS

Preparation and sterilization of glass wares

All the glasswares were boiled in 2.5 per cent solution of Extran-neutral (Merck) and washed thoroughly in tapwater and double distilled water, dried and sterilzed in hot air oven at 160°C for 1 h.

Sterile disposable syringes and needles were mostly used. When glass syringes and needles were used, they were sterilized by autoclaving.

Blood collection sets, rubber and plastic items

All the rubber and plastic items including micropipette tips and microfuge tubes were boiled in Extran-neutral solution, washed thoroughly in tap water and distilled water and dried in air overnight and autoclaved.

Blood collection sets were assembled after thorough washing, wrapped in aluminium foil and sterilized by autoclaving. After sterilization, the sets were dried at 42-43°C overnight.

Collection of milk samples

Mid-stream milk samples were collected into sterile vials using aspetic precautions from 70 clinical/ subclinical cases of bovine mastitis brought to the Veterinary Hospitals at Mannuthy and Trichur and Veterinary Clinic attached to University Livestock Farm, Mannuthy.

3.1 Isolation of staphylococci

The milk samples were immediately plated onto Mueller-Hinton Agar (Hi-media) and incubated at 37°C overnight. Colonies which were Gram-positive on staining and arranged as bunch of grapes were subcultured onto Staphylococcus Agar No. 110 (Hi-media) and incubated as earlier. After testing the purity of the culture, the colonies were subcultured on Tryptic Soy Agar (Hi-media) slants in duplicate tubes and incubated at 37°C for 24 h. and stored at 4°C for further studies (Mathew, 1986).

3.2 Characterization of staphylococci

The isolates were characterised by the following tests:

Catalase test, Oxidase test, Nitrate-reduction test, Voges-Proskauer test (V-P Test), Anaerobic fermentation of

glucose (using Hugh-Leifson medium) (Cowan, 1974; Benson, 1984).

3.3 Biotyping of staphylococci

The biotyping of the staphylococcal isolates were carried out by examining their ability to produce coagulase/ clumping factor, haemolysin, pigment and urease and to hydrolyse Tween-80 and casein (Hajek and Balusek, 1988; Paul, 1992).

3.3.1 Coagulase production

The coagulase production (free and bound) of the isolates were tested using rabbit plasma and bovine plasma as per the method described by Cowan (1974) and Benson (1984).

3.3.2 Haemolysin production

The production of alpha, beta and delta-haemolysins were tested on 5 per cent sheep and rabbit blood agar. Alpha and delta - haemolysins were detected by incubating the plates inoculated with the isolates, for 24 h. at 37°C and beta-haemolysin was detected by keeping the plates at 4°C for 24 h. after overnight incubation at 37°C.

3.3.3 Pigment production

The production of pigments by the staphylococcal isolates was tested on Nutrient Agar (Hi-media) containing 1 per cent Tween-80 (CDH) and on Milk Agar (Cruickshank *et al.*, 1975; Paul, 1992).

3.3.4 Urease production

The production of urease by the staphylococcal isolates was tested using Christensen's Urea Agar containing 2 per cent urea.

3.3.5 Tween-80 hydrolysis

Hydrolysis of Tween-80 was studied by incorporating 1 per cent Tween-80 in Nutrient Agar and incubating for 4 days at 37°C as per the method described by Cowan (1984).

3.3.6 Casein hydrolysis

Hydrolysis of casein by the isolates was studied on Milk Agar plates after inoculating the medium with the isolates and incubating at 37°C for 4 days as per the method described by Cruickshank *et al.* (1975).

3.4 Antibiogram of the isolates

Antibiotic sensitivity of the isolates of staphylococci was detected by Agar diffusion method (Kirby-Baur method) (Brown and Blowers, 1978).

3.4.1 Media

Mueller-Hinton Agar (Hi-media) was used throughout the study.

3.4.2 Antibiotic Sensitivity Discs

Antibiotic sensitivity discs supplied by M/s. Hi-media Lab, Bombay; M/s. Rhone-Poulenc Lab, Bombay and M/s. Difco Lab, USA were used in the study. The antibiotic discs used and their strength are furnished below:

	Antibiotic used	Abbreviations used	Strength (mcg or I.U./ disc)	Diameter of zone of inhibition indicating sensitivity (mm or more)
1.	Amoxycillin	Ax	10 mcg	20
2.	Ampicillin	Am	10 "	20
3.	Chloramphenicol	C	30 "	12
4.	Cloxacillin	Cx	5 "	9
5.	Erythromycin	E	15 "	13
6.	Flumequine	F	30 "	21

Contd.

Antibiotic used	Abbreviations used	Strength (mcg or I.U./ disc)	Diameter of zone of inhibition indicating sensitivity (mm or more)
7. Gentamicin	G	10 "	12
8. Kanamycin	K	30 "	13
9. Methicillin	M	5 "	9
10. Nalidixic acid	Na	30 "	13
11. Nitrofurantoin	Ni	300 "	14
12. Novobiocin	No	30 "	17
13. Pefloxacin	Pf	5 "	20
14. Penicillin-G	Pg	10 I.U	28
15. Polymixin-B	Pb	300 "	8
16. Rifampicin	R	5 mcg	16
17. Streptomycin	St	10 "	11
18. Sulfurazole	Sf	300 "	12
19. Tetracycline	T	30 "	14
20. Vancomycin	V	30 "	9

The discs were stored at 4°C

3.4.3 Procedure

Preparation of culture plates, preparation of inoculum, inoculation of plates, application of antibiotic discs, incubation, reading of zone of inhibition and reading the results were done as per method described by Brown and Blowers (1978).

3.5 Resistogram of the isolates

The resistance of staphylococci to heavy metal ions and certain chemical agents was detected using the method described by Novick and Roth (1968). The heavy metal salts and chemical agents with their strength are furnished below:

Metal salts/ chemical agents used	Abbrevi-ations used	Conc. of Solution	Strength (mcg/disc)
1. Acriflavin	Ac	0.024%	4.8
2. Ammonium molybdate	Ao	0.1 M	2,472
3. Antimony trichloride	At	0.1 M	456
4. Barium chloride	B	0.1 M	488
5. Boric acid	Bo	0.5 M	620
6. Cadmium chloride	Cd	0.001 M	3.68
7. Cetrimide (Cetyl trimethyl ammonium bromide)	Ct	3.64%	728
8. Copper sulphate	Cs	0.1 M	500
9. Crystal violet	Cv	0.000002	0.04
10. Ferrous sulphate	Fs	2 M	11,120
11. Iodine	I	0.1 M	508
12. Lead oxide	L	0.1 M	1,372
13. Mercuric chloride	Mc	0.001 M	5.44
14. Pot. permanganate	Pp	1 M	3,160
15. Pot. tellurite	Pt	Saturated solution	
16. Silver nitrate	Sn	0.1 M	340
17. Sodium arsenate	Sa	0.1 M	624

3.5.1 Preparation of discs

The salts of heavy metals and chemical agents were dissolved in double distilled water, kept at 4°C and used within a week. Sterile blank discs supplied by Hi-media were used in this study. The discs were impregnated with 20 mcl of the stipulated concentration of metal salt/chemical agent. The discs were dried at 37°C for 18 h. and stored at 4°C.

3.5.2 Testing the susceptibility/resistance

Testing the susceptibility/resistance of the isolates was done on Mueller-Hinton Agar using the method described for antibiotic susceptibility testing of bacteria by Kirby-Bauer method (Brown and Blowers, 1978).

3.5.3 Reading the results

The organism was considered resistant when there was profuse growth in contact with the disc containing the heavy metal salt/chemical agent and the organism was considered sensitive when there was wide zone of inhibition of bacterial growth around the discs (>10 mm).

3.6 Plasmid profile analysis

3.6.1 Buffers and reagents

The following buffers and reagents were prepared as per Maniatis *et al.* (1982), Sambrook *et al.* (1989) and Paul (1992).

(i) Luria-Bertani (LB) Medium (Maniatis *et al.*, 1982)

Bacto-tryptone	-	10 g
Bacto-yeast extract	-	5 g
NaCl	-	10 g
Distilled water	-	1000 ml

pH adjusted to 7.3, autoclaved at 121°C for 15 min. and stored at 4°C.

(ii) Tris EDTA Glucose Buffer (TEG) (Solution I)

Glucose	-	50 mM
Tris HCl (ph-8.00)	-	25 mM
EDTA (pH - 8.00)	-	10 mM

Autoclaved for 20 min. at 10 lb/sq.in. and stored at 4°C.

(iii) SDS-NaOH (Solution II)

NaOH - 0.2 N (freshly diluted from 10 N stock)

SDS - 1% (freshly diluted from 20% stock)

Taken in equal volumes and mixed together

(iv) Potassium Acetate Solution (Solution III)

5 M Potassium acetate	-	60 ml
Glacial acetic acid	-	11.5 ml
Distilled water	-	28.5 ml

pH adjusted to 4.8 with glacial acetic acid and autoclaved at 121°C for 15 min.at 15 lb/sq.in. and stored at 4°C.

(v) Tris EDTA Buffer (TE)

Tris HCl (pH - 8.00)	-	10 mM
EDTA (pH - 8.00)	-	1 mM

Sterilized by autoclaving at 121°C for 15 min. at 15 lb/sq.in. and stored at 4°C.

TE butter of pH - 7.5 also prepared, sterilized and stored at 4°C.

(vi) Tris Borate Electrophoresis Buffer (TBE) (5x)

Tris base	-	54.0 g
Boric acid	-	27.5 g
0.5 M EDTA (pH-8.00)	-	20 ml
Distilled water	-	1000 ml

The concentrated stock solution (5x) was diluted to 1x before use. The stock solution was stored at 4°C.

(vii) Gel-loading Buffer

Bromophenol Blue	-	0.25%
Sucrose in Water	-	40% (W/V)

Stored at 4°C

(viii) Ethidium Bromide Solution (10 mg/ml)

One gram of ethidium bromide was added to 100 ml of distilled water. The solution was stirred on a magnetic stirrer for several hours till the dye was dissolved. The solution was transferred to a dark bottle, wrapped with aluminium foil and stored at room temperature.

(ix) Sodium Dodecyl Sulphate (10%)

Hundred gram of electrophoresis grade SDS was dissolved in 900 ml of double distilled water and heated to 68°C. The

pH was adjusted to 7.2 with concentrated HCl. The volume was adjusted to 1000 ml and dispensed into 50 ml aliquots.

(x) Distilled ethanol(xi) Lysozyme solution

Stock solution of lysozyme at the concentration of 50 mg/ml was prepared in double distilled water and dispensed in aliquots of 2 ml and stored at -20°C. Each aliquot was discarded after one. Working solution was prepared to contain lysozyme at the rate of 5 mg/ml in TEG buffer (pH-8.0).

(xii) Lysostaphin solution

Stock solution of lysostaphin was prepared to contain lysostaphin at the rate of 1 mg/ml in TE buffer (pH-7.5) and stored at -20°C. The working solution was prepared so as to have a final concentration of 100 mcg of lysostaphin per ml.

3.6.2 Plasmid isolation and characterization

Plasmid isolation was done by alkaline lysis method of Brinboim and Doly (1979) modified by Sambrook *et al.* (1989). The additions of lysozyme and lysostaphin were carried out as per Paul (1992).

3.6.2.1 Harvesting and lysis of bacteria

3.6.2.1.1 Harvesting

A single colony of staphylococcal isolate was inoculated in 5 ml of LB medium and incubated overnight at 37°C on a bacteriological shaker.

The culture was transferred to 1.5 ml microfuge tube and centrifuged at 7,500 x g for 10 min. at 4°C.

The medium was removed by aspiration, leaving the pellet as dry as possible.

3.6.2.1.2 Lysis by alkali

The lysozyme (SRL) was added from the stock solution to the ice cold solution I of TEG buffer to a final concentration of 5 mg/ml. The Lysostaphin (Sigma) was added from the stock solution to the TE buffer (pH-7.5) so as to have a final concentration of 100 mcg/ml. The bacterial pellet was resuspended in 70 mcl of lysozyme solution and 70 mcl of lysostaphin solution by vortexing. Incubated in a water bath at 37°C for 45 min.

Afterwards 200 mcl of freshly prepared solution II of 0.2 N NaOH and 1 per cent SDS was added. The tubes were closed tightly and the contents were mixed by inverting the

tubes 3-4 times, while the tubes were incubating over the ice for 15 min.

Then 150 mcl of ice cold solution III of 5 M potassium acetate was added. The tubes were closed and mixed by inverting for 3-4 times, while the tubes were incubating over the ice for 15 min.

The tubes were then centrifuged at 13,000 x g for 10 min. at 4°C. The measured volume of supernatent was transferred to a fresh tube.

The supernatent was precipitated with double volume of cold ethanol and mixed by tapping. Covered with 'Parafilm M' (American Can Co.) and kept at -20°C overnight.

On the next day, the tubes were centrifuged at 16,000 x g for 10 min. at 4°C and the supernatent was removed.

The pellet was aspirated thoroughly and the pellet was vacuum dried for 15 min.

The pellet was dissolved in 20 mcl of TE buffer (pH-8) and plasmid DNA was stored at -20°C till used, after wrapping with 'Parafilm M'.

3.6.2.2 Agarose gel electrophoresis

The method described by Meyers *et al.* (1976) was used. The agarose gel electrophoresis was done using submarine gel electrophoretic system.

The gel tray (13x13 cm) was sealed with adhesive cellophane tape on either sides and a comb with 11 teeth (5 x 1.5 mm/tooth) was clamped on it, 0.5 to 1 mm above the tray surface, so as to form completely sealed wells. To prepare 0.9 per cent of agarose gel with 4 mm thickness, 630 mg of low EEO agarose (Sigma) was dissolved in 70 ml of Tris-borate buffer (TBE, pH-8).

The slurry was heated in a boiling water bath until the agarose was dissolved. After cooling to 50°C, the agarose solution was poured into sealed gel tray and allowed to set for 30 min.at room temperature. The comb and adhesive tape were removed and the tray was kept submerged in TBE (pH-8) in the electrophoretic tank.

To 20 mcl each of plasmid DNA preparations, 2 mcl of Bromophenol blue gel-loading buffer was mixed and the mixture was loaded in the 11 wells of the submerged gel by a micropipette.

The electrophoresis was carried out along with marker DNA at 100 V and 35-40 mA for 3 h. or until the tracking dye

(Bromophenol blue) reached the appropriated distance through the gel.

The gel was stained by immersing in distilled water containing ethidium bromide (0.5 mcg/ml) for 30-45 min. at room temperature and then destained by soaking in distilled water for 20 min.

The gel was visualised under UV illumination using UV trans-illuminator.

3.6.2.3 Plasmid DNA photography

The photographs of plasmid DNA containing gels were taken under UV illumination using 100 ASA (Konica) 135/36 colour film at '4' aperture for an exposure time of 6-10 sec using ordinary 35 mm. Single Lense Reflex (SLR) photographic camera with or without yellow filter. Developing of the film and printing of the photographs were done on film and print processors (Karpe, 1993).

3.6.2.4 Molecular size determination of plasmids

Molecular sizes of the plasmids were examined by employing *E. coli* V 517 strains of Marcina *et al.* (1978). The plasmid DNA separated from *E. coli* V 517 containing 8 plasmid types ranging from 2.1 to 54 kb size was run simultaneously in each gel. The distance migrated by each

plasmid of *E. coli* V 517 from the bottom of the well to the leading edge of each plasmid band was measured in centimeter from the gel. By plotting the values of distance migrated by plasmid DNA fragments (X-axis) versus the log 10 kb values of the molecular sizes of the plasmid DNA (Y-axis) on ordinary graph paper a linear curve was obtained. The molecular sizes of plasmids from staphylococcal isolates were determined by interpolation of linear curve from the values of distance migrated in cm. The log value so obtained on Y-axis (log 10 kb) was calculated to antilog so as to obtain the molecular size in kilo base (kb) of the plasmids of staphylococcal isolates under investigation.

3.7 *In vitro* transfer of plasmids

3.7.1 Conjugation studies

The method described by Datta (1978) with modifications suggested by Mathew and Punnoose (1986) was used.

3.7.1.1 Donor strains

Six multiple-drug resistant staphylococci, which are sensitive to rifampicin were selected as donors.

Sl. No.	Donor	Coagulase production	Haemolysin production (Hly)	Drug resistance
1.	987	+	A and B	Am^r, Pg^r
2.	A	-	A	Am^r, Pg^r, G^r, S^r
3.	303-R	+	A	Am^r, Pg^r, S^r
4.	228	-	A	Am^r, Pg^r, G^r, S^r, E^r
5.	2Y	+	A	Am^r, Pg^r, G^r, S^r, E^r
6.	352	-	'-' on sheep blood agar	Am^r, S^r, E^r

3.7.1.2 Recipient strains

S. aureus RN450 RF phage-free, plasmid-free, haemolysin-negative, having chromosomal-resistance to rifampicin and fusidic acid (Rif^r and Fus^r).

3.7.1.3 Antibiotic stock solutions

Pure antibiotics obtained from M/s. Central Drug Research Lab., Calcutta were used in the experiment.

A stock solution of the antibiotic containing 2000 mcg/ml or I.U/ml was prepared and stored at -20°C in 1 ml aliquots for subsequent use (Barry, 1976).

The activity standards of different antibiotics are as follows:

Antibiotics	Activity standard (mcg or IU/mg)
Ampicillin	995
Penicillin-G	1658 IU
Gentamicin	641
Streptomycin sulphate	748
Erythromycin	920
Rifampicin	959.4

$$\text{Volume of solvent (ml)} = \frac{\text{Wt. of sample (mg) x Activity Std. of antibiotic (mcg or IU/mg)}}{\text{Desired concentration (mcg/ml)}}$$

The following solvents and diluents were used (Barry, 1976):

Sl. No.	Antibiotic	Solvent	Diluent
1.	Ampicillin	Phosphate buffer (pH-8; 0.1 M)	Phosphate buffer (pH-6; 0.1 M)
2.	Penicillin-G	Water	Water
3.	Gentamicin	Phosphate buffer (pH-8; 0.1 M)	Water
4.	Streptomycin	Water	Water
5.	Erythromycin	Ethanol	Water
6.	Rifampicin	Dimethyl sulfoxide	Phosphate buffer (pH-7)

3.7.1.4 Media

(i) Tryptic soy broth (Hi-media)

(ii) Selective Media I and II

a. Selective Medium I

Mueller-Hinton Agar was reconstituted and sterilized by autoclaving and kept at 45-50°C waterbath. The selective media contained rifampicin and any one of the antibiotic to which the donor was resistant. The selective media were prepared by adding Rifampicin and Ampicillin (Ia), Rifampicin and Penicillin-G (Ib), Rifampicin and Gentamicin (Ic), Rifampicin and Streptomycin (Id), Rifampicin and Erythromycin (Ie) into Mueller-Hinton Agar contained in different flasks and kept at 45°C and mixed by rotating the flask and poured into sterile petridishes. When solidified, the moisture on the surface of agar was removed by placing them at 37°C for 30 minutes. The concentration of different antibiotics contained in different media is as follows: (Barry, 1976; Mathew, 1986).

Sl. No.	Antibiotic	Abbreviations used	Concentration (mcg or IU/ml)
1.	Ampicillin	Am	32
2.	Penicillin-G	Pg	1.67 IU
3.	Gentamicin	G	6
4.	Streptomycin	S	15
5.	Erythromycin	E	8
6.	Rifampicin	R	2.5

b. Selective Medium II (Sheep blood agar for haemolysin detection)

Haemolysin production by transconjugants was detected on 5 per cent sheep blood agar plates containing 2.5 mcg of rifampicin per ml.

3.7.1.5 Procedure

The donor and recipient organisms were cultivated separately in Tryptic Soy broth for 6 h. Then 0.1 ml of the donor culture and 0.2 ml of recipient culture were co-cultivated in 10 ml of Tryptic Soy broth at 37°C overnight. This was centrifuged at 3000 rpm at 4°C for 30 min. The sediment was plated on selective medium I and incubated at 37°C for 24 h. The donor and recipient controls

were plated separately on selective media I and also on ordinary Mueller-Hinton Agar. The colonies coming up on selective medium I - transconjugants - were purified by subculturing on selective medium I. The drug resistance pattern of the transconjugants was detected by Agar diffusion method of Kirby and Bauer (Brown and Blowers, 1978).

The transfer of haemolysins (alpha and beta) in conjugation experiment was detected by subculturing the transconjugants on selective medium II.

3.7.1.6 Experiment to exclude the possibility of transduction and transformation

Cell free filtrate of an overnight culture of the donors in Tryptic Soy broth were prepared by filtering through 450 nm (Millipore filter) and 200 nm (Sartorius filter) membrane filters. Three milliliter of cell free filtrate of each donor was mixed separately with three milliliters of an overnight culture of the recipient and incubated at 37°C for 6-8 h. The mixture was plated onto selective medium I (a, b, c, d and e) and selective medium II and incubated at 37°C. After 24-48 h. incubation, the plates were examined for the presence of any colonies. Recipient and filtrate controls were plated separately, in

selective medium I (a, b, c, d and e) and selective medium II (Mathew and Punnoose, 1986).

3.7.2 Protoplast fusion studies

Protoplast fusion studies of 6 donor field strains and recipient *S. aureus* RN450 RF were carried out as per Gotz *et al.* (1981) with slight modification suggested by Stahl and Pattee (1983).

3.7.2.1 Donor strains : Same as conjugation studies.

3.7.2.2 Recipient strains : Same as conjugation studies.

3.7.2.3 Antibiotic stock solutions: Same as conjugation studies.

3.7.2.4 Media and chemicals.

(i) Hypertonic Buffer (Sucrose-Magnesium-Tris buffer) SMTB (Stahl and Pattee, 1983)

Tris - 100 m M

$MgSO_4$ - 40 m M

Sucrose - 800 mM

(ii) Penassay Broth (Difco manual, 1960)

Beef extract - 1.5 g

Yeast extract	-	1.5 g
Peptone	-	5 g
Dextrose	-	1 g
NaCl	-	3.5 g
K_2HPO_4	-	3.68 g
KH_2PO_4	-	1.32 g
Distilled water	-	1000 ml
pH	-	7.0

(iii) Hypertonic Buffer Medium (HBM) (Gotz *et al.*, 1981)

Penassay broth powder	-	35 g
Hypertonic buffer	-	1000 ml
pH	-	7.0

Sterilized by autoclaving at 10 lb/sq.in. for 20 min. and stored at 4°C.

(iv) Polyethylene Glycol Solution (PEG 6000-40%) (Gotz *et al.*, 1981)

Polyethylene glycol 6000	-	40 g
Hypertonic buffer (pH 7.6)	-	100 ml

Polyethylene glycol and hypertonic buffer were sterilized separately by autoclaving at 10 lb/sq.in. and later mixed thoroughly with aseptic precautions.

(v) Bovine Serum Albumin Solution (BSA-0.08%) (Gotz *et al.*, 1981)

Hypertonic buffer medium	-	100 ml
Bovine serum albumin (BSA)	-	80 mg

Sterilized by filtration through membrane filter of 450 nm (millipore filter).

(vi) Regeneration (R) Medium (Stahl and Pattee, 1983)

Trypticase Soy Broth (Hi-media)	-	30 g
Sucrose (SRL)	-	273 g
$C_6H_5O_7Na_3.2H_2O$	-	0.5 g
Starch	-	2.1 g
Agar (Hi-media)	-	25 g
Deionised double distilled water	-	1000 ml
pH	-	7.2

Sterilized by autoclaving at 115°C (10 lb/sq.in.) for 20 min.

(vii) IM $CaCl_2$ Solution

$CaCl_2.2H_2O$	-	14.7 g
Distilled water	-	100 ml

Sterilized by autoclaving at 15 lb/sq.in. for 15 min.

(viii) DNase I from bovine pancreas (Sigma) was prepared in HBM at the concentration of 50 mcg/ml, lysozyme (SRL) was prepared in Tris EDTA glucose buffer (pH 8.0) at the concentration of 2000 mcg/ml and lysostaphin (Sigma) was prepared in Tris EDTA buffer (pH 7.5) at the concentration of 1 mg/ml. Stock solutions of these agents were filter-sterilized and stored in aliquots of 1 ml at -20°C.

3.7.2.5 Procedure

3.7.2.5.1 Preparation of protoplasts

Ten millilitre samples of donor and recipient strains of staphylococci grown in Trypticase Soy Broth (Hi-media) to the stationary phase was harvested by centrifugation at 10,000xg for 25 min at 4°C and suspended to the same volume of HBM. Lysostaphin (Sigma) and lysozyme (SRL) were added to a final concentration of 20 and 2,000 mcg/ml, respectively and cell suspension were incubated at 37°C for 4-5 h. with gentle shaking. The intact bacteria and cell debris were pelleted by centrifugation at 2,500xg for 8 min at 4°C. The supernatants were collected and centrifuged again at 16,000xg for 10 min. at 4°C. The pelleted protoplasts were resuspended in HBM to 1/10 the volume of starting culture. In control experiments with DNase treatment, 50 mcg of DNase I (Sigma) per ml was added during

both preparation and fusion of the protoplasts (Gotz *et al.*, 1981).

3.7.2.5.2 Protoplast fusion

For protoplast fusion, equal amounts of protoplasts suspensions were mixed, and to 0.4 ml of the mixture was added 0.04 ml of IM $CaCl_2$ solution followed by 2 ml of PEG (SRL) 40 per cent solution. The fusion mixture was then gently, but thoroughly mixed and incubated in a water bath at 20°C without shaking for 3 min. After 3 min. of incubation the protoplast mixtures were diluted with 7 ml of HBM containing 0.08 per cent BSA. For each donor strain 0.1 ml of the fusion mixture was spread gently with glass spreaders on Regeneration (R) Medium agar plates, which were containing Rifampicin and any one of the 5 antibiotics to which the donor organism was resistant, in the same concentration as in selective medium I (a, b, c, d and e) for conjugation experiments. Thus these media served as Regeneration cum selective medium I for detecting the transfer of plasmid-coded drug resistance by protoplast fusion. The agar plates were incubated at 35°C (Rh - 70%) for 7 days (Gotz *et al.*, 1981).

The fusion mixtures obtained in the control experiments also were treated in the above fashion to exclude the false-positive results.

The colonies developed on the Regeneration cum selective medium I - biparental strains - were further confirmed for transfer of drug resistance by agar diffusion method (Brown and Blowers, 1978).

The plasmid-coded haemolysin transfer by protoplast fusion was detected by plating the fusion mixtures on plain Regeneration medium agar and incubating at 35°C (Rh - 70%) for 7 days. The biparental strains were subsequently inoculated on 5 per cent defibrinated sheep blood agar plates and incubated at 37°C for 48 h. and then kept at 4°C for 24 h to detect haemolysin transfer (alpha and beta) by protoplast fusion.

3.8 Numerical Index of Discrimination of typing methods

The ability of different typing methods used alone, or in combination, to discriminate staphylococcal strains can be calculated by the Numerical Index of Discrimination (D) (Gaston and Hunter, 1989). This value measures the probability that two isolates of different clonal origin chosen at random will be assigned to different types, by the typing methods used (Aarestrup *et al.*, 1995a). The formula used for finding this index is as follows:

$$D = 1 - \frac{1}{(N\ (N-1))} \sum_{j=1}^{s} nj\ (nj-1)$$

D = Numerical index of discrimination

s = Total number of types

nj = Number of isolates belonging to the j^{th} type

N = Number of isolates tested

Results

RESULTS

4.1 and 4.2 Isolation and characterization of staphylococci

Bacterial organisms were isolated from 52 (74.29%) milk samples, out of the 70 samples tested and out of this, 26 isolates (50%) were Gram-positive cocci appearing as bunch of grapes.

The Gram-positive coccal organisms appearing as bunch of grapes, were subjected for further studies and all the 26 isolates were identified as staphylococci (Table 1).

4.3 Biotyping of staphylococci

Biotyping of the isolates was done based on different biochemical tests and the results of the tests, viz., coagulase production, haemolysin production, pigment production, urease production, Tween-80 hydrolysis and casein hydrolysis are presented in Table 2 and 3.

4.3.1 Coagulase production

Out of the 26 isolates, 4 (15.38%) and 12 (46.15%) isolates were positive for tube and slide tests respectively, using bovine plasma. All the four isolates

positive for the tube test were also positive for slide test except one isolate. The 13 (50%) isolates were CPS and the rest were CNS using bovine plasma.

With rabbit plasma, 9 (34.62%) and 8 (30.76%) isolates were positive for tube and slide tests, respectively. Seven (26.92%) isolates were positive for both tube and slide tests. Two isolates were positive for only tube test, while one isolate was found positive for only slide test. A total of 10 isolates (38.46%) were CPS and the rest were CNS with rabbit plasma.

Six (23.07%) isolates were positive for tube or slide test using both rabbit and bovine plasma. Altogether 17 (65.38%) isolates were found positive for coagulase production (Plate 1).

4.3.2 Haemolysin production

Haemolysin production by the 26 isolates was detected on 5 per cent sheep and rabbit blood agar plates. Sixteen (61.53%) isolates were found positive for haemolysin production.

Out of the 26 isolates, 11 (42.31%) were positive for haemolysins on sheep blood agar. Nine (34.62%) isolates were positive for alpha-haemolysin, 4 (15.38%) isolates

were positive for beta-haemolysin and one (3.85%) isolate was positive for delta-haemolysin. Three isolates (11.54%) were positive for both alpha and beta-haemolysins on sheep blood agar.

On rabbit blood agar, 11 (42.31%) isolates were positive for alpha-haemolysin. But beta and delta-haemolysin production could not be detected on rabbit blood agar plates.

Six isolates (23.08%) produced haemolytic activity on both sheep and rabbit blood agar plates.

Altogether 16 (61.54%) isolates were found to be haemolysin producers (Plate 2).

4.3.3 Pigment production

The pigments produced by different isolates ranged from light yellow, cream, golden yellow or orange red. Some buffy coloured isolates also could be obtained.

Only 16 (61.54%) isolates were producing pigments, both on Tween-80 agar and Milk agar. But 2 (7.69%) isolates each, though produced pigment on Milk agar, did not produce pigment on Tween-80 agar and vice versa. Altogether 20 (76.92%) isolates were pigment producers (Plate 3 and 4).

4.3.4 Urease production

Out of 26 isolates of staphylococci, 17 (65.38%) were positive for urease production (Plate 5).

4.3.5 Tween-80 hydrolysis

Out of 26 isolates, 10 (38.46%) were positive for hydrolytic action on Tween-80 (Plate 3).

4.3.6 Casein hydrolysis

Out of 26 isolates, 8 (30.76%) isolates were positive for hydrolytic action on casein (Plate 4).

The criteria for grouping the staphylococcal isolates into different biotype groups are shown in Table 4. An isolate is considered positive for a particular character/ reaction when it gives positive reaction in any one of the different tests carried out to find out the reaction.

Nineteen biotypes (Biotype-A to S) were formed based on different characteristics of the isolates like production of coagulase, haemolysin, pigment and urease and hydrolysis of Tween-80 and casein on the respective media. The difference in the reaction of any one of these 6 characters was the basis for the formation of a biotype.

The details of biotyping of the staphylococcal isolates into 19 biotypes (Biotype-A to S) are presented in Table 5. The biotype containing the maximum number of the isolates is Biotype-B, with 5 isolates (19.23%). This is followed by Biotype-H with 3 isolates (11.54%) and Biotype-P with 2 isolates (7.69%). All the other 16 biotypes have got single isolate (3.85%) only. Biotype-A and B contain the most virulent isolates among the 19 biogtypes, as the isolates in these two biotypes were positive for almost all the criteria used in biotyping.

4.4 Antibiogram of staphylococci

The results of antibiotic sensitivity tests are presented in Table 6. The percentage of resistance to various antibiotics are presented in Table 7.

The maximum resistance (61.54%) was observed in the case of ampicillin followed by nalidixic acid (50.00%) and methicillin and amoxycillin (42.31% each). The minimum resistance was noticed to chloramphenicol and vancomycin (0% each); cloxacillin, nitrofurantoin, pefloxacin and polymixin-B (7.69% each); gentamicin and tetracycline (15.38% each) followed by kanamycin (19.23%).

Among the 26 isolates, one was sensitive to all the antibiotics (3.85%), 3 isolates were monoresistant (11.54%)

and 22 (84.61%) were multiple resistant. Among the multiple resistant organisms, 4 isolates were double antibiotic resistant (15.38%), 3 isolates were triple resistant (11.54%), 4 isolates were quadruple resistant (15.38%), one isolate each was quintuple and sextuple resistant (3.85%), 2 isolates were septuple resistant (7.69%), one isolate was octuple resistant (3.85%) and 3 isolates were nonuple resistant (11.54%). One isolate (3.85%) each was resistant to 10, 11 and 14 antibiotics tested. Twenty-six different patterns of antibiotic resistances were obtained. Among the 25 drug-resistant isolates 25 different patterns of antibiotic resistances ranging from one to fourteen antibiotics were observed. One isolate (Isolate No.460) was sensitive to all the antibacterial agents tested and so its antibiogram was without resistance against any antibacterial agent.

4.5 Resistogram of staphylococci

The resistogram of 26 staphylococcal isolates are presented in Table 8. the maximum degree of resistance (96.15%) was noticed against barium chloride and potassium permanganate followed by lead oxide (92.31%), boric acid (88.46%), sodium arsenate (53.85%), acriflavin (42.31%), ammonium molybdate (26.92%), silver nitrate (19.23%), cadmium chloride and mercuric chloride (11.54% each) and

crystal violet (7.69%). None of the isolates was found resistant to antimony trichloride, centrimide, copper sulphate, ferrous sulphate, iodine and potassium tellurite. The details of percentage of resistance to metal ions and chemical agents are furnished in Table 9.

Among the 26 staphylococcal isolates, one isolate (3.85%) was resistant to single metal salt/chemical agent, 2 isolates (7.69%) each were triple and quadruple resistant, 8 isolates (30.77%) were quintuple resistant, 6 isolates (23.08%) were sextuple resistant, 5 isolates (19.23%) were septuple resistant and 2 isolates (7.69%) were octuple resistant.

Twenty different patterns of resistance to metal salts/ chemical agents were recognised among 26 staphylococcal isolates. The details are presented in Table 10. The most common pattern of resistance was the multiple resistance noticed among 15.38 per cent of the isolates against acriflavin, barium chloride, boric acid, lead oxide and potassium permanganate. This was followed by multiple resistance against barium chloride, boric acid, lead oxide, potassium permanganate and sodium arsenate shown by 11.54 per cent of the isolates.

4.5.1 Comparison between resistance to antibacterial drugs and metal salts/chemical agents

Of the 25 antibiotic resistant isolates 16, 13, 11, 11, 10, 10, 9, 8, 8, 7, 7, 5, 4 4, 2, 2, 2, 2, 0 and 0 isolates were resistant to Am, Na, Ax, M, E, Sf, St, F, Pg, No, R, K, G, T, Cx, Nf, Pf, Pb, C and V respectively, making 131 individual resistances (Table 11). Among these Pp revealed 99.24 per cent resistance, B (98.47%), L (97.71%), Bo (96.18%), Sa (63.36%), Ac (48.85%), Ao (34.35%), Sn (17.56%), Mc (12.21%), Cr (11.45%) and Cd (8.4%).

Similarly Ax, Cx, E, F, G, K, M, Nf, No, Pf, Pg, Pb, R, St and T resistant isolates also revealed 100 per cent correlation between B, Bo and Pp resistances of the isolates. The percentages of correlation between resistances to different antibiotics and that of metal salts/chemical agents are shown in the Table 11.

The comparison of the coagulase production, haemolysin production, antibiogram and resistogram of the isolates are presented in Table 12.

4.6 Plasmid profile analysis

Only 16 staphylococcal isolates revealed the presence of plasmids by AGE out of the 26 isolates studied and these

isolates contained 1, 2, 4 or 5 plasmids. Among the 16 isolates carrying plasmids, no two isolates had same plasmid profile, i.e., all the 16 profiles were different in nature (Plate 6). But totally 17 plasmid profile types were formed. All the 10 isolates lacking plasmid were grouped into a single type and this type was having the maximum number of isolates.

The molecular size in kilobase (kb) were calculated by plotting the values of distance migrated in centimetres (cm) by the plasmid DNA fragments, versus the log 10 kb values of the molecular sizes of corresponding plasmids obtained from *E. coli* V 517, the molecular weight reference plasmid strain. The standard smooth linear curve was obtained on the graph paper (Fig.1).

The molecular size of the plasmids of staphylococcal isolates was determined by interpolation of standard linear curve from values of distance migrated in centimetres by the plasmid DNA fragments. The log value so obtained after interpolation on Y-axis (log 10 kb), was converted into antilogarithmic values, so as to estimate the molecular size in kb, of the particular plasmid DNAs. The details are given in Table 13.

The molecular size of the plasmids from the staphylococcal isolates are listed in Table 14. Sixteen

isolates were carrying 1 to 5 numbers of plasmids of different molecular sizes ranging from 2.45 to 47.32 kb (Plate 6). The 2.45 and 47.32 kb plasmids were the smallest and largest plasmids respectively, obtained in the study and were seen in the same isolate (JA).

4.6.1 Comparison between plasmid profile and virulence factors (coagulase and haemolysin productions)

Eight isolates (80%) out of the 10 isolates which were producing both coagulase and haemolysin, were found harbouring one or more plasmids and most of these isolates were multiple drug resistant. The 2 isolates (987 and A) harbouring 3.89 kb plasmid, were found to be haemolysin producers. The isolates 383 and 483, harbouring 3.55 kb plasmid, were found to be positive for coagulase and haemolysin production. Similarly another two isolates 1025 and 352, possessing 3.63 kb plasmid found to be positive for haemolysin, but negative for coagulase (Table 15).

4.6.2 Comparison between plasmid profile, antibiogram and resistogram

The comparison between plasmid profile, antibiogram and resistogram of the staphylococcal isolates are presented in Table 15.

In general, it is seen that, isolates carrying plasmids were multiple-drug resistant and multiple metal salt/chemical agent resistant, except for isolates 383, 483, 460 and 369. The isolate 383 carries a 3.55 kb plasmid, but it is single antibiotic resistant (streptomycin-resistant). Isolate 483 carries 2 plasmids of 4.39 and 3.55 kb and it is resistant to 6 metal salt/chemical agents and one antibacterial agent i.e., sulfurazole only. Isolate 460 carries a 3.35 kb plasmid, but it is sensitive to all the drugs tested. Similarly 369 carries 2 plasmids of 13.8 and 4.37 kb, but resistant to nalidixic acid, sulfurazole and chemical agent barium chloride only.

4.7 Plasmid transfer techniques

Results of conjugation and protoplast fusion experiments are presented in Table 16.

4.7.1 Conjugation experiment

All the six staphylococcal isolates transferred resistances against more than one drug to the recipient, by conjugation. All of them transferred their Am-resistance. All the five isolates, which were penicillin-resistant, could transfer this resistance to the recipient. Among the 5 streptomycin resistant isolates, 4 transferred the resistance. Three isolates each were resistant to

gentamicin and erythromycin, but only one isolate each transferred these resistances.

Regarding transfer of haemolysin production, only one isolate (987) transferred its beta-haemolysin character. None of the isolates producing alpha-haemolysin (including 987) transferred their alpha-haemolysin character to the recipient.

4.7.2 Protoplast fusion experiment

Only two isolates (228 and 2Y) transferred their resistances to ampicillin, penicillin and streptomycin to the recipient by protoplast fusion, but none of them transferred gentamicin and erythromycin resistances. Both these isolates transferred alpha-haemolysin to the recipient.

The pattern of transfer of antibiotic resistances in these two isolates 228 and 2Y were similar in conjugation and protoplast fusion experiments.

4.8 Determination Numerical Index of Discrimination of different typing methods

Different methods employed for typing the staphylococcal isolates, were evaluated based on their

Numerical Indices of Discrimination. There were 4 methods and 11 combinations of these 4 methods. The Numerical Index of Discrimination, number of types formed and prevalence of dominating types in each method of typing and combination of methods are furnished in Table 17. The antibiogram typing and all combinations formed with antibiogram typing have got maximum index (i.e. one) and these methods grouped the 26 isolated into 26 different types with only single isolate in each type (i.e. prevalence of dominating type is 3.85%). It is seen that when number of types increases for a particular typing method, its 'D' value also increases. The least efficient typing method among the different methods and their combinations is plasmid profiling and it has got least 'D' value (0.862), least number of types (17) and highest prevalence of dominating type (38.46%) among all the methods and their combinations.

Table 1. Characteristics of Gram-positive cocci appearing as bunch of grapes

Isolates	Gram's staining	Oxidase test	Catalase test	Nitrate reduction test	Anaerobic fermentation of glucose	V-P test
2	+	-	+	-	+	+
799	+	-	+	+	+	+
987	+	-	+	+	+	+
A	+	-	+	-	+	+
461	+	-	+	+	+	+
303-R	+	-	+	+	+	+
KLA	+	-	+	+	+	+
9/94	+	-	+	-	+	+
117-M	+	-	+	-	+	+
228	+	-	+	-	+	+
970	+	-	+	-	+	-
TUBE	+	-	+	+	+	+
303-L	+	-	+	+	+	+
383	+	-	+	+	+	+
NEW	+	-	+	+	+	+
2Y	+	-	+	+	+	-
483	+	-	+	+	+	+
460	+	-	+	+	+	+
369	+	-	+	+	+	+
488	+	-	+	+	+	+
1025	+	-	+	-	+	-
1042	+	-	+	+	+	+
1274	+	-	+	+	+	+
JA	+	-	+	+	+	+
352	+	-	+	+	+	-
374	+	-	+	+	+	-

+ positive reaction; - negative reaction

Table 2. Coagulase production, haemolysin production and pigment production of staphylococcal isolates

Isolates	Coagulase production				Haemolysin production				Pigment production	
	Rabbit plasma		Bovine plasma		Sheep blood			Rabbit blood	Tween-80 agar	Milk agar
	Tube test	Slide test	Tube test	Slide test	alpha	beta	delta	alpha		
2	-	-	-	-	+	-	-	+	-	-
799	-	-	-	+	-	-	-	-	+	+
987	+	+	+	+	+	+	-	-	+	+
A	-	-	-	-	+	-	-	+	-	-
461	+	+	-	-	-	-	-	+	+	+
303-R	+	+	-	+	+	-	-	-	+	+
KLA	+	-	-	-	-	-	-	+	-	-
9/94	-	-	-	-	-	-	-	-	+	+
117-M	-	-	-	-	-	-	-	-	+	+
228	-	-	-	-	+	-	-	+	-	-
970	-	-	-	-	-	-	-	-	+	+
TUBE	+	-	-	-	-	-	-	-	+	+
303-L	+	+	-	+	+	-	-	-	+	+
383	+	+	+	+	+	+	-	+	+	+
NEW	-	+	-	-	-	-	-	-	-	-
2Y	-	-	-	+	+	-	-	+	+	-
483	+	+	+	-	-	-	+	+	+	+
460	+	+	+	+	+	+	-	-	+	+
369	-	-	-	+	-	-	-	-	-	-
488	-	-	-	+	-	-	-	+	+	+
1025	-	-	-	-	-	+	-	-	+	-
1042	-	-	-	+	-	-	-	-	+	+
1274	-	-	-	-	-	-	-	+	-	+
JA	-	-	-	+	-	-	-	-	-	+
352	-	-	-	-	-	-	-	+	+	+
374	-	-	-	+	-	-	-	-	+	+

Table 3. Biochemical characteristics of staphylococcal isolates

Isolates	Coagulase production	Haemolysin production	Pigment production	Urease production	Tween-80 hydrolysis	Casein hydrolysis
2	-	+	-	-	-	-
799	+	-	+	-	-	+
987	+	+	+	+	+	-
A	-	+	-	-	-	-
461	+	+	+	-	-	-
303-R	+	+	+	+	+	-
KLA	+	+	-	-	+	+
9/94	-	-	+	-	-	-
117-M	-	-	+	+	+	-
228	-	+	-	+	-	-
970	-	-	+	-	-	+
TUBE	+	-	+	+	-	-
303-L	+	+	+	+	+	-
383	+	+	+	+	+	-
NEW	+	-	-	+	+	-
2Y	+	+	+	+	-	-
483	+	+	+	+	+	-
460	+	+	+	+	+	+
369	+	-	-	+	-	-
488	+	+	+	+	-	+
1025	-	+	+	-	-	+
1042	+	-	+	+	-	+
1274	-	+	+	+	+	+
JA	+	-	+	+	-	-
352	-	+	+	-	-	-
374	+	-	+	+	-	-

+ positive reaction; - negative reaction

Table 4. Biotypes of staphylococci

Biotypes	Characteristics					
	Coagulase	Haemolysin	Pigment	Urease	Tween-80 hydrolysis	Casein hydrolysis
A	+	+	+	+	+	+
B	+	+	+	+	+	-
C	+	+	+	+	-	+
D	+	+	+	+	-	-
E	+	+	+	-	-	-
F	+	+	-	-	+	+
G	+	-	+	+	-	+
H	+	-	+	+	-	-
I	+	-	+	-	-	+
J	+	-	-	+	+	-
K	+	-	-	+	-	-
L	-	+	+	+	+	+
M	-	+	+	-	-	+
N	-	+	+	-	-	-
O	-	+	-	+	-	-
P	-	+	-	-	-	-
Q	-	-	+	+	+	-
R	-	-	+	-	-	+
S	-	-	+	-	-	-

+ positive reaction; - negative reaction

Table 5. Biotyping of staphylococcal isolates

Biotypes	Isolates	Number of isolates (n=26)	Percentage (%)
A	460	1	3.85
B	987,303-R,303-L,383,483	5	19.23
C	488	1	3.85
D	2Y	1	3.85
E	461	1	3.85
F	KLA	1	3.85
G	1042	1	3.85
H	TUBE,JA,374	3	11.54
I	799	1	3.85
J	NEW	1	3.85
K	369	1	3.85
L	1274	1	3.85
M	1025	1	3.85
N	352	1	3.85
O	228	1	3.85
P	2,A	2	7.69
Q	117-M	1	3.85
R	970	1	3.85
S	9/94	1	3.85

Table 6. Antibiogram of staphylococcal isolates

Isolates	Antibiogram
2	Ax,Am,E,G,K,Pg,St,Sf
799	Am,E,F,M,No
987	Am,Na,Pg
A	Ax,Am,G,K,M,Na,Pg,St,Sf
461	Ax,Am,E,M
303-R	Ax,Am,F,Pg,Pb,St
KLA	Na,R
9/94	Na,Nf,No,Pf
117-M	Am,F,M
228	Ax,Am,E,G,K,M,Pg,St,Sf,T
970	Am,M,Na,No
TUBE	Ax,Na,No,R
303-L	Am,F,Pg,Pb,R,St,Sf
383	St
NEW	Na
2Y	Ax,Am,E,F,G,K,M,Na,Nf,Pf,Pg,St,Sf,T
483	Sf
460	Sensitive to all drugs tested
369	Na,Sf
488	Ax,Na
1025	Am,Cx,E,F,M,No,R,St,Sf
1042	Am,E,M
1274	Na,R
JA	Ax,Am,E,F,M,Na,No,R,Sf
352	Ax,Am,E,K,St,Sf,T
374	Ax,Am,Cx,E,F,M,Na,No,Pg,R,T

Abbreviations

Ax - Amoxycillin
Am - Ampicillin
Cx - Cloxacillin
E - Erythromycin
F - Flumequine
G - Gentamicin
K - Kanamycin
M - Methicillin
Na - Nalidixic acid
Ni - Nitrofurantoin
No - Novobiocin
Pf - Pefloxacin
Pg - Penicillin-G
Pb - Polymixin-B
R - Rifampicin
St - Streptomycin
Sf - Sulfurazole
T - Tetracycline

Table 7. Percentage of antibiotic resistance of staphylococcal isolates

Antibacterial agent	Number of resistant isolates (n=26)	Percentage (%)
Amoxycillin	11	42.31
Ampicillin	16	61.54
Chloramphenicol	Nil	0
Cloxacillin	2	7.69
Erythromycin	10	38.46
Flumequine	8	30.77
Gentamicin	4	15.38
Kanamycin	5	19.23
Methicillin	11	42.31
Nalidixic acid	13	50.00
Nitrofurantoin	2	7.69
Novobiocin	7	26.92
Pefloxacin	2	7.69
Penicillin-G	8	30.77
Polymixin-B	2	7.69
Rifampicin	7	26.92
Streptomycin	9	34.62
Sulfurazole	10	38.46
Tetracycline	4	15.38
Vancomycin	Nil	0

Table 8. Resistogram of staphylococcal isolates

Isolates	Resistogram
2	Ao,B,Bo,L,Pp,Sa
799	Ac,B,Bo,L,Pp
987	Ac,Ao,B,Bo,Cd,L,Pp
A	Ao,B,Bo,L,Pp,Sn,Sa
461	Ac,Ao,B,Bo,L,Pp
303-R	Ac,B,Bo,L,Pp
KLA	B,Bo,L,Pp,Sn
9/94	Ao,B,Bo,L,Pp,Sa
117-M	Ao,B,Bo,L,Mc,Pp,Sa
228	B,Bo,L,Pp,Sa
970	Ac,B,Bo,Cd,L,Pp,Sn
TUBE	B,Bo,Cd,L,Mc,Pp,Sn,Sa
303-L	Ac,B,Bo,L,Pp
383	Ac,B,Bo,L,Pp
NEW	B,L,Pp
2Y	Ac,Ao,B,Bo,Cv,L,Pp,Sa
483	B,Bo,Cv,L,Pp,Sa
460	B,Bo,L,Pp,Sa
369	B
488	Ac,L,Pp
1025	Ac,B,Bo,L,Pp,Sa
1042	B,Bo,Pp,Sa
1274	B,Bo,L,Pp,Sa
JA	Ac,B,Bo,L,Mc,Pp,Sa
352	B,Bo,L,Pp,Sn,Sa
374	B,Bo,L,Pp

Abbreviations

Ac - Acriflavin
Ao - Ammonium molybdate
B - Barium chloride
Bo - Boric acid
Cd - Cadmium chloride
Cv - Crystal violet
L - Lead oxide
Mc - Mercuric chloride
Pp - Potassium permanganate
Sn - Silver nitrate
Sa - Sodium arsenate

Table 9. Percentage of metal salt/chemical agent resistance of staphylococcal isolates

Metal salt/ chemical agent	Number of resistant isolates (n=26)	Percentage (%)
Acriflavin	11	42.31
Ammonium molybdate	7	26.92
Antimony trichloride	Nil	0.00
Barium chloride	25	96.15
Boric acid	23	88.46
Cadmium chloride	3	11.54
Cetrimide	Nil	0.00
Copper sulphate	Nil	0.00
Crystal violet	2	7.69
Ferrous sulphate	Nil	0.00
Iodine	Nil	0.00
Lead oxide	24	92.31
Mercuric chloride	3	11.54
Potassium permanganate	25	96.15
Potassium tellurite	Nil	0.00
Silver nitrate	5	19.23
Sodium arsenate	14	53.85

Table 10. Resistance pattern of staphylococcal isolates against metal salts and chemical agents

Resistance pattern	Number of isolates (n=26)	Percentage (%)
Ac,B,Bo,L,Pp	4	15.38
B,Bo,L,Pp,Sa	3	11.54
Ao,B,Bo,L,Pp,Sa	2	7.69
B	1	3.85
Ac,L,Pp	1	3.85
B,L,Pp	1	3.85
B,Bo,L,Pp	1	3.85
B,Bo,Pp,Sa	1	3.85
B,Bo,L,Pp,Sn	1	3.85
Ac,Ao,B,Bo,L,Pp	1	3.85
Ac,B,Bo,L,Pp,Sa	1	3.85
B,Bo,Cv,L,Pp,Sa	1	3.85
B,Bo,L,Pp,Sn,Sa	1	3.85
Ac,Ao,B,Bo,Cd,L,Pp	1	3.85
Ac,B,Bo,Cd,L,Pp,Sn	1	3.85
Ac,B,Bo,L,Mc,Pp,Sa	1	3.85
Ao,B,Bo,L,Mc,Pp,Sa	1	3.85
Ao,B,Bo,L,Pp,Sn,Sa	1	3.85
Ac,Ao,B,Bo,Cv,L,Pp,Sa	1	3.85
B,Bo,Cd,L,Mc,Pp,Sn,Sa	1	3.85

Table 11. Comparison between antibacterial drugs and metal salt/chemical agent resistance in staphylococcal isolates

Anti-bacterial agent	Number of resistant isolates (25)	Number resistant to metal salt/chemical agents																
		Ac	Ao	At	B	Bo	Cd	Ct	Cs	Cv	Fs	I	L	Mc	Pp	Pt	Sn	Sa
		(11)	(7)	(0)	(25)	(23)	(3)	(0)	(0)	(2)	(0)	(0)	(24)	(3)	(25)	(0)	(5)	(14)
Amoxycillin	11	5	4	-	10	10	1	-	-	1	-	-	11	2	11	-	2	7
Ampicillin	16	9	6	-	16	16	2	-	-	1	-	-	15	2	16	-	3	9
Chloramphenicol	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cloxacillin	2	1	-	-	2	2	-	-	-	-	-	-	2	-	2	-	-	1
Erythromycin	10	5	3	-	10	10	-	-	-	1	-	-	9	1	10	-	-	7
Flumequine	8	6	2	-	8	8	-	-	-	1	-	-	8	2	8	-	-	4
Gentamicin	4	1	3	-	4	4	-	-	-	1	-	-	4	-	4	-	1	4
Kanamycin	5	1	3	-	5	5	-	-	-	1	-	-	5	-	5	-	2	5
Methicillin	11	6	4	-	11	11	1	-	-	1	-	-	11	2	11	-	2	7
Nalidixic acid	13	5	4	-	12	10	3	-	-	1	-	-	13	2	13	-	4	6
Nitrofurantoin	2	1	2	-	2	2	-	-	-	1	-	-	2	-	2	-	-	2
Novobiocin	7	4	1	-	7	7	2	-	-	-	-	-	7	2	7	-	2	4
Pefloxacin	2	1	2	-	2	2	-	-	-	1	-	-	2	-	2	-	-	2
Penicillin-G	8	4	4	-	8	8	1	-	-	1	-	-	8	-	8	-	1	4
Polymixin-B	2	2	-	-	2	2	-	-	-	-	-	-	2	-	2	-	-	-
Rifampicin	7	3	-	-	7	7	1	-	-	-	-	-	7	2	7	-	2	4
Streptomycin	9	5	3	-	9	9	-	-	-	1	-	-	9	-	9	-	2	6
Sulfurazole	10	4	3	-	10	9	-	-	-	2	-	-	9	1	9	-	2	8
Tetracycline	4	1	1	-	4	4	-	-	-	1	-	-	4	-	4	-	-	3
Vancomycin	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total number of resistances	131	64	45	-	129	126	11	-	-	15	-	-	128	16	130	-	23	83
% of resistance	100	48.85	34.35	0	98.47	96.18	8.4	0	0	11.45	0	0	97.71	12.21	99.24	0	17.56	63.36

Table 12. Comparison of coagulase production, haemolysin production, antibiogram and resistogram of staphylococcal isolates

Isolates (n=26)	Coagulase production	Haemolysis production	Antibiogram	Resistogram
2	-	+	Ax,Am,E,G,K,Pg,St,Sf	Ao,B,Bo,L,Pp,Sa
799	+	-	Am,E,F,M,No	Ac,B,Bo,L,Pp
987	+	+	Am,Na,Pg	Ac,Ao,B,Bo,Cd,L,Pp
A	-	+	Ax,Am,G,K,M,Na,Pg,St,Sf	Ao,B,Bo,L,Pp,Sn,Sa
461	+	+	Ax,Am,E,M	Ac,Ao,B,Bo,L,Pp
303-R	+	+	Ax,Am,F,Pg,Pb,St	Ac,B,Bo,L,Pp
KLA	+	+	Na,R	B,Bo,L,Pp,Sn
9/94	-	-	Na,Nf,No,Pf	Ao,B,Bo,L,Pp,Sa
117-M	-	-	Am,F,M	Ao,B,Bo,L,Mc,Pp,Sa
228	-	+	Ax,Am,E,G,K,M,Pg,St,Sf,T	B,Bo,L,Pp,Sa
970	-	-	Am,M,Na,No	Ac,B,Bo,Cd,L,Pp,Sn
TUBE	+	-	Ax,Na,No,R	B,Bo,Cd,L,Mc,Pp,Sn,Sa
303-L	+	+	Am,F,Pg,Pb,R,St,Sf	Ac,B,Bo,L,Pp
383	+	+	St	Ac,B,Bo,L,Pp
NEW	+	-	Na	B,L,Pp
2Y	+	+	Ax,Am,E,F,G,K,M,Na,Nf,Pf,Pg,St,Sf,T	Ac,Ao,B,Bo,Cw,L,Pp,Sa
483	+	+	Sf	B,Bo,Cw,L,Pp,Sa
460	+	+	Sensitive to all drugs tested	B,Bo,L,Pp,Sa
369	+	-	Na,Sf	B
488	+	+	Ax,Na	Ac,L,Pp
1025	-	+	Am,Cx,E,F,M,No,R,St,Sf	Ac,B,Bo,L,Pp,Sa
1042	+	-	Am,E,M	B,Bo,Pp,Sa
1274	-	+	Na,R	B,Bo,L,Pp,Sa
JA	+	-	Ax,Am,E,F,M,Na,No,R,Sf	Ac,B,Bo,L,Mc,Pp,Sa
352	-	+	Ax,Am,E,K,St,Sf,T	B,Bo,L,Pp,Sn,Sa
374	+	-	Ax,Am,Cx,E,F,M,Na,No,Pg,R,T	B,Bo,L,Pp

Table 13. Estimation of molecular sizes of plasmids of staphylococcal isolates

Isolates	No. of plasmids	Distance migrated (cm) x	Log 10 kb. y	Antilog 10 kb. (Mol. size in kb.)
987	1	5.8	0.590	3.89
1274	1	4.1	1.050	11.22
	2	5.7	0.640	4.39
JA	1	2.9	1.675	47.32
	2	4.2	1.000	10.00
	3	5.6	0.651	4.48
	4	6.4	0.525	3.35
	5	7.5	0.389	2.45
E. coli V 517 (Reference strain)	1	2.8	1.7324	54.00
	2	4.6	0.8633	7.30
	3	5.1	0.7482	5.60
	4	5.4	0.7160	5.20
	5	5.8	0.5911	3.90
	6	6.7	0.4771	3.00
	7	7.4	0.4314	2.70
	8	8.0	0.3222	2.10

Table 14. Plasmid profiles of staphylococcal isolates

Isolates	Molecular size of plasmids (kb)
2	-
799	-
987	3.89
A	5.15,4.39,3.89,2.82
461	5.31,3.98
303-R	-
KLA	25.7
9/94	-
117-M	5.13,3.35
228	-
970	-
TUBE	-
303-L	-
383	3.55
NEW	-
2Y	3.91,2.82
483	4.39,3.55
460	3.35
369	13.8,4.37
488	-
1025	3.63
1042	5.37
1274	11.22,4.39
JA	47.32,10.00,4.47,3.35,2.45
352	3.63,3.35
374	4.39,2.95

Table 15. Comparison of coagulase production, haemolysin production antibiogram, resistogram and plasmid profile of staphylococcal isolates

Isolates (n=26)	Coagulase production	Haemolysin production	Antibiogram	Resistogram	Plasmid profile (kb)
2	-	+	Ax,Am,E,G,K,Pg,St,Sf	Ao,B,Bo,L,Pp,Sa	-
799	+	-	Am,E,F,M,No	Ac,B,Bo,L,Pp	-
987	+	+	Am,Na,Pg	Ac,Ao,B,Bo,Cd,L,Pp	3.89
A	-	+	Ax,Am,G,K,M,Na,Pg,St,Sf	Ao,B,Bo,L,Pp,Sn,Sa	5.15,4.39,3.89,2.82
461	+	+	Ax,Am,E,M	Ac,Ao,B,Bo,L,Pp	5.31,3.98
303-R	+	+	Ax,Am,F,Pg,Pb,St	Ac,B,Bo,L,Pp	-
KLA	+	+	Na,R	B,Bo,L,Pp,Sn	25.7
9/94	-	-	Na,Nf,No,Pf	Ao,B,Bo,L,Pp,Sa	-
117-M	-	-	Am,F,M	Ao,B,Bo,L,Mc,Pp,Sa	5.13,3.35
228	-	+	Ax,Am,E,G,K,M,Pg,St,Sf,T	B,Bo,L,Pp,Sa	-
970	-	-	Am,M,Na,No	Ac,B,Bo,Cd,L,Pp,Sn	-
TUBE	+	-	Ax,Na,No,R	B,Bo,Cd,L,Mc,Pp,Sn,Sa	-
303-L	+	+	Am,F,Pg,Pb,R,St,Sf	Ac,B,Bo,L,Pp	-
383	+	+	St	Ac,B,Bo,L,Pp	3.55
NEW	+	-	Na	B,L,Pp	-
2Y	+	+	Ax,Am,E,F,G,K,M,Na,Nf,Pf,Pg,St,Sf,T	Ac,Ao,B,Bo,Cv,L,Pp,Sa	3.91,2.82
483	+	+	Sf	B,Bo,Cv,L,Pp,Sa	4.39,3.55
460	+	+	Sensitive to all drugs tested	B,Bo,L,Pp,Sa	3.35
369	+	-	Na,Sf	B	13.8,4.37
488	+	+	Ax,Na	Ac,L,Pp	-
1025	-	+	Am,Cx,E,F,M,No,R,St,Sf	Ac,B,Bo,L,Pp,Sa	3.63
1042	+	-	Am,E,M	B,Bo,Pp,Sa	5.37
1274	-	+	Na,R	B,Bo,L,Pp,Sa	11.22,4.39
JA	+	-	Ax,Am,E,F,M,Na,No,R,Sf	Ac,B,Bo,L,Mc,Pp,Sa	47.32,10.00,4.47,3.35,2.45
352	-	+	Ax,Am,E,K,St,Sf,T	B,Bo,L,Pp,Sn,Sa	3.63,3.35
374	+	-	Ax,Am,Cx,E,F,M,Na,No,Pg,R,T	B,Bo,L,Pp	4.39,2.95

Table 16. Antibiotic resistance and haemolysin transfer by conjugation and protoplast fusion

Donor			Recipient	Conjugation		Protoplast fusion	
Isolates	Antibiotic resistance	Haemolysin (Hly)		Antibiotic resistance	Haemolysin (Hly)	Antibiotic resistance	Haemolysin (Hly)
987	Am^r, Pg^r	alpha & beta	*S. aureus* RN450 RF phage-free, plasmid free, haemolysin negative	Am^r, Pg^r	beta	-	-
A	Am^r, Pg^r, G^r, S^r	alpha	" "	Am^r, Pg^r, G^r, S^r	-	-	-
303-R	Am^r, Pg^r, S^r	alpha	" "	Am^r, Pg^r	-	-	-
228	$Am^r, Pg^r, G^r, S^r, E^r$	alpha	" "	Am^r, Pg^r, S^r	-	Am^r, Pg^r, S^r	alpha
2Y	$Am^r, Pg^r, G^r, S^r, E^r$	alpha	" "	Am^r, Pg^r, S^r	-	Am^r, Pg^r, S^r	alpha
352	Am^r, S^r, E^r	'-'on sheep blood agar	" "	Am^r, S^r, E^r	-	-	-

Table 17. Numerical Indices of Discrimination for biotyping, antibiogram typing, resistogram typing, plasmid profiling and their combinations

Methods	Numerical index of discrimination (D)	Number of types (s)	Prevalence of dominating type (%)
Biotyping (BIO)	0.957	19	19.23
Antibiogram typing (ANTI)	1	26	3.85
Resistogram typing (RESI)	0.969	20	15.38
Plasmid profiling (PLAS)	0.862	17	38.46
BIO + ANTI	1	26	3.85
BIO + RESI	0.991	24	11.54
BIO + PLAS	0.997	25	7.69
ANTI + RESI	1	26	3.85
ANTI + PLAS	1	26	3.85
RESI + PLAS	0.988	23	11.54
BIO + ANTI + RESI	1	26	3.85
BIO + ANTI + PLAS	1	26	3.85
BIO + RESI + PLAS	0.997	25	7.69
ANTI + RESI + PLAS	1	26	3.85
BIO + ANTI + RESI + PLAS	1	26	3.85

Plate 1. Coagulase production of staphylococcal isolates (Tube test with rabbit plasma)

A. Coagulase-negative isolate (228)
B. Coagulase-positive isolate (383)
C. Coagulase-positive isolate (460)

Plate 2. Haemolysin production of staphylococcal isolates (sheep blood agar)

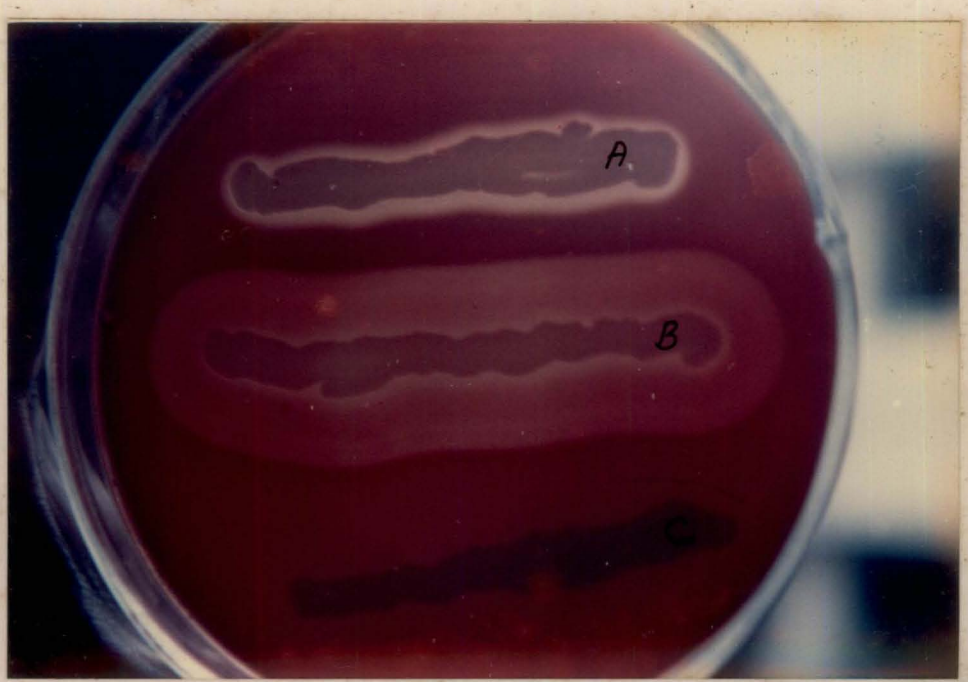
A. Alpha-haemolysin positive isolate (2)
B. Beta-haemolysin positive isolate (1025)
C. Non-haemolytic isolate (1042)

Plate 3. Pigment production and Tween-80 hydrolysis of staphylococcal isolates (Tween-80 agar)

A. Pigment positive, Tween-80 hydrolysis negative isolates (799, 461 and 374)

B. Pigment negative, Tween-80 hydrolysis positive isolates (NEW, KLA and 1274)

C. Pigment and Tween-80 hydrolysis negative isolates (228 and 369)



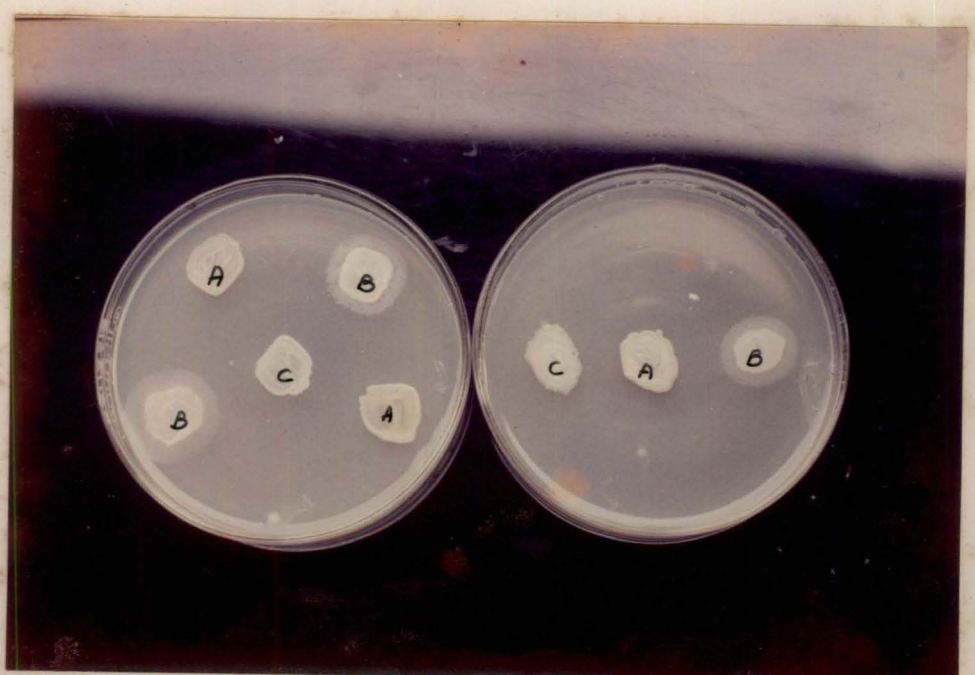


Plate 4. Pigment production and casein hydrolysis of staphylococcal isolates (Milk agar)

A. Pigment positive, casein hydrolysis negative isolates (987 and 461)

B. Pigment negative, casein hydrolysis positive isolates (KLA and 1025)

C. Pigment and casein hydrolysis negative isolates (369 and 2Y)

Plate 5. Urease production of staphylococcal isolates (Christensen's urea agar)

A. Urease-positive isolates (488, NEW and TUBE)
B. Urease-negative isolate (2)


A
A
C
B
C
B


2
NEW
488
TUBE
B
A
A
A

Plate 6. Plasmid profile analysis

Lane A. *E. coli* V 517 (molecular size marker) with 8 plasmids 54, 7.3, 5.6, 5.2, 3.9, 3.0, 2.7 and 2.1 kb

Lane B. Isolate 'JA' with 5 plasmids, 47.32, 10.00, 4.47, 3.35 and 2.45 kb

Lane C. *S. aureus* RN450 RF (recipient strain) with no plasmid

Lane D. Isolate 970 with no plasmid

Lane E. Isolate 228 with no plasmid

Lane F. Isolate 'A' with 4 plasmids, 5.15, 4.39, 3.89 and 2.82 kb

Lane G. Isolate 2 with no plasmid

Lane H. Isolate 799 with no plasmid

Lane I. Isolate 1274 with 2 plasmids, 11.22 and 4.39 kb

Lane J. Isolate 374 with 2 plasmids, 4.39 and 2.95 kb

Lane K. *E. coli* V 517 (molecular size marker)

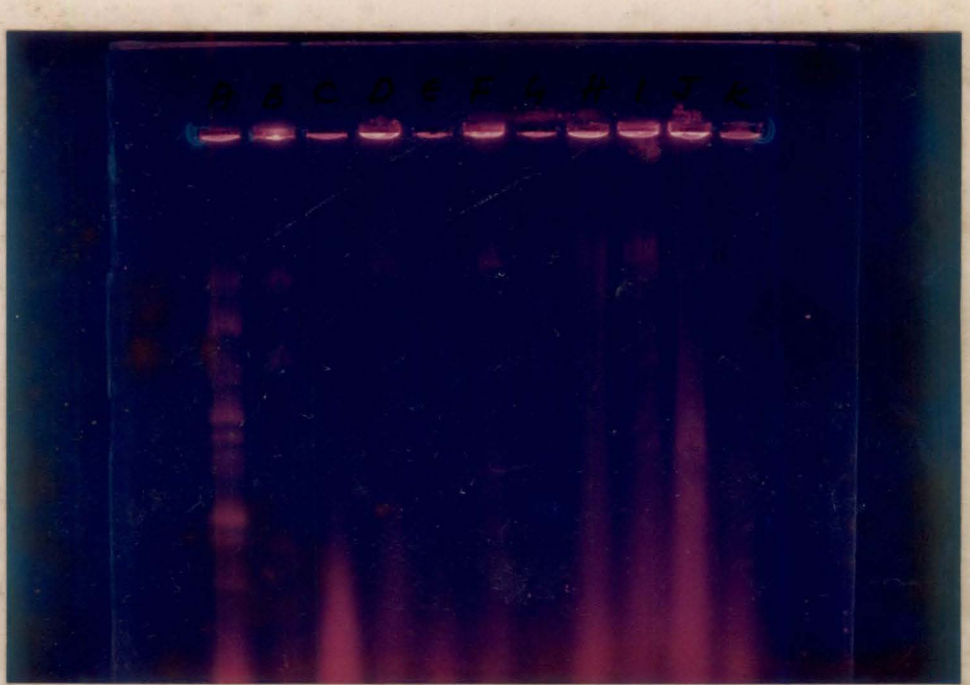
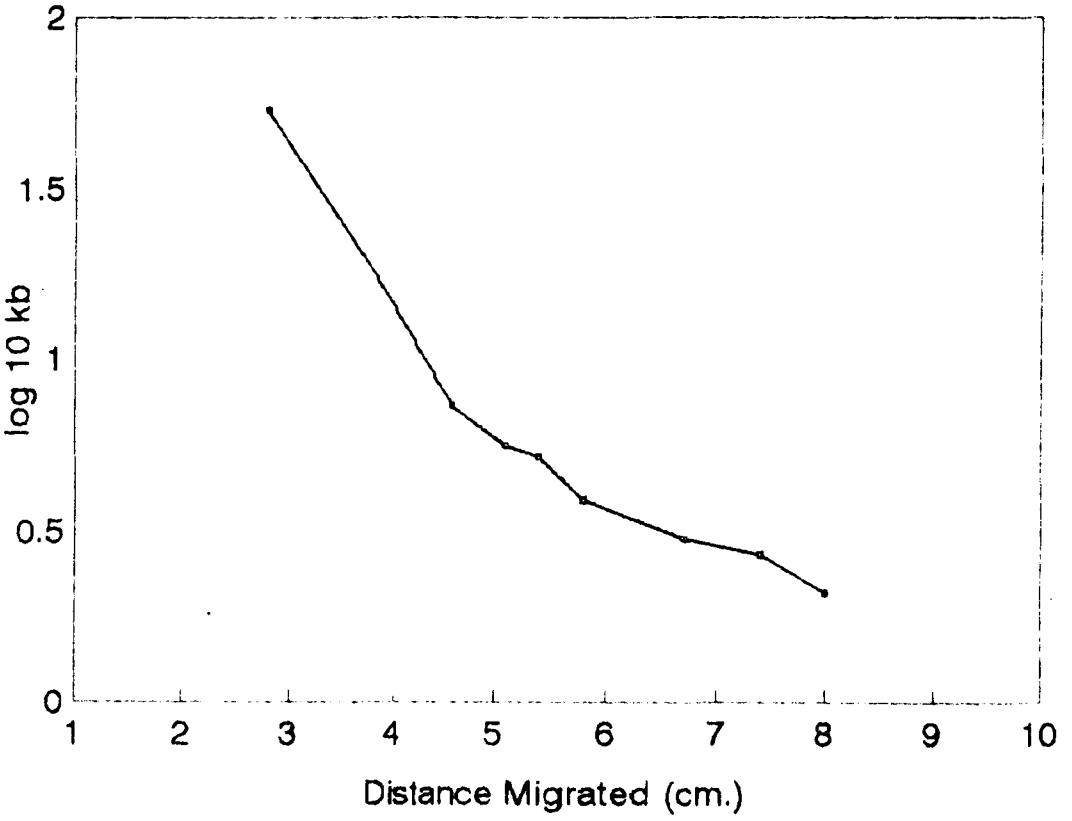


Fig.1 Molecular size Vs. Migration of *E. coli* V 517 Plasmid DNA



Discussion

DISCUSSION

5.1 and 5.2 Isolation and characterization of staphylococci

A total of 70 milk samples from bovine clinical/ subclinical mastitis cases were screened and bacterial organisms could be isolated from 52 cases (74.29%). Out of this 52 cases, 50 per cent of the isolates were Gram-positive cocci appearing in bunch of grapes and all of them were identified as staphylococci based on oxidase test, catalase test and anaerobic fermentation of glucose.

In the present study, no 'micrococci' could be identified, which is in agreement with the observation made by Mathew (1986). The results of the present study also indicate that the most important single causative agent of bovine mastitis is staphylococci and this result is similar to the observations of Jayappa *et al.* (1977), Watts and Owens (1989) and Saini *et al.* (1994).

5.3 Biotying of staphylococci

In this study, six criteria were used for biotying the isolates. So, comparatively large number of biotypes (19 biotypes) were obtained from 26 staphylococcal isolates

(Tables 4). So reliability of this typing method in differentiating the isolates is very high (73.08%) when compared with reliability of 8 per cent obtained by Paul (1992) and 13.33 per cent by Aarestrup *et al.* (1995b) in differentiating the isolates of *S. aureus* of bovine mastitis origin.

Altogether 17 isolates (65.38%) were identified as CPS, either by rabbit plasma, bovine plasma or both and nine isolates (34.62%) were CNS. The results obtained in the present study are not in agreement with the results obtained by Mathew (1986), who got 17 (32.69%) CPS and 35 (67.31%) CNS from cases of bovine mastitis, using only rabbit plasma. But results of the present study are comparable with the results of Saini *et al.* (1994) who could isolate 34 (73.91%) CPS and 12 (26.09%) CNS from staphylococci of bovine subclinical mastitis origin.

It can be inferred from the present study that bovine plasma is more dependable, for detecting coagulase production by staphylococci from bovine mastitis as it has given the maximum number of positivity (Table 2) and hence bovine plasma also should be included along with rabbit plasma, for detecting coagulase production.

Production of haemolysins was detected on sheep and rabbit blood agar plates and 11 isolates (42.31%) were

positive on each media. However, only 6 isolates (23.08%) were positive on both the media. Ten isolates (38.46%) were positive only on one among the two media. Altogether 16 isolates (61.54%) were positive for haemolysin production.

Matsunaga *et al.* (1993) observed that 74.10 per cent of the *S. aureus* isolates of bovine mastitis origin, produced alpha-haemolysin, 65.50 per cent beta-haemolysin and 12.10 per cent delta-haemolysin, whereas in the present study only 57.69 per cent of the staphylococcal isolates produced alpha-haemolysin, 15.38 per cent beta-haemolysin and 3.85 per cent delta-haemolysin, when the two systems were used in combination (Table 2). So in both the studies alpha-haemolysin is produced by maximum number of isolates followed by beta and delta-haemolysins.

It is interesting to note that out of the 17 CPS isolates of the present study, ten isolates (58.82%) were alpha-haemolysin-positive, 3 isolates (17.65%) beta-haemolysin positive and one isolate (5.88%) delta-haemolysin positive. From this observation, it can be concluded that CPS are better producers of haemolysins than CNS. The beta-haemolysin production by the CPS isolates of the present study is comparatively less (17.65%) when compared with the proportion of *S. aureus* isolates (72.38%) of Aarestrup *et al.* (1995b) from bovine mastitis cases in

Denmark. The reason for this difference could be probably due to the fact that in the present work, the coagulase-positive strains would include *S. aureus* and other CPS like *S. hyicus* and *S. intermedius*, whereas Aarestrup *et al.* (1995b) used only *S. aureus* strains.

It was observed that sheep blood is capable of detecting alpha, beta and delta-haemolysins whereas rabbit blood could only detect alpha-haemolysin. Hence it is better to use sheep as well as rabbit blood agar to detect haemolysin production by staphylococci.

The pigments produced by the isolates ranged from light yellow, cream, golden yellow, orange red and buffy colours. Sixteen (61.54%) isolates were pigment-producers, on both Tween-80 agar and milk agar. But two isolates (7.69%) were able to produce pigment on milk agar, but unable to produce on Tween-80 agar and vice versa. Altogether 20 (76.92%) isolates were pigment producers. Out of the 17 CPS isolates, 14 (82.35%) isolates were pigment producers. Hajek and Balusek (1988) reported that out of the total 111 CPS isolated from rooks and gulls, only 78 (70.27%) isolates were pigment producers and De Centorbi *et al.* (1988) in their studies found that all the 36 *S. aureus* isolates (100%) from young goats were pigment-producers. While comparing these two results with that of present study it

can be concluded that majority of CPS are in general produce pigments and this can be considered as one of the characters indicating virulence (Minor and Marth, 1976).

Urease production was noticed among 17 (65.38%) of the 26 staphylococcal isolates and among 14 (82.35%) of the 17 CPS isolates. Hajek and Balusek (1988) reported urease producdtion by 104 (93.69%) of the 111 CPS isolates. The results obtained in the present study indicate that comparatively lesser number of the CPS isolates (82.35%) produce urease than the isolates of Hajek and Balusek (1988). Paul (1992) reported comparatively lesser proportion (72%) of the 50 *S. aureus* from bovine mastitis cases produced urease, than the CPS isolates of the present study. The urease production by the staphylococcal isolates can be considered as one of the characters indicating virulence as 82.35 per cent of the CPS isolates in the present study produced urease.

Hydrolysis of Tween-80 was noticed in 10 (38.46%) isolates of staphylococci and 8 (47.06%) isolates among CPS. Hajek and Balusek (1988) reported the hydrolysis of Tween-80 by 74 (66.67%) of the 111 CPS and Paul (1992) reported 32 (64%) of the 50 *S. aureus* isolates. The results obtained in the present study showed a low percentage of Tween-80 hydrolysis, when compared with the above results. No

conclusion could be drawn between coagulase production and hydrolysis of Tween-80.

Hydrolysis of casein was noticed among 8 (30.77%) isolates of the 26 staphylococci and 5 (29.41%) isolates of the 17 CPS isolates. But these results are comparatively less when compared with the result obtained for Hajek and Balusek (1988), wherein 106 (95.50%) isolates of the CPS from rooks and gulls, were able to hydrolyse casein out of the total 111 CPS isolates. No conclusion could be drawn between coagulase production and hydrolysis of casein.

Based on the characteristics of the 26 isolates, 19 biotypes were found to occur. The predominant biotype was Biotype-B with 5 isolates (19.23%) followed by Biotype-H with 3 isolates (11.54%), Biotype-P with 2 isolates (7.69%) and all the other 16 biotypes were with single isolate (3.85%) only. Biotype-A was positive for all the six criteria used for biotyping. Eventhough the isolates grouped in Biotype-A and B were the most virulent ones, they were sensitive to most of the antibacterial drugs tested. The isolates belonging to Biotype-A and B were having many common properties with the *S. aureus* isolates belonging to Biotype-C of De Centorbi *et al.* (1988). The Biotype-C of De Centorbi *et al.* (1988) shared common properties with *S. aureus* isolates obtained by Lachica *et al.* (1971) and Smith

and Roguinsky (1977) from bovine and caprine mastitis, respectively. In the present study the biotyping system was found to be a fairly good technique in discriminating the isolates and this technique was easy to perform on a limited number of staphylococcal isolates.

5.4 Antibiogram of staphylococci

The antibiogram of staphylococci revealed 26 different resistance patterns and one isolate (3.85%) was showing sensitivity to all the 20 antibacterial drugs tested (Isolate 460). No two isolates with the same antibiogram was seen. The reliability of this method in distinguishing the isolates was to the tune of 96.15 per cent. The reliability value obtained in the present study compared well with the value obtained by Paul (1992) i.e., 88 per cent.

It was seen that all the 26 isolates (100%) were sensitive to chloramphenicol and vancomycin and thus these drugs could be recommended as the most effective drugs to treat bovine staphylococcal mastitis. The results obtained by Mathew (1986), Trinidad *et al.* (1990), Saini *et al.* (1994) and Aarestrup *et al.* (1995b) fully agree with the present findings.

Cloxacillin was 92.31 per cent effective and result obtained by Mathew (1986) support this result. The present study revealed that nitrofurantoin was 92.31 per cent effective. Mathew (1986) and Saini *et al.* (1994) reported similar results.

The result of the present finding that pefloxacin was 92.31 per cent effective, corroborate with the finding of Dasgupta *et al.* (1993).

Gentamicin was 84.62 per cent effective in the present study and this agrees with the finding of Saini *et al.*, 1994 (91.30%); Mathew, 1986 (98.08%) and Trinidad *et al.*, 1990 (100%).

Tetracycline was 84.62 per cent effective in the present study. The value obtained by different scientists are variable between 61.54 to 99.7 per cent (Mathew 1986; Trinidad *et al.*, 1990 and Saini *et al.*, 1994).

Flumequine was 69.23% effective, but Dasgupta *et al.* (1993) obtained only 20 per cent sensitivity in caprine staphylococci.

Over 69 per cent of the isolates was sensitive to penicillin and this generally agrees with the findings of Saini *et al.*, 1994 (58.7%); Mathew, 1986 (76.92%); Aarestrup

et al., 1995b (85.71%) and Trinidad *et al.*, 1990 (92.30%).

Streptomycin was effective in 65.38 per cent cases and results obtained by various workers ranged from 50 to 96.1 per cent (Mathew, 1986 and Trinidad *et al.*, 1990). Sulfurozole was 61.54 per cent effective and this value is in agreement with Mathew, 1986 and Trinidad *et al.*, 1990.

The sensitivity of staphylococcal isolates to erythromycin (61.54%), amoxycillin (57.69%), methicillin (57.69%) and ampicillin (38.46%) obtained in the present study were not in agreement with the results obtained by Sudharma *et al.*, 1985; Mathew, 1986 and Saini *et al.*, 1994. This may be due to the indiscriminate use of these antibiotics in animals, which resulted in the emergence of drug-resistant staphylococci.

On comparison of the results of antibiogram studies of staphylococci isolated from bovine mastitis in and around Thrissur by Sudharma *et al.* (1985) and Mathew (1986), with that of the present work, it is seen that the sensitivity of isolates to chloramphenicol, tetracycline, streptomycin and sulpha drugs have increased during the period of last 10 years, but sensitivity to erythromycin, amoxycillin, methicillin and ampicillin have decreased during this period.

When studying the *S. aureus* isolated from bovine mastitis in Denmark, Aarestrup *et al.* (1995b) had observed that 85 isolates (81%) were sensitive to all the 11 antibiotics tested, while in the present study only one isolate (3.85%) was susceptible to all the 20 antibiotics tested. Eight antibiotics were common in both the studies. Judicious use of antibiotics could be attributed to the increased sensitivity of *S. aureus* isolated from bovine mastitis in Denmark.

5.5 Resistogram of staphylococci

The resistogram of staphylococcal isolates from bovine mastitis was studied using 17 different metal salts/chemical agents. The reliability of this method was found to be slightly high (76.92%) when compared with the result (70%) obtained by Paul (1992) to distinguish *S. aureus* isolates of bovine mastitis origin.

The resistance pattern Ac B Bo L Pp was shown by 15.38 per cent of the isolates, followed by B Bo L Pp Sa showed by 11.54 per cent of the isolates and Ao B Bo L Pp Sa by 7.69 per cent of the isolates.

Maximum degree of resistance (96.15%) was noticed against potassium permanganate and barium chloride. Similar observations were made by Novick and Roth (1968).

The isolates revealed 92.31 per cent resistance against lead oxide which agrees with the finding of Novick and Roth (1968). A high degree of resistance (88.46%) was observed in the present study against boric acid, but Paul (1992) could obtain only 50 per cent resistance.

In the present study 53.85 per cent of the isolates were resistant to sodium arsenate which fully concur with the results obtained by Paul (1992).

Resistance to acriflavin (42.31%) obtained in the present study was in close agreement with the result (40%) obtained by Paul (1992).

Ammonium molybdate (26.92%), silver nitrate (19.23%), cadmium chloride and mercuric chloride (11.54% each) and crystal violet (7.69%) showed comparatively low degree of resistances. But Novick and Roth (1968) obtained high degree of resistance against silver nitrate. Novick and Roth (1968) obtained 100 per cent resistance against cadmium, while Paul (1992) observed only 48 per cent and so the resistance to cadmium was found to be highly variable among staphylococci. Mathew (1986) could not obtain a single isolate of staphylococci, resistant to mercuric chloride, which compare favourably with the very low percentage obtained in the present study.

All the isolates were sensitive to antimony trichloride, cetrimide, copper sulphate, ferrous sulphate, iodine and potassium tellurite in the present study, which is in almost agreement with Novick and Roth (1968). Paul (1992), who used only cetrimide from among the metal salts/chemical agents mentioned above, also observed low degree of resistance against cetrimide.

As all the 26 staphylococcal isolates were sensitive to 6 agents (Out of the 17 metal salts/chemical agents) used in the study, the reliability of this typing method got reduced to 76.92 per cent.

5.5.1 Comparison between resistance to antibacterial drugs and metal salts/chemical agents

In the present study, it was observed that there was a correlation existing between the resistances against certain antibacterial drugs and resistance against certain metal salts/chemical agents (Table 11). There was a high degree of correlation between the resistances to the following drug and resistances to metal salts/chemical agents.

Resistances to Ax, E, R and Sf and resistances to B, Bo, L, Pp and Sa; resistance to Na and resistances to B, Bo, L and Pp; resistances to Am, F, M and S and resistances to

Ac, B, Bo, L, Pp and Sa; resistance to Pg and resistances to Ac, Ao, B, Bo, L, Pp and Sa.

Similar observations were made by Novick and Roth (1968), who reported the existence of resistance against metal ions/ chemical agents and antibacterial drugs simultaneously on plasmids of *S. aureus*.

Karpe (1993) observed the existence of resistance against antibacterial drugs and metal ions/chemical agents on plasmids of *E. coli* isolated from bovine mastitis, which concur with the observations made in the present study.

5.6 Plasmid profile analysis

In the present study, 16 staphylococcal isolates (61.54%) revealed the presence of plasmids. Among the 17 CPS isolates, 11 isolates (64.71%) revealed the presence of one or more plasmids. One CPS isolate (JA) carried maximum number of plasmids (5) and possessed both the largest and smallest plasmids obtained in the study. There was no appreciable difference between the CPS and CNS regarding the number and molecular size of plasmids.

It is observed that the reliability of this method in differentiating the isolates is 61.54 per cent and this is in agreement with the result (64%) obtained by Paul (1992).

Ten isolates revealed the presence of more than one plasmid. Only one isolate (JA) possessed large plasmid of size more than 30 kb. All other isolates possessed only small plasmids and this finding concur with the observation of Matthews *et al.* (1992), who observed a high proportion of smaller plasmids in the staphylococci isolated from bovine mammary glands. But Paul (1992) observed large plasmids in 60 per cent of *S. aureus* isolates.

In this study, no plasmid with same molecular size was seen common to all the isolates. But some plasmids with same molecular size were seen in two or more isolates. One 4.39 kb plasmid was seen in isolates A, 483, 1274 and 374 and another 3.89 kb plasmid in 987 and A; 3.63 kb plasmid in isolates 1025 and 352; 3.55 kb plasmid in isolates 383 and 483; 3.35 kb plasmid in 117-M, 460, JA and 352 and 2.82 kb plasmid in A and 2Y. Similar observation was also made by Matthews *et al.* (1992), who got some small plasmids of molecular weights 2.0 MDa, 2.6 MDa, 3.7 MDa, and 4.8 MDa in different species of staphylococci isolated from bovine mammary secretions.

There was also some coincidence seen between the presence of a particular plasmid and antibiotic resistance (Table 15). Two of the four isolates carrying a 4.39 kb plasmid were resistant to Sf, two isolates possessing a

3.89 kb plasmid were resistant to Am, Na and Pg, one 3.63 kb plasmid was commonly seen in two isolates, which were resistant to Am, E, St and Sf and another small plasmid of 2.82 kb was seen commonly in two isolates which were resistant to Ax, Am, G, K, M, Na, Pg, St and Sf. But Matthews *et al.* (1992) reported that no relationship was found between antibiotic resistance and presence of a specific plasmid. Generally it is seen that most of the isolates carrying plasmid were resistant to Am, Ax, M and E.

In the present study one of the isolates (460), which possessed one plasmid was found sensitive to all the 20 drugs tested, while another isolate (483) revealed two plasmids, was resistant to single antibacterial drug. Hence the plasmid profile study appear to be frustrating because some of the genes on 'cryptic plasmids' would mediate antibacterial drug resistance and other markers of interest, as suggested by Noble and Allaker (1992). However, further studies are needed to establish the relationship between plasmid profile and antibiotic resistance.

The present study indicated that the plasmid profiling did not appear to be an adequate method for delineating the staphylococci causing bovine mastitis, and this is in agreement with Matthews *et al.* (1992). But the use of antibiogram in conjunction with plasmid profile analysis was

found to be efficient to some extent, in delineating the staphylococcal isolates under study and this is in agreement with Paul (1992).

5.6.1 Comparison between plasmid profile and virulence factors (coagulase, haemolysin and pigment productions)

In the present study, it was seen that among the 10 isolates, which were coagulase and haemolysin producers, 7 (70%) isolates were carrying plasmids (Table 15). Among 7 coagulase-positive and haemolysin-negative isolates, 4 (57.14%) isolates were carrying plasmids. Among the 6 isolates which were coagulase-negative and haemolysin positive, 4 isolates (66.67%) were carrying plasmids. Among the 3 isolates which were non-producers of both coagulase and haemolysin, only one isolate (33.33%) was carrying plasmids.

Similarly among 20 isolates, which were pigment-producers, 13 isolates (65%) were carrying plasmids and among 6 isolates which were non-producers of pigment, 3 isolates (50%) were carrying plasmids.

The results of the present study, i.e., majority of coagulase positive, haemolysin positive and pigment producing strains have plasmids, in general agree with the

findings of Muth (1971), Falkow (1975) and Brock and Madigan (1988).

5.6.2 Comparison between plasmid profile, antibiogram and resistogram

Generally it is seen that the isolates which were MDR and multiple metal salt/chemical agent resistant, carried one or more plasmids (Table 15). But certain isolates like 2, 799, 9/94, TUBE, 228, 970, 303-R and 303-L which were multiple drug and metal salt/chemical agent resistant, no plasmid could be isolated by routine procedures.

The genes for these characters may be carried by small plasmids, which could not be detected by the routine procedures. So further work is required to draw a conclusion regarding correlation between plasmid profile, antibiogram and resistogram.

5.7 Plasmid transfer techniques

5.7.1 Conjugation studies

The study conducted in mixed cultures revealed that the three CPS isolates could transfer ampicillin and penicillin-resistances to the recipient, *S. aureus* RN450 RF. Among the CPS isolates, only two isolates were streptomycin-resistant and among them only one isolate could

transfer streptomycin-resistance. Gentamicin and erythromycin resistances were not transferred by the CPS isolate having these resistances. Eventhough, all the CPS isolates were alpha-haemolysin producers, none of them could transfer this character. But, it is interesting to note that one beta-haemolysin producing CPS could transfer the character.

Mathew and Punnoose (1988) reported the transfer of penicillin and streptomycin resistances from *S. aureus* to *S. aureus* RN450 RF, in mixed cultures and the present study also revealed similar results.

Isolate 303-R was plasmid-free, but able to transfer ampicillin and penicillin resistances and unable to transfer streptomycin resistance and alpha-haemolysin production. The possible reason for the transfer of some resistances and non-transfer of other resistance and characters might be due to the fact that they are located on conjugative and non-conjugative small plasmids, respectively. The very small plasmids are not routinely detected by standard procedures. Mathew and Punnoose (1988) also explained such a phenomenon on the basis of transduction and transformation, even after taking proper precautions to exclude the same.

Isolate 987 having only a 3.89 kb plasmid, transferred ampicillin and penicillin resistances along with beta-haemolysin production by conjugation, suggest the existance of all these characters in one and the same plasmid. This finding concur with the findings of Falkow (1975) that beta-haemolysin production is a plasmid-mediated character.

Isolate 2Y carried two plasmids of 3.91 kb and 2.82 kb. But could transfer only ampicillin, penicillin and streptomycin resistances and unable to transfer erythromycin and gentamicin-resistances. This suggest that ampicillin, penicillin and streptomycin-resistances are located on a single plasmid and conjugation of the organisms resulted in the transfer of plasmid carrying these resistances and resistances to erythromycin and gentamicin may be located in the plasmid, which is non-conjugative.

Among the three CNS isolates, all the isolates could transfer ampicillin and streptomycin-resistances. Two penicillin resistant isolates could transfer penicillin-resistance also. Among the two gentamicin-resistant, only one could transfer this resistance. Similarly among the two erythromycin-resistant isolate, only one could transfer this resistance. None of the two alpha-haemolysin producers could transfer this character.

The ampicillin, streptomycin, and penicillin resistances were transferred by the CNS isolates to the *S. aureus* isolates. Mathew (1986) and Muhammad *et al.* (1993) reported that only streptomycin-resistance could be transferred and not penicillin and erythromycin resistance from CNS to *S. aureus*. The result of the present work only partially agree with the result of the above authors.

Plasmids of 5.15 kb, 4.39 kb, 3.89 kb and 2.82 kb were seen in isolate 'A' and transfer of ampicillin, penicillin, streptomycin and gentamicin resistance together suggest that these may be plasmid-mediated characters.

Regarding isolate 228, which is plasmid-free, ampicillin, penicillin and streptomycin resistance were found to be transferred by conjugation. The inability to detect the plasmid by the routine procedures might be due to the fact that the plasmids responsible for the antibiotic-resistance are of very small size. But unlike, isolate 303-R, isolate 228 could transfer streptomycin resistance. The possible reason for the non-transfer of streptomycin resistance from isolate 303-R was that, the resistance gene might be located on a non-conjugative plasmid or that plasmid may be a 'suicide plasmid'.

Isolate 352 had 2 plasmids of 3.63 kb and 3.35 kb and the transfer of ampicillin, streptomycin and erythromycin resistances together suggest that they are linked.

The inability to transfer alpha-haemolysin indicate that the genes responsible may be located on a non-conjugative plasmid or on the chromosome.

The transfer of plasmid-mediated characters by isolates-303-R and 228- (which were found plasmid free) suggests the existence of smaller plasmids which could not be detected by routine methods.

5.7.2 Protoplast fusion studies

Among six isolates of staphylococci (3 CPS and 3 CNS), only one isolate each of CPS and CNS was able to produce biparental strains and it appears that the formation of the clones of biparental strains were to the tune of 33.33 per cent of the fusion studies conducted.

In two cases of formation of biparental strains, only ampicillin, penicillin and streptomycin resitances and alpha- haemolysin production were expressed. But gentamicin and erythromycin resistances were not transferred/expressed.

No plasmid was detected in Isolate 228, but resistances to ampicillin, pencillin and streptomycin and

alpha-haemolysin production were expressed in biparental strain. But gentamicin and erythromycin resistances were not expressed. This could probably due to the different restriction and modification system in the donor and recipients, as suggested by Gotz *et al.* (1981).

But in case of Isolate 2Y, which was carrying 2 plasmids of 3.91 kb and 2.82 kb, the resistances against ampicillin, penicillin and streptomycin and alpha-haemolysin production were transferred.

From the results of the above studies, it is concluded that the transfer of phenotypic characters from coagulase-positive and coagulase-negative strains are of similar nature.

Since alpha-haemolysin was transferred only by protoplast fusion and not by conjugation, the possibility of chromosome-mediated nature of this character could not be excluded.

Further studies are warranted as to why, beta-haemolysin and resistances to gentamicin and erythromycin were not expressed in biparental strains.

5.8 Numerical Index of Discrimination of different typing methods

Numerical Index of Discrimination (D) of a typing method indicates the ability of that typing method to discriminate two strains of bacteria of different clonal origin chosen at random, to be assigned to different types. In this study both definitive (antibiogram, resistogram and biotyping) and comparative (plasmid profiling) techniques were used as adapted by Aarestrup *et al.* (1995b).

Antibiogram and all possible combinations, all having the value for Numerical Index of Discrimination as one (1) appeared to have maximum efficacy (Table 17). Unlike observations made by Aarestrup *et al.* (1995b), in the present study, antibiogram typing and its combinations were found to be most efficient typing methods to discriminate the isolates of different clonal origin into different types. This is due to the fact that a large number (20) of drugs were tested in this study and a good proportion of isolates were found resistant to these drugs. This finding is not in full agreement with that of Aarestrup *et al.* (1995b). The probable reason might be the fact that he had used lesser number of antibiotics (11) and low proportion of isolates resistant to these drugs.

Resistogram typing alone had a D value of 0.969. Eventhough 17 metal salts/chemical agents were used in this study, all the isolates were found to be sensitive to six of them. All combinations of resistogram, also had high D values.

Biotyping alone showed less D value, i.e., 0.957, when comparing with that of first two methods and their combinations.

Plasmid profiling was found to be the least efficient with a D value of 0.862, and all its combinations were better than plasmid profiling alone.

In the present study, eventhough less number of staphylococcal isolates were compared, all the methods were having good D values than the D values obtained by Aarestrup *et al.* (1995b) while studying 105 *S. aureus* isolates. This can be substantiated on the basis that staphylococcal isolates of the present study were well separated among them than the *S. aureus* isolates of Aarestrup *et al.* (1995b) which were more related.

The Isolate 303-R and 303-L belonged to Biotype-B, Resistogram type 1 and Plasmid profile type that contained 'no plasmid'. These isolates were found inseparable with these three methods of typing and they were found to be well

related than the other isolates. But antibiogram of these isolates were different. This suggest that antibiotic resistance may be acquired or lost by a strain of staphylococci during the course of time, and this observation agrees with Novick (1980). Isolate 303-R acquired amoxycillin resistance and 303-L acquired rifampicin and sulfurazole-resistances, during the course of time.

The antibiogram typing and all its combinations were showed the maximum discriminatory power and they were found to be the best typing methods for typing the isolates of staphylococci of bovine mastitis origin. The isolates like 303-R and 303-L which were highly related could be separated only by antibiogram typing. But Aarestrup *et al.* (1995b) obtained the least D value for antibiogram typing, as they have obtained 81 per cent of the strains sensitive to all the antibiotics tested, contrary to the present findings. Plasmid profiling was found to be the least efficient among the individual typing methods. But plasmid profiling may be useful in solving epidemiological problems concerning isolates, that are assigned to the same type by other typing techniques. These findings are in confirmation with the observations made by Aarestrup *et al.* (1995b).

Summary

SUMMARY

This study revealed that bacterial organisms were responsible in 52 out of 70 cases of bovine clinical/sub-clinical mastitis, and 26 isolates of staphylococci were obtained. Biotyping of the isolates were carried out based on different tests, viz. coagulase production, haemolysin production, pigment production, urease production, Tween-80 hydrolysis and casein hydrolysis. Antibiogram of the isolates was studied by agar diffusion method of Kirby and Bauer, using 20 antibacterial drugs. Resistogram of the isolates was studied using 17 metal salts/chemical agents, by agar diffusion method.

Plasmid profile of the 26 staphylococcal isolates was obtained by extracting the plasmids by alkaline lysis method and subsequent agarose gel electrophoresis of the plasmid DNA preparations. Plasmid transfer by conjugation and protoplast fusion, from the selected drug-resistant and haemolysin-positive staphylococcal isolates was carried out with *S. aureus* RN450 RF as the recipient, which is plasmid-free, phage-free, Hly^-; Rif^r/Fus^r.

The Numerical Index of Discrimination of biotyping, antibiogram typing, resistogram typing and plasmid profiling

and all possible combinations was determined using the formula of Gaston and Hunter.

From the study, following observation were made:

From the 70 milk samples tested, 74.29 per cent were culturally positive for various bacterial organisms, from which 26 isolates (50%) of staphylococci were obtained and no *Micrococcus* was detected.

All the staphylococcal isolates were positive for Gram's staining, catalase test and anaerobic fermentation of glucose. Nineteen and 21 isolates were positive for nitrate-reduction and V-P tests, respectively and the rest of them were negative for these tests. All the isolates were negative for oxidase test.

Nineteen biotypes were formed by biotyping the 26 isolates. Among them Biotype-B was having the maximum number of isolates (5), followed by Biotype-H with three isolates, Biotype-P with two isolates and all the remaining 16 biotypes were having single isolates only. Biotype-A was positive for all the criteria used for biotyping. Biotype-A and B were containing the most virulent isolates. Biotyping was having a reliability of 73.08 per cent in differentiating the isolates.

Thirteen and 10 isolates were positive for coagulase production in bovine and rabbit plasma, respectively. Altogether 17 isolates were found to be CPS.

Eleven isolates each were positive for haemolysin production both on sheep and rabbit blood agar. Altogether 16 isolates were positive for haemolysin production.

Sixteen isolates were producing pigments on both Tween-80 agar and Milk agar. Altogether 20 isolates were positive for pigment production. Pigment production can be considered as one of the characters indicating virulence.

Seventeen isolates were urease producers and this character also can be considered as one of the characters indicating virulence.

Tween-80 was hydrolysed by 10 isolates and casein was hydrolysed by 8 isolates.

Antibiogram studies revealed that chloramphenicol and vancomycin were cent per cent effective. Followed of Cx, Ni, Pf and Pb (92.31% each); G and T (84.62% each); K (80.77%); R and No (73.08% each); F and Pg (69.23% each); St (65.38%); E and Sf (61.54%) and Ax and M (57.69%). The least effective drugs were nalidixic acid and ampicillin which were effective against only 50 per cent and 38.46 per cent

of the isolates, respectively. The efficacy of C, T, St and Sf were found to be increased and that of E, Ax, M and Am were found to be decreased when compared to the results of previous studies. The reliability of this method in distinguishing the isolates was to the tune of 96.15 per cent.

Resistogram studies revealed that the maximum degree of resistance was noticed against B and Pp (96.15% each), followed by L (92.31%). Resistance against Bo was to the tune of 88.46 per cent, Sa (53.85%), Ac (42.31%), Ao (26.92%), Sn (19.23%), Cd and Mc (11.54% each) and Cv (7.69%). All the isolates were sensitive to At, Ct, Cs, Fs, I and Pt. The reliability of resistogram typing was to the tune of 76.92 per cent in distinguishing the isolates.

There was a high degree of correlation existing between the resistances to the drugs, viz. Ax, E, R, Sf, Na, Am, F, M, S and Pg and resistances to metal salts/chemical agents like Ac, B, Bo, L, Pp and Sa.

Only 16 isolates revealed plasmids (61.54%). No plasmid with same molecular size was seen common to all the isolates. Maximum number of plasmids seen in an isolate was five, which possessed both the largest and smallest plasmids obtained in the study. Some plasmids with same molecular size were seen common in more than one isolates. Some

coincidence was seen between presence of a particular plasmid and resistance against a particular antibiotic. More than one plasmids were seen in isolate that was resistant to single antibacterial drug and one plasmid was present in an isolate, that was sensitive to all the drugs. No appreciable difference could be detected between CPS and CNS regarding the number and molecular size of plasmids. The reliability of this method in differentiating the isolates was 61.54 per cent. Ordinary SLR camera was used for taking photograph of plasmid profile instead of polaroid camera and colour photographs with better resolution were obtained.

Conjugation studies revealed that CPS could transfer resistances against ampicillin, penicillin and streptomycin and one beta-haemolysin-positive isolate could transfer that character also. But gentamicin and erythromycin resistances and alpha-haemolysin were not transferred. CNS isolates could transfer ampicillin, penicillin, streptomycin, gentamicin and erythromycin resistances. But none could transfer alpha-haemolysin.

The formation of clones of biparental strains in protoplast fusion were to the tune of 33.33 per cent only. Ampicillin, penicillin, streptomycin resistances and

alpha-haemolysin were expressed in biparental strains. But gentamicin and erythromycin resistances were not expressed.

Antibiogram and all its combinations with biotyping, resistogram and plasmid profiling, were having the maximum efficacy, with all of them having the value of Numerical Index of Discrimination as one. Resistogram and its combinations and biotyping and its combinations were having better values than plasmid profiling alone, which was having 0.862. Antibiogram typing and all its combination were found to be the best typing methods for typing staphylococci of bovine mastitis origin.

From the studies, following conclusion were made:

1. Both bovine plasma and rabbit plasma should be included in the study to detect coagulase production by staphylococci causing bovine mastitis.

2. Both sheep and rabbit blood agars should be used to detect haemolysin production by staphylococci.

3. Indiscriminate use of antibacterial drugs should be avoided to prevent the emergence of drug-resistant strains. Antibiogram should be obtained prior to therapy. The drugs of choice for staphylococcal

mastitis are chloramphenicol, vancomycin, cloxacillin, nitrofurantoin, pefloxacin and polymixin-B.

4. Antibiogram, resistogram and biotyping could serve as an adjunct to plasmid profiling in epidemiological studies. These typing methods in combinations are recommended as epidemiological tool in differentiating and identifying staphylococci of bovine mastitis origin.

5. Transfer of antibiotic resistance and haemolysin production by conjugation and protoplast fusion was established.

6. Correlation between resistances to certain antibiotics and metal salts/chemical agents was found in the present study.

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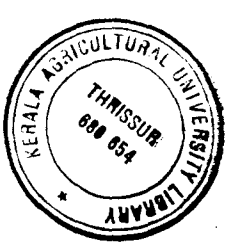

CERTAIN PLASMID - MEDIATED CHARACTERS OF STAPHYLOCOCCI ISOLATED FROM BOVINE MASTITIS

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ABSTRACT OF A THESIS

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ABSTRACT

Twenty-six staphylococci were isolated from 70 cases of clinical/sub-clinical bovine mastitis. They were characterised by various biochemical tests and biotyped using coagulase production, haemolysin production, pigment production, Tween-80 hydrolysis and casein hydrolysis. These 26 isolates comprised 19 biotypes of which, most preponderating one was Biotype-B comprising of 5 isolates. The reliability of biotyping in distinguishing the isolates was found to be 73.08 per cent.

The antibiogram study revealed that chloramphenicol and vancomycin were cent per cent effective. Cloxacillin, nitrofurantoin, pefloxacin and polymixin-B were also effective. Ampicillin and nalidixic acid were found to be least effective. The reliability of this method was found to be 96.15 per cent. Resistogram study revealed that maximum degree of resistance was noticed against barium chloride and potassium permanganate. All the isolates were found to be sensitive to antimony trichloride, cetrimide, copper sulphate, ferrous sulphate, iodine and potassium tellurite. The reliabiling of resistogram study was found to be 76.92 per cent.

Production of haemolysin and resistance to antibiotics were found to be plasmid-mediated. Correlation between resistances to certain antibiotics and metal salts/chemical agents was also found.

The plasmid profiling revealed only 16 isolates carrying plasmids. No plasmid was found common to in all the isolates. The maximum number of plasmids in an isolate was five, and this isolate carried both the largest and smallest plasmids. The reliability of plasmid profiling was 61.54 per cent.

Conjugation studies revealed transfer of ampicillin, penicillin, streptomycin, erythromycin and gentamicin resistances and beta-haemolysin production to the recipient. But alpha-haemolysin was not transferred. Protoplast fusion studies revealed the expression of only ampicillin, penicillin and streptomycin resistance and alpha-haemolysin production, by the biparental strains.

Determination of Numerical Index of Discrimination indicated that antibiogram typing and all its combinations were having the maximum 'D' value of one. Plasmid profiling was having the least value, i.e. 0.862. So it is suggested that antibiogram typing and its combinations can be used in differentiating and identifying the staphylococci causing bovine mastitis, more accurately.