CHARACTERIZATION OF STAPHYLOCOCCI ISOLATED FROM CASES OF MASTITIS AND STUDY OF THEIR R PLASMIDS

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled "CHARACTERIZATION OF STAPHYLOCOCCI ISOLATED FROM CASES OF MASTITIS AND STUDY OF THEIR R PLASMIDS" 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.

Mannuthy,
18-2-1986.

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CERTIFICATE

Certified that this thesis, entitled "CHARACTERIZATION OF STAPHYLOCOCCI ISOLATED FROM CASES OF MASTITIS AND STUDY OF THEIR R PLASMIDS" is a record of research work done independently by Sri. Mathew, E.S. 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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MATHEW, E.S.

Dedicated to

my

Beloved parents

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INTRODUCTION

INTRODUCTION

Bovine mastitis is one of the major obstacles in the development of dairy industry as it leads to heavy economic loss. Among the various microorganisms causing mastitis, staphylococci have an important role (Jhala, 1976; Verma and Mishra, 1977; Singh and Baxi, 1982). One of the baffling phenomenon in the control of staphylococcal infections is the emergence of strains resistant to chemotherapeutic agents. Prior to 1942, staphylococcal isolates from various infections were sensitive to penicillin and other antibiotics (Wilson and Miles, 1975). But the indiscriminate use of these drugs has resulted an increase in moderately or completely drug resistant staphylococci.

For successful chemotherapy of mastitis selection of proper antimicrobial agent is essential. As such antibiotic sensitivity test which is a very useful tool in assessing the effectiveness of the antibiotics against specific microorganisms, has assumed great importance. Mechanisms of emergence of drug resistant staphylococci has not been elucidated 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 resistances

(Chopra and Howe, 1978). Though there are a number of reports on the antibiotic sensitivity pattern of staphylococci, little is known about the R plasmids of staphylococci isolated from clinical cases of mastitis. Bacterial drug resistance mediated through R plasmids is transmitted from one strain to another by conjugation, transduction and transformation. The resistance transfer in staphylococci is mainly mediated by transduction and conjugation.

Phage typing is an important method in the epidemiological studies of staphylococcal infections. The virulence of staphylococci has also been found to be related to their phage types (Koiranen, 1969).

In India staphylococci isolated from cases of bovine mastitis have not been compared with respect to their mannitol fermentation, coagulase production, hemolysin production, mercuric resistance, lysostaphin sensitivity, susceptibility to various phages and antibiogram. Hence the present investigation was designed to study the staphylococci isolated from cases of bovine mastitis giving importance to the following aspects.

1. A comparative study of mannitol fermentation, coagulase production, hemolysin production, lysostaphin sensitivity, susceptibility to various phages and mercuric resistance.

2. Determine the antibiotic resistance patterns of staphylococci by agar diffusion and agar dilution methods.

3. Find out the correlation between phage patterns and antibiogram.

4. Investigate the possibility of transfer of R plasmid DNA through conjugation *in vitro*.

5. Recommend the most suitable chemotherapeutic agent-active against staphylococci isolated from cases bovine mastitis.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

I. Characterisation of staphylococci

Ogston (1880) discovered the cluster forming coccus from pyogenic abscesses in man, while Louis Pasteur made similar conclusions in France at about the same time (Pasteur, 1880). During the decade 1950-60, the name Micrococcus pyogenes was in common use when discussing staphylococcal mastitis in animals (Schalm et al., 1971). There was considerable objection in accepting Micrococcus in place of Staphylococcus as a generic name (Shaw et al., 1951). In the mid-fifties there was renewed interest in the classification of staphylococci and micrococci and a number of papers presented arguments for and against the separation of these organisms into two separate genera (van Eseltine, 1935; Thatcher and Simon, 1957). However Evans et al.,(1955) proposed that staphylococci should be separated from micrococci on the basis of their ability to grow anaerobically and to form acid from glucose. In 1957, the official name for the genus was reverted to Staphylococcus from Micrococcus in the Bergey's manual (Breed et al., 1957). Family Micrococcaceae was divided into three genera, namely, Micrococcus, Staphylococcus and Planococcus in the 8th edition of Bergey's Manual of determinative bacteriology (Buchanan and Gibbons, 1974).

The use of the standard glucose fermentation test for distinguishing Staphylococcus from Micrococcus (Evans et al., 1955) had been criticised by several investigators (Gibson, 1967; Mortenson and Kocur, 1967). Baird and Parker (1966) proposed a double-tube oxidation/fermentation test to differentiate staphylococci and micrococci.

Reid and Wilson (1959) found that 100% of staphylococci isolated from cases of acute mastitis, 89.7% recovered from cases of chronic mastitis and 73.1% of isolates from normal udders fermented mannitol. Anaerobic fermentation of mannitol was suggested to differentiate between Staphylococcus aureus and Staphylococcus epidermidis (Cown, 1974). This was used by a number of investigators for the characterisation of staphylococci (McDonald and Anderson, 1981; Evans, 1983).

Use of catalase test in the characterization of staphylococci isolated from bovine mastitis cases was reported by various authors (McDonald and Anderson, 1981; Hodges et al., 1984).

Staphylococci produced at least four hemolysins, viz., alpha, beta, gamma and delta. On sheep blood agar, a wide zone of incomplete or partial hemolysis was reported around colonies of staphylococci after incubation at 35°C (Minor and Marth, 1976). The hemolysin production as a parameter in the characterisation of staphylococci had been reported

by a number of workers (Loken and Harvey, 1962; McDonald and Anderson, 1981; Walia et al., 1981; and Poutrel, 1984).

Loeb (1903) discovered that Staphylococcus pyogenes could coagulate goose plasma in vitro. Two types of coagulase had been described - the bound coagulase and free coagulase. The term bound coagulase was introduced by Duthie (1954) to designate a factor that he considered was responsible for the clumping of cells that was associated with slide coagulase test. Gupta et al. (1978) made a comparative study on the efficacy of plasma from rabbit, human, bovine, sheep and horse, and found that horse plasma gave better results than others. Sheep plasma was considered unreliable for coagulase test, whereas rabbit, human and bovine plasma showed this activity in decreasing order. Singh et al. (1981) compared coagulase test with egg yolk reaction, polymyxin B agar growth, tellurite glycine agar growth, protease activity and mercuric chloride agar growth using 230 staphylococcal strains isolated from bovine mastitis and found that the coagulase test could not be substituted by any of these tests used in the study. Hodges et al. (1984) used rabbit plasma for conducting coagulase test on staphylococcal strains isolated from bovine mastitis. They have identified 900 strains of staphylococci/micrococci from samples of bovine milk examined for mastitis producing organisms and found that 831 were coagulase-positive and 65 coagulase-negative.

The discovery and characterization of the staphylolytic enzyme, lysostaphin, by Schindler and Schuhardt (1964) provided a mild and rapid method for liberating the nucleic acids and other cytoplasmic contents from lysostaphin susceptible members of family Micrococcaceae. After testing a large number of organisms under Micrococcaceae and other families of bacteria, the lytic activity of lysostaphin was found specific for members of the genus Staphylococcus (Kloosius and Schuhardt, 1968). Gutierrez et al. (1981) have examined 195 gram positive catalase positive cocci isolated from ovine mastitis, abscesses in slaughtered animals and pulmonary lesions in lambs for the susceptibility to the lytic action of lysostaphin and by other biochemical tests. They could identify 192 out of the 195 strains, as staphylococci on the basis of lysostaphin sensitivity. The studies also indicated that sensitivity to lysostaphin could not be used to differentiate coagulase-positive and coagulase-negative strains.

Phage-typing

The accumulation of knowledge concerning the staphylococcal bacteriophages and their lytic action had led to a technique for the differentiation of staphylococcal strains on the basis of phage susceptibility. Early phage isolates were not of a type suitable for differentiation of host strains, for they had broad lytic power, lysing most

staphylococcal cultures (Koiranen, 1969). Callow (1922) was the first to obtain a series of phages which differed in lytic activity. From 14 cases of acute purulent infections she obtained six phages with varying ability to lyse stock strains of staphylococci isolated from similar sources. Fisk (1942) laid the foundation for a practical typing technique utilising lysis on solid media. He had used undiluted phage lysate, whereas Wilson and Atkinson (1945) had adopted the use of the "routine test dilution" (RTD) i.e., the highest dilution giving confluent lysis of the propagating strain. Bacteriophage typing was considered particularly significant for the staphylococci which were so difficult to distinguish on any other basis and this could be used in the study of the epidemiology of staphylococcal infections (Oeding and Williams, 1958; Wentworth, 1963; Koiranen, 1969).

The subcommittee on bacteriophage typing of staphylococci, of the International Committee on Bacteriological Nomenclature, London had established a basic series of phages that were divided into four groups (Coles and Eisenstark, 1959.

Group I, phages 29, 52, 52A and 79

Group II, phages 3A, 3B, 3C and 55

Group III, phages 6, 7, 42E, 47, 53, 54, 70, 73, 75 and 77

Group IV, phages 42D.

Blair and Williams (1961) worked for the development of standard methods of phage typing in order to improve the comparability of the results obtained by different laboratories. In this way a basic international phage set was developed which included 21 phages.

Loken and Harvey (1962) isolated staphylococci from bovine udders and reported that the lysis by phage 42D (group IV) was the most common observation. The International Subcommittee on Staphylococcus Phage Typing decided at its third meeting in 1962 that a standard set should be composed for the typing of bovine staphylococci (Subcommittee, 1963). Ian Davidson, of Weybridge, England performed the comparison of different phages by typing 454 strains with bovine phages and the phages of the human set at RTD. Based on the comparison, he had chosen a set consisting of 17 phages and suggested an extensive international experiment on its use in the typing of bovine staphylococci (Davidson, 1964, 1966).

Using the international series of phages and Davidson's set of phages, attempts were made by Bonin and Blobel (1967) to type 1073 cultures of coagulase positive staphylococci isolated from bovine milk samples. It was observed that only 73% of the cultures could be typed with the international series as against 84% typed with Davidson's set. Using a modified Davidson's set the authors were able to type 90% of

878 isolates. Of the typable strains 84.4% could be assigned to the various lysogroups and 63% of them to group IV. Frost (1970) studied the action of 20 selected phages on 1187 strains of Staphylococcus aureus isolated from dairy cattle. The phage patterns of 933 strains were analysed and 45 simple patterns could be accounted for 69% of strains and 7% were untypable. The phages were separated into following phage groups, group I -11.4%, group II -0.8%, group III -2.2%, group III/IV 20.9%, group IV -51.4%, miscellaneous 5.8% and others 7.5%.

Bhatia et al. (1981) isolated six new phages from untypable strains of Staphylococcus aureus. In the study 456 strains of Staphylococcus aureus were included. All these phages were employed to type the 456 untypable strains of S. aureus at RTD and 100RTD. At 100 RTD, these phages could type 165 (36.18%) strains which included 83 (18.20%) strains that could be typed at RTD also. These phages were found to give better results than the standard experimental control phages.

In India the phages used at the Staphylococcal Phage Typing Centre, New Delhi, included an International basic set of 23 and 4 experimental typing phages (Rahman and Baxi, 1983a) as shown below:

Group	Phages
I	29/52/52A/79/80
II	3A/3C/55/71
III	6/42E/47/53/54/75/77/83A/84/85
IV	81/94/95/96
Experimental	A/B/C/F

Rahman and Daxi (1983b) reported the predominance of phage group III among Staphylococcus aureus isolated from cows and buffaloes suffering from mastitis.

II. Staphylococci as the cause of mastitis

The presence of staphylococci in bovine udders had been known for many years. Evans (1916) examined the bacterial flora of 192 milk samples from five dairy herds and recovered "micrococci" from 58.8% of the samples. On the basis of hemolysis, fermentation of mannite and pigmentation, one half of the isolates was considered to be typical of S. aureus. Ten of 17 selected strains injected into rabbits were shown to be virulent staphylococci. So they opined that pathogenic staphylococci could be isolated from milk drawn from apparently normal udder.

Dhanda and Sethi (1962) found that 41.2% of mastitis in cows and 30.5% of mastitis in buffaloes were caused by S. aureus. The characteristics of 1312 coagulase-positive and 109 coagulase-negative staphylococci isolated from bovine mastitis were reported by Koiranen (1969). Hopkrik

(1972) reported S. aureus as the predominant organism in mastitis in cows, followed by Streptococcus agalactiae, S. epidermidis and Streptococcus uberis. Misra et al. (1973) reported that 33.1% of the udder infections in 305 cows were due to S. aureus. Jhala (1976) reported 103 (45.38%) of S. aureus from 227 milk samples in a study conducted in Gujarat. A survey performed during 1965-66 showed that 40.7% of 3956 composite milk samples collected randomly from cows in 399 New Zealand herds yielded coagulase-positive haemolytic staphylococci (Elliot et al., 1976a; Elliot et al., 1976b and Tattersfield et al., 1976). Kohler-samouilidis (1977) demonstrated 71 strains of S. aureus, 82 strains of S. epidermidis and 17 strains of Micrococcus from 170 milk samples obtained from cows with suspected mastitis. Suribabu et al. (1979) studied the milk samples from clinical cases of mastitis from cows and buffaloes for determining the bacterial etilogy and found that 55% was S. aureus. Pearson and Mackie (1979) examined 477 quarters effected for the first time and found that 139 (29%) were due to S. aureus. Kapur et al. (1979) found that among the 304 staphylococcal strains isolated from bovine mammary glands, 103 were coagulase-positive and 201 as coagulase-negative. Bacteriological examination of 1262 milk samples from cases of mastitis was carried out by Ramachandra et al. (1984) and observed that staphylococcal

and streptococcal mastitis ranked high. Sudharma et al. (1985) examined 483 milk samples from cases of bovine mastitis from different parts of Kerala and different types of bacteria were isolated from 240 cases, 31.67% of the organisms isolated were found to be staphylococci.

III. Antibiotic resistance of staphylococci

From 1945 onwards, penicillinase forming strains became increasingly common, first in hospitals and subsequently in the general population and among animals (Parker, 1983).

Van and Devriese (1971) tested 132 mastitis strains from 63 herds and showed that 38% produced penicillinase, 26% were resistant to streptomycin, 21% to tetracycline, 3.5% to chloramphenical and 3% possessed multiple drug resistance to four antibiotics. They also observed that all the strains were sensitive to neomycin, virginiamycin, and rifamycin. Janetschke and Rizk (1973) reported in Syria that S. aureus was the most common organism isolated from bovine mastitis and it was observed that 78% of the isolates were penicillin resistant followed by streptomycin (29%) and chloramphenicol (6.6%). 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. In mid Western USA 813 cultures from milk of

infected udders from 24 herds were examined by McDonald and Anderson (1981) for antibiotic sensitivity to S. aureus and coagulase-negative staphylococci and found that over 90% was sensitive to bacitracin, cephalothin, chloramphenicol, cloxacillin, erythromycin, gentamicin, kanamycin, linomycin, neomycin, penicillin, oleandomycin and vancomycin. S. aureus displayed greater antibiotic resistance than the coagulase-negative staphylococci.

Tests on 1657 strains of S. aureus isolated from bovine mastitis collected by 13 Australian laboratories between 1974-79 showed that the commonest resistance was to penicillin and streptomycin, and multiple resistance was rare. No strain was resistant to methicillin. Resistance to penicillin declined from 35% in 1974-75 to 7% in 1979 (Frost and O'Boyle, 1981). Nag and Ghosh (1982) studied the sensitivity to antibiotics of 390 coagulase-positive staphylococci from cows and buffaloes with mastitis, endometritis, ostomyelitis and surgical wounds and found that 12.3% of strains were resistant to all seven antibiotics (benzylpenicillin, ampicillin, streptomycin, oxytetracycline, chloramphenicol, bacitracin and kanamycin) tested; 62% was single resistant, others were multiple resistant. Among these 76% of the strains were resistant to benzylpenicillin, 66% to ampicillin, 38% to oxytetracycline, 46% to streptomycin, 34% to bacitracin, 18% to chloramphenicol and 16% to kanamycin.

In a study on the incidence, epidemiology and antibiotic sensitivity of staphylococci isolated from mastitis cases in bovines, Rahman and Baxi (1983b) reported the susceptibility pattern of S. aureus and S. epidermidis to various antimicrobial agents like neomycin, chloramphenicol and nitrofurantoin as equal and significantly greater number of organisms were found resistant to penicillin, and streptomycin. Franklin and Ratzion (1983) determined the minimum inhibitory concentration (MIC) of ten antimicrobial drugs to 287 S. aureus strains isolated from bovine mastitis. Thirtyseven strains produced betalactamase. All strains were sensitive to oxacillin, neomycin and more than 90% to streptomycin, trimethoprim, chloramphenicol, erythromycin and tetracycline, whereas all were resistant to sulfamethoxazole.

Craven and Anderson (1983) examined 24 isolates of S. aureus from bovine mastitis cases and found that all strains produced penicillinase and none showed intrinsic resistance (methicillin resistance) to cloxacillin. In a study of S. aureus isolated from bovine mastitis Kapur et al. (1984) observed sensitivity to various chemotherapeutic agents in the following order; Furadantin (98.18%), cloxacillin (97.56%), chloramphenicol (90.20%), oleandomycin (80.77%); neomycin (79.25%), streptomycin (71.82%), oxytetracycline (40.54%), polymyxin B (49.50%), ampicillin (33.33%) and penicillin (36.36%). Sudharma et al. (1985) studied the

antibiogram of staphylococci isolated from bovine mastitis cases, by agar diffusion method and found that 84% of the organisms were sensitive to gentamycin, 78.46% to chloramphenicol, 62.5% to neomycin, 60.96% to tetracycline, 55.56% to furadantin, 50% to ampicillin and 43.17% to penicillin. None of the isolates was resistant to all the antibiotics tested.

IV. Mercuric resistance

Mercury and organomercurial-resistant bacteria were first isolated from mercury contaminated soil in Japan (Robinson and Tuovinen, 1984). Bacterial resistance to mercury and organomercurials was reported to be determined by plasmids which in many instances also encode resistance to other heavy metals and antibiotics (Novick and Roth, 1968). The penicillinase plasmids or S. aureus carry determinants for resistance to mercury as well as arsenate, lead, cadmium and bismuth ions all grouped in one region (Kondo et al., 1974). The Hg^r phenotype was reported to be closely associated with tetracycline resistance, a lack of mannitol fermentation and coagulase-positive phenotype (Groves and Young, 1975). 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 mercury acetate.

The plasmid determined nature of resistance to mercury compounds was established by determining the ability for cotransduction (with other plasmid encoded determinants) and high frequency of conjugal transfer of the Hg^r determinant. Isolation of covalently closed circular DNA from the Hg^r strains and its ability to transform Hg^s recipients to the Hg^r phenotype provided further evidence that the Hg^r determinant was plasmid encoded, as did curing the Hg^r strains with agents such as ethylmethane sulfonate. There appeared to be a strong correlation between antibiotic resistance and resistance to mercury and several other metals (Novick and Roth, 1968; Nakahara et al., 1977a; Nakahara et al., 1977b).

Stanisich et al. (1977) and Bennett et al. (1978) reported the occurrence of mercuric resistance genes on transposons, as for other resistance genes carried by plasmids. The evolution of multiple metal and antibiotic resistant strains might be the result of the sequential acquisition of individual transposons to form composite transposable units. Eventhough mercuric resistance transposon had been reported in other species of bacteria, no mercury resistance transposon had been demonstrated in any of the broad-spectrum Hg^r strains of S. aureus (Robinson and Tuovinen, 1984).

V. R plasmids of staphylococci

The term "plasmid" was introduced by Lederberg (1952) as a generic name for extrachromosomal genetic element.

A plasmid is a replicon that is stably inherited (i.e., readily maintained without specific selection) in an extra-chromosomal state. Naturally occurring plasmids of prokaryotes are generally dispensable. An R plasmid is a plasmid that carries genetic information for resistance to antibiotics and/or antibacterial drugs.(Novick et al., 1976).

Resistance (R) factors mediated resistance to various antibiotics was reported originally in species of shigella in Japan (Watanabe, 1963). Novick (1963) had shown that both the genes for determining the synthesis of penicillinase and control of its production were very probably carried by one plasmid. Chabbert et al. (1964) reported that all the evidence pointed to a plasmid locous for the genes determining chloramphenicol resistance in all chloramphenicol resistant strains studied. The evidence for plasmid inheritance of tetracycline resistance was reported by Ashoshov (1966) and Novick and Bouanchaud, 1971).

Drug resistance associated with R factors was originally thought to result from decreased permeability to the drugs. But, later this view was changed. Harwood and Smith (1969) presented evidence that cells bearing R factors contained an enzyme located in the periplasmic space which inactivated streptomycin by adenylation. In many cases, streptomycin resistance in staphylococci was reported to be plasmid mediated (Grinsted and Lacey, 1973).

Resistance of staphylococci to penicillin, erythromycin, tetracycline, chloramphenicol, kanamycin, methicillin and fusidic acid was suspected to be or known to be plasmid linked (Falkow, 1975). Lacey (1975) opined that most antibiotic resistance in S. aureus, as in Enterobacteriaceae, could be plasmid mediated.

Bastos et al. (1980) isolated a plasmid with a molecular weight of 1.4×10^6 daltons which determined constitutive resistance to erythromycin and lincomycin in S. aureaus. It was reported to be the smallest naturally occurring element coding for antibiotic resistance in this species. A study was conducted by Cohen et al. (1982) on S. aureus which was resistant to many antibiotics like oleandomycin, erythromycin, gentamicin, methicillin, penicillin and tetracycline. A single 32-megadalton (md) plasmid was detected in all the strains and the endonuclease restriction patterns of all isolates with the epidemic antibiogram were identical.

VI. Conjugation in staphylococci

Conjugation 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' (Willetts and Skurray, 1980).

The literature on conjugation in staphylococcal species

is scant. Iordanescu et al. (1978) and Sjostrom et al.(1979) described similar types of resistance plasmids from S. aureus and S. epidermidis. 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). The interspecific spread of resistance to antibiotics between strains of staphylococci in nature was suspected to occur by cell-to-cell contact, i.e., phage-mediated conjugation (Lacey, 1971,a,b, 1980a). According to Novick (1980) staphylococcal R factors could not apparently promote their own transfer by conjugation.

The rarity of gene transfer between animal and human strains of S. aureus in vitro have been reported by Lacey (1980b). Fawcett, et al. (1981) reported the failure of coagulase-negative staphylococci to transfer antibiotic resistance to S. aureus 1030 in mixed culture. Schaberg et al. (1982) demonstrated the intergeneric transfer of conjugal resistance plasmids from Streptococcus faecalis to S. aureus. Emergence of antibiotic resistance in S. aureus was suspected to occur by genetic transfer from S. epidermidis (Cohen et al. 1982). 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 both cultures. Mathew and Punnoose (1986) have reported for the

first time, the conjugal transfer of streptomycin resistant plasmid DNA from coagulase-negative staphylococci to S. aureus RN 450 RF in mixed cultures.

MATERIALS AND METHODS

MATERIALS AND METHODS

Milk samples from cases of bovine mastitis were collected from the Kerala Agricultural University Livestock Farm, Mannuthy and the University Veterinary Hospitals at Mannuthy and Trichur. A total of 360 milk samples were screened.

I. Isolation of staphylococci

The milk samples were plated on Mueller-Hinton Agar (HI-Media) and incubated at 37°C for 24 hours for isolating bacterial organisms. Those colonies suggestive of staphylococci were examined microscopically after Gram staining. The gram positive cocci, arranged as bunches of grapes were subcultured on Mannitol Salt Agar and incubated for 24-36 hours at 37°C. After incubation the colonies were subcultured on Tryptic soy Agar (Difco) slants in duplicate tubes and stored at 4°C for further experimental studies.

A. Typing of staphylococci

The typing of staphylococci was done by using the following tests.

i) Gram staining, anaerobic fermentation of glucose, aerobic and anaerobic utilisation of mannitol, catalase test (Cowan, 1974).

ii) Haemolysin production:

The haemolytic ability of the strains were studied using sheep blood agar.

iii) Coagulase test (Cowan, 1938)

0.5 ml undiluted rabbit plasma was mixed with an equal volume of an 18-24 hour broth culture and incubated at 37°C for 4 hours and examined after 1 and 4 hours for a coagulum. Negative tubes were left at room temperature over night and then re-examined.

iv) Lysostaphin sensitivity (Bennett, 1978)

1. An isolated colony from an agar plate was transferred using an inoculation loop to 0.2 ml of phosphate buffered saline (PBS) and emulsified.

2. Half of suspended cells was transferred to another tube (13 x 100 mm) and was mixed with 0.1 ml of PBS and kept as control.

3. 0.1 ml lysostaphin (Sigma Chemical Co., St. Louis, USA) dissolved in 0.02 M phosphate buffer containing 1% NaCl was added to the original tube to give a final concentration of 25 µm lysostaphin per ml.

4. Both the tubes were incubated at 35°C for not more than 2 hours. A clearing of turbidity in the test mixture was considered positive.

v) Phage typing

The phage typing of S. aureus was done at the Staphylococcal Phage Typing Centre, ICMR, Maulana Azad Medical College, New Delhi. The phages used for typing were as follows:

Group	Phages
I	29/52/52A/79/80
II	3A/3C/55/71
III	6/42E/47/53/54/75/77/83A/84/85
IV	81/94/95/96

II. Antibiotic sensitivity

A. Agar diffusion method

Kirby-Bauer method (Brown and Blowers, 1978) was used for testing the sensitivity/resistance of the staphylococcal strains.

i) Medium

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

ii) Antibiotic discs

Antibiotic sensitivity discs manufactured by M/s. Pasteur Biologicals, Gujarat and M/s. Span Diagnostics, Surat were used in the study. The antibiotic discs used with their strength are furnished below:

1. Amoxycillin - 10 mcg
2. Ampicillin - 10 mcg
3. Bacitracin - 10 u
4. Chloramphenicol - 30 mcg
5. Cloxacillin - 1 mcg
6. Erythromycin - 15 mcg
7. Gentamicin - 10 mcg
8. Methicillin - 5 mcg
9. Neomycin - 30 mcg
10. Nitrofurantoin - 300 mcg
11. Penicillin - 10 units
12. Streptomycin - 10 mcg
13. Sulphamethoxazole - 25 mcg
14. Tetracycline - 30 mcg

iii) Preparation of culture plates

The M.H. Agar was reconstituted and sterilised by autoclaving at 121°C for 15 minutes and cooled to 45-50°C and poured into sterile glass petridishes of 100 mm size to obtain a depth of approximately 4 mm. When cooled the moisture on the agar surface was removed by keeping them at 37°C for 30 minutes and were used after sterility test.

iv) Preparation of inoculum

At least four morphologically similar colonies from an agar medium were touched with a wire loop and the growth

was transferred to a test tube containing 4 ml of sterile Tryptic Soy broth (Difco). The tubes were inoculated for 5 hours at 37°C to produce a bacterial suspension of moderate cloudiness. The density of the suspension was standardised by dilution with sterile saline to a density visually equivalent to a barium sulphate standard. The standard was prepared by adding 0.5 ml of 0.048 M barium chloride (1.175% W/V of $BaCl_2 2H_2O$) to 99.5 ml of 0.18 M sulphuric acid (1% W/V). The standard was kept in screw capped tube of the same size as that used for the broth cultures and stored in the dark at room temperature. Before use the standard was shaken vigorously.

v) Inoculation of plates

Plates were inoculated within 15 minutes of preparation of the suspension so as to prevent any change in the density of the inoculum. A sterile cotton-wool swab was dipped in the suspension and surplus fluid was removed by rotation of the swab against the side of the test tube above the fluid level. The medium was inoculated by swabing over the entire surface of the plate.

vi) Application of antibiotic discs

After the inoculum has dried, antibiotic discs were applied with a sterile forceps and pressed gently to ensure even contact with the medium. Seven discs were placed on a

100 mm plate. The spatial arrangement of the discs was such that they were not closer than 15 mm from the edge of the plate and far apart to prevent overlapping of zone of inhibition.

vii) Incubation

Plates were inverted and placed singly in the incubator at 37°C within 15 minutes of application of the discs, as long delay before incubation would allow excess prediffusion of antibiotic. The plates were incubated for 16-18 hours.

viii) Reading of zone of inhibition

The diameter of zone of inhibition was measured to the nearest millimetre with a millimetre rule. The point of abrupt diminution of growth, which in most cases corresponded with the point of complete inhibition of growth, was taken as the zone edge.

ix) Interpretation

Each zone size was interpreted, by reference to a table (Table 1) into one of the three categories as sensitive, intermediate or resistant.

B. Agar dilution method (Barry, 1976)

All the strains were tested by agar dilution method using single concentration of antibiotic.

i) Medium: Mueller-Hinton Agar (HI-media) was used.

ii) Antibiotics

Erythromycin, chloramphenicol, tetracycline, streptomycin, rifampicin, gentamicin, ampicillin (Central Drug Research Lab., Calcutta) and benzyl penicillin (Glaxo Laboratories, Bombay) were used.

The activity standard of the antibiotics used were provided by the manufacturers (Table 2).

iii) Antibiotic stock solutions

The standard or reference obtained from the above laboratories were stored at -20°C unless otherwise specified. A stock solution containing 2000 mcg/ml or 1.U/ml was prepared (Barry, 1976). The actual weight of active substance in the sample was calculated by multiplying the weight of the sample (in mg) by the weight (mcg or IU) of active substance per mg. The volume of solvent required to achieve the desired concentration was calculated by dividing the weight in mcg of active substance in the sample by desired concentration.

$$\text{Volume of solvent} = \frac{\text{Weight of sample (in mg)} \times \text{activity standard (mcg or I.U/ml)}}{\text{Desired concentration (mcg or I.U/ml)}}$$

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

Antibiotic	Solvent	Diluent
1. Ampicillin	Phosphate buffer pH 8.00, 0.1 M	Phosphate buffer pH 6.0, 0.1 M
2. Chloramphenicol	Ethanol	Water
3. Rifampicin	Dimethyl sulfoxide	Phosphate buffer, pH 7.0
4. Streptomycin	Water	Water
5. Tetracycline	Water	Water
6. Gentamicin	Phosphate buffer pH 8.0, 0.1 M	Water
7. Erythromycin	Ethanol	Water
8. Penicillin	Water	Water

Stock solutions were prepared and distributed in small aliquots required for one antibiotic incorporation and stored at -20°C.

iv) Preparation of antibiotic containing medium

Mueller-Hinton agar was reconstituted and sterilized by autoclaving at 121°C for 15 minutes. The antibiotic stock solutions prepared and stored at -20°C were taken out and thawed. When the agar has cooled down to 45-50°C a fixed quantity of antibiotic solution was incorporated into the agar and mixed by rotating and poured into sterile petri-dishes. When solidified, the moisture on the agar surface was removed by placing them at 37°C for 30 minutes. The following were the final concentration of antibiotics per ml of medium (Barry, 1976).

Tetracycline	-	12 mcg/ml
Streptomycin	-	15 mcg/ml
Chloramphenicol	-	25 mcg/ml
Erythromycin	-	8 mcg/ml
Gentamicin	-	6 mcg/ml
Benzyl Penicillin		1 mcg/ml
Rifampicin	-	2.5 mcg/ml
Ampicillin	-	32 mcg/ml

v) Inoculation of medium and incubation

An 18 hour old, 4 to 5 ml Tryptic Soy broth culture containing approximately 10^9 colony forming unit (CFU) per ml was used. This was diluted to 1 in 20 and 0.02 ml of it was used as the inoculum (Barry, 1976). To facilitate a number of strains to be tested at the same time, petri-dish was divided into 12 regions and each region was inoculated with a particular strain and incubated at 37°C for 18-24 hours. The results were read after the incubation and the sensitivity of the organisms at the particular concentration of antibiotic used, was indicated by absence of growth on the inoculated region. A definite dense film of growth or more than one colony at the site of inoculation was considered evidence to show that the antibiotic failed to inhibit growth adequately at that concentration.

III. Mercuric resistance

The mercuric resistance of all the isolates were determined as per the procedure described by Moore (1960).

The medium for the preparation of mercuric chloride agar was made from a peptone agar base containing peptone 2%, sodium chloride 0.5% and agar 1%. The pH of the medium was adjusted to 7.4 and was autoclaved at 10 lbs pressure for 15 mts. The mercuric chloride was added from a stock solution just before plates were poured. The stock solution was not sterilized. To prepare the medium 100 ml of the peptone agar was melted and allowed to cool to 50°C. Then, 3.63 ml of the agar was pipetted off and replaced by the same volume of 1 in 1000 mercuric chloride. The plates were poured and dried at 37°C for 30 mts and used immediately. The medium was inoculated with a large loop full of an overnight broth culture of the test strain. Twelve strains were tested on each peptone agar plates. After inoculation the plates were incubated at 37°C overnight and were examined for a circle of confluent growth or no growth.

IV. Conjugation experiments

For the conjugation experiment the method described by Datta (1978) with slight modifications was used.

i) Media used:

a) Tryptic Soy broth (Difco)

b) Mueller-Hinton Agar (Hi-Media).

ii) Antibiotics

The antibiotics and the concentrations used were same as that used for determining the sensitivity/resistance by agar dilution method.

iii) Recipient strains

Both the recipients used were having chromosomal resistance as detailed below.

a) S. aureus RN 450RF, phage-free, plasmid-free Rif^r/Fus^r*

b) S. epidermidis 131S, plasmid-free, str^r*

iv) Donor strains

In the mating experiments using S. aureus as recipient, 20 strains (Table 3) and in the mating experiments using S. epidermidis as recipient, 7 strains (Table 4) were selected after studying the drug resistance pattern of all the strains by agar diffusion and agar dilution methods. All the donor strains were rifampicin sensitive. In the conjugation experiments with S. epidermidis 131S as the recipient, only streptomycin-sensitive donors were used.

v) In vitro transfer

a) Using S. aureus RN 450RF as recipient

From six hour old cultures, 0.1 ml of rifampicin sensitive donor (Table 3) and 0.2 ml of the recipient were

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* Kindly provided by DR. Dennis, R. Schaberg of the University of Michigan, USA.

co-cultivated in 10 ml of Tryptic soy broth (Difco) and incubated overnight at 37°C and was centrifuged at 3000 rpm for 30 mnts at 4°C. The sediment was plated on to Mueller-Hinton agar containing 2.5 mcg/ml of rifampicin and one of the antibiotics to which the donor was resistant (selective media) and incubated at 37°C for 24 hours. Donor and recipient controls were plated separately. The concentrations of the antibiotics were the same as used in agar dilution method of sensitivity tests. The colonies coming up on the selective media - transcipients - were purified by subculturing on 'selective media' and the resistances transferred were further confirmed by agar diffusion method.

b) Using S. epidermidis 1318 as recipient

Since the chromosomal marker for the recipient was streptomycin resistance, only streptomycin-sensitive donors were used in the experiment. From six hour old cultures, 0.1 ml of the streptomycin-sensitive donor (Table 4) and 0.2 ml of the recipient were co-cultivated in 10 ml of Tryptic soy broth (Difco) and incubated at 37°C for 24 hours and was centrifuged at 3000 rpm for 30 mnts at 4°C. The sediment was plated on to Mueller-Hinton agar containing 150 mcg/ml of streptomycin and one of the antibiotics to which the donor was resistant (selective media) and incubated at 37°C for 24 hours. The concentration of erythromycin and tetracycline used was the same as used in agar dilution method of

sensitivity tests. The donor and recipient controls were always plated separately.

vi) Experiment to exclude the possibility of transduction and transformation

Cell-free filtrates 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 millilitre of cell-free filtrate of each donor was mixed separately with three millilitre of an overnight culture of the recipient and incubated at 37°C for 6-8 hours and the mixture was plated on to appropriate 'selective media' and incubated at 37°C. After 24-48 hours incubation the plates were examined for the presence of any colonies. Recipient and filtrate controls were plated separately.

RESULTS

RESULTS

A total of 360 milk samples from cases of bovine mastitis were examined for the presence of staphylococci. Bacterial organisms could be isolated from 195 (54.17%) samples. From these, 52 strains of (26.67%) staphylococci were isolated which consisted of 17 (32.69%) S. aureus strains and 35 (67.31%) coagulase-negative staphylococci.

I. Isolation and identification

a) Gram's staining: All the 52 isolates were Gram positive.

b) Anaerobic fermentation of glucose: All the strains were able to ferment glucose anaerobically. No micrococci could be detected.

c) Mannitol fermentation: Mannitol was fermented anaerobically by 39 strains while 13 strains were unable to ferment mannitol anaerobically. Mannitol was utilised aerobically by 40 strains while 12 strains were unable to do so. Ten strains could not utilise mannitol both aerobically and anaerobically.

d) Catalase test: All the isolates were positive for the catalase test.

e) Haemolysin production: Among the 52 strains of staphylococci 21 (40.38%) were found to produce haemolysis

on solid media containing 5% defibrinated sheep blood. Among the 17 coagulase-positive staphylococci four strains (23.52%) (M19, M51, M79 and M87) could not produce haemolysis on sheep blood agar while all other coagulase-positive strains (76.48%) produced haemolytic zone on sheep blood agar. Nine out of the 35 (25.71%) coagulase-negative staphylococci were found to produce haemolysis of sheep red blood cells.

f) Coagulase test: Out of the 52 strains 17 (32.69%) were coagulase-positive and 35 (67.31%) were coagulase-negative. The coagulase-positive strains are referred here as S. aureus. Of the 17 coagulase-positive isolates, six gave positive reaction in one hour, two strains in four hours and the remaining nine strains gave a positive reaction after keeping at room temperature overnight.

g) Lysostaphin sensitivity: Fortyseven (90.38%) out of the 52 staphylococcal strains were sensitive to lysostaphin. All the coagulase-positive staphylococci were found sensitive to lysostaphin. The results of anaerobic fermentation of glucose, aerobic and anaerobic utilization of mannitol, catalase test, haemolysin production, coagulase test and lysostaphin sensitivity are presented in table 5.

h) Bacteriophage typing: All the coagulase-positive strains (S. aureus) were phage typed at routine test dilution

(RTD) and the untypable strains were retyped at 100 RTD. The strains which could not be typed at both dilutions were classified as non-typable (NT). Seven (41.17%) of the 17 coagulase-positive strains could be typed with one or other of the phages of the International basic set of 23 phages. Ten strains were grouped as non-typable. Of the typable strains group III showed predominance (28.57%) over other groups. 57.14% of the strains belonged to mixed groups. Only one strain belonged to group II. The details of the phage pattern, phage group(s) and the antibiotic resistance pattern are presented in table 6.

II. Antibiotic sensitivity

a) Agar diffusion method: All the 17 S. aureus strains were sensitive to bacitracin, while majority of them were resistant to streptomycin (64.70%), amoxycillin (52.94%), ampicillin (52.94%), sulphamethoxazole (42.86%) and tetracycline (41.18%). Only 5.88% of the S. aureus strains were resistant to cloxacillin, nitrofurantoin, neomycin, methicillin and gentamicin; 11.76% to chloramphenicol and 23.53% to erythromycin and penicillin.

All the 35 coagulase-negative staphylococci were sensitive to cloxacillin, gentamicin, methicillin, neomycin and nitrofurantoin. 42.86% of the coagulase-negative strains were resistant to streptomycin and sulphamethoxazole while only 37.14% was resistant to tetracycline. The resistance

pattern to other antibiotics were to ampicillin and penicillin (22.86%); amoxycillin (11.43%); erythromycin (17.14%) and bacitracin and chloramphenicol (2.86%).

Out of the 52 staphylococcal strains studied, ten (19.23%) (M10, M19, M29, M31, M36, M43, M56, M64, M65 and M79) were sensitive to all the 14 antibiotics tested (Table 7). The details of the results of antibiotic sensitivity of all the strains by agar diffusion method are given in table 8.

b) Agar dilution method: All the 17 S. aureus isolates were sensitive to gentamicin, erythromycin and rifampicin; 47.06% of the S. aureus strains were resistant to penicillin and streptomycin followed by tetracycline (35.29%), ampicillin (11.77%) and chloramphenicol (5.88%).

All the 35 coagulase-negative staphylococci were sensitive to ampicillin, gentamicin, chloramphenicol and rifampicin and 31.43% of the coagulase-negative strains were resistant to tetracycline, 28.57% to streptomycin, 22.86% to benzyl penicillin and 14.29% to erythromycin. The results of the drug sensitivity/resistance of the isolates by agar dilution method are presented in table 9.

III. Mercuric resistance

All the 52 strains of staphylococci were sensitive to mercuric chloride at a concentration of 1 in 27,500.

IV. Conjugation experiments

In the conjugation experiments, ten S. aureus and ten coagulase-negative strains were used as donors when S. aureus RN 450RF was used as the recipient. One S.aureus and six coagulase-negative strains of staphylococci were used as donors when S. epidermidis 1316 was used as the recipient.

1) Using S. aureus RN 450RF as recipient

a) S. aureus as donors: Six of the ten S. aureus donor strains used in the study could transfer either one or more drug resistance markers to the recipient. Of these one strain (M51) transferred tetracycline resistance only, while two strains (M12 and M18) transferred streptomycin resistance, two strains (M35 and M80) transferred penicillin and streptomycin resistances jointly while one strain (M48) transferred streptomycin and tetracycline resistances together. No chloramphenicol, ampicillin or erythromycin resistances were found transferred from the donors to the recipient in the study. Details of the donors, recipient, selective donor-markers and the transcipients obtained are presented in table 10.

b) Coagulase-negative staphylococci as donors: All the four streptomycin resistant donor strains (M9, M38, M41 and M49) were found to transfer the resistance to the recipient

S. aureus RN 450RF. But the two multiple resistant strains (M41 and M49) could transfer only the streptomycin resistance and not the penicillin and tetracycline resistances. Erythromycin, tetracycline and penicillin resistances were not transferred from any of the donors to the recipient (Table 10).

ii) Using S. epidermidis 1315 as recipient

a) S. aureus as donor: Only one S. aureus strain (M87) was used in the study. It was observed that the tetracycline resistance could not be transferred from the donor to the recipient (Table 11).

b) Coagulase-negative staphylococci as donor: The selective donor markers used were tetracycline and erythromycin resistances. All the six donor strains studied in the experiment failed to transfer any of these resistance markers (Table 11).

V. Experiment to exclude the possibility of transduction and transformation

No recipient strain could acquire the antibiotic resistance markers when mixed with the filtrate of the donors. The donor filtrate controls were unable to produce any growth on the Mueller-Hinton agar. Similarly the recipient controls plated on to selective media containing the appropriate antibiotics also did not show any growth.

TABLES

Table 1. Zone-size Interpretative chart for the agar diffusion method

Sl. No.	Antimicrobial Agent	Disc content	Dia. of zone of inhibition indicating		
			Resistance (mm or less)	Inter-mediate	Sensitive (mm or more)
1	Ampicillin	10 mcg	20	21-28	29
2	Bacitracin	10 units	8	9-12	13
3	Amoxycillin	10 mcg	12	13-17	18
4	Penicillin-G	10 units	20	21-28	29
5	Methicillin	5 mcg	9	10-13	14
6	Tetracycline	30 mcg	14	15-18	19
7	Chloramphenicol	30 mcg	12	13-17	18
8	Erythromycin	15 mcg	13	14-17	18
9	Streptomycin	10 mcg	11	12-14	15
10	Cloxacillin	1 mcg	10	11-12	13
11	Neomycin	30 mcg	12	13-16	17
12	Gentamicin	10 mcg	12	-	13
13	Sulphamethoxazole	25 mcg	10	11-15	16
14	Nitrofurantoin	300 mcg	14	15-16	17

(concl.)

Table 2. Activity standard of the antibiotics used

Sl.No.	Antibiotic	Activity standard (mcg or IU/mg)
1	Tetracycline Hydrochloride	983
2	Streptomycin sulphate	748
3	Erythromycin	920
4	Chloramphenicol (Assay 100%)	1000
5	Rifampicin	900
6	Gentamicin	641
7	Ampicillin	855
8	Benzyl penicillin	1658

(concl.)

Table 3. Details of the donors and selective donor markers when using S. aureus RN 450RF as recipient

Sl.No.	Donors	Selective donor markers
	a) S. aureus strain numbers	
1	M2	Str, Tet
2	M12	Str
3	M17	Chl
4	M18	Pen, Str, Amp
5	M34	Str, Amp
6	M35	Str, Tet, Amp, Pen
7	M48	Str, Tet
8	M51	Str, Tet, Pen
9	M80	Em, Pen, Str, Tet
10	M87	Tet
	b) Coagulase-negative staphylococcal strain numbers	
11	M9	Str
12	M22	Em, Tet
13	M37	Tet
14	M38	Str
15	M41	Pen, Str, Tet
16	M49	Pen, Str, Tet
17	M67	Em, Tet
18	M68	Em
19	M70	Em, Tet
20	M89	Em, Tet

Abbreviations used: Str- Streptomycin, Pen - Penicillin
Tet - Tetracycline, Em - Erythromycin
Amp - Ampicillin

(Concl)

Table 4. Details of the donors and selective donor markers when using *S. epidermidis* 131S as recipient

Sl.No.	Donors	Selective donor marker(s)
	a) *S. aureus* strain number	
1	M87	Tet
	b) Coagulase-negative staphylococcal strain number	
2	M22	Em, Tet
3	M37	Tet
4	M67	Em, Tet
5	M68	Em
6	M70	Em, Tet
7	M89	Em, Tet

Abbreviations used: Tet - Tetracycline, Em - Erythromycin

(Concl.)

Table 5. Results of the anaerobic fermentation of glucose, mannitol utilisation, catalase test, haemolysin production, coagulase test and lysostaphin sensitivity

Sl.No.	Strain number	Anaerobic fermentation of glucose	Mannitol utilisation		Catalase test	Haemolysin production	Coagulase test	Lysostaphin sensitivity
			Aerobic	Anaerobic				
1	2	3	4	5	6	7	8	9
1	M2	+	+	+	+	+	+ 1h	+
2	M5	+	+	+	+	-	-	+
3	M6	+	-	-	+	-	-	+
4	M7	+	+	+	+	+	+ 4h	+
5	M9	+	+	-	+	+	-	+
6	M10	+	-	-	+	-	-	+
7	M11	+	-	-	+	-	-	+
8	M12	+	+	+	+	+	+ 1h	+
9	M13	+	-	-	+	-	-	+
10	M14	+	-	-	+	-	-	+
11	M17	+	-	-	+	-	+	+
12	M18	+	+	+	+	+	+ 1h	+
13	M19	+	+	+	+	-	+	+
14	M22	+	+	+	+	-	-	-
15	M25	+	-	+	+	-	-	+
16	M26	+	+	+	+	-	-	+

(Contd.)

1	2	3	4	5	6	7	8	9
17	M29	+	+	+	+	-	-	+
18	M31	+	+	+	+	-	-	+
19	M34	+	+	+	+	+	+	+
20	M35	+	+	+	+	+	+ 1h	+
21	M36	+	+	+	+	-	-	-
22	M37	+	+	+	+	+	-	+
23	M38	+	+	+	+	+	-	+
24	M39	+	+	+	+	+	+	+
25	M41	+	+	+	+	+	-	+
26	M43	+	+	+	+	-	-	+
27	M44	+	+	+	+	-	-	+
28	M45	+	+	+	+	+	-	+
29	M46	+	+	+	+	-	-	+
30	M48	+	+	+	+	+	+	+
31	M49	+	+	+	+	-	-	+
32	M50	+	+	+	+	-	-	+
33	M51	+	+	+	+	-	+	+
34	M52	+	+	+	+	+	-	+
35	M56	+	+	-	+	+	+	+
36	M57	+	+	+	+	-	-	+
37	M64	+	+	+	+	-	-	-
38	M65	+	-	-	+	-	-	+

(Contd.)

1	2	3	4	5	6	7	8	9
39	M66	+	+	+	+	+	+	+
40	M67	+	+	+	+	-	-	-
41	M68	+	+	+	+	-	-	-
42	M69	+	+	+	+	+	+ 1h	+
43	M70	+	+	+	+	-	-	+
44	M78	+	-	-	+	-	-	+
45	M79	+	-	-	+	-	+ 1h	+
46	M80	+	+	+	+	+	+ 4h	+
47	M81	+	+	-	+	+	-	+
48	M82	+	-	-	+	+	-	+
49	M84	+	+	+	+	+	-	+
50	M85	+	-	+	+	-	-	+
51	M87	+	+	+	+	-	+	+
52	M89	+	+	+	+	-	-	+

(Concl.)

Table 6. The phage pattern, phage groups and antibiotic resistance pattern of coagulase-positive staphylococci

Sl.No.	Strain No.	Phage pattern	Phage group(s)	Antibiotic resistance (Agar diffusion method)
1	M2	3A/47/6/42E/47/75	II/III	Tet; Str; Amp; Nit
2	M7	N.T	-	Sul; Amp
3	M12	3A/3C/6/42E/47/75/81/94	II/III/IV	Str
4	M17	N.T	-	Str; Em; Pen; Amp; Chl
5	M18	N.T	-	Str; Pen; Sul; Amp; Amo
6	M19	N.T	-	Nil
7	M34	47	III	Str; Sul; Amp
8	M35	3A/3C/6/47/75/81/94	II/III/IV	Str; Tet; Amp
9	M39	N.T	-	Sul
10	M48	3A/3C/55/71	II	Str; Tet; Em
11	M51	6/42E/47/54/75/77/83A/84/81	III/IV	Str; Tet; Pen; Sul; Amp
12	M56	6/42E/54	III	Nil
13	M66	N.T.	-	Chl; Sul
14	M69	N.T	-	Pen; Amp; Em
15	M79	N.T	-	Nil
16	M80	N.T	-	Str; Tet; Em; Amp; Gen; Amo; Sul
17	M87	N.T	-	Tet; Sul

Abbreviations used: N.T. - Non-typable, Str - Streptomycin, Tet - Tetracycline, Amp - ampicillin, Pen - penicillin, Chl - chloramphenicol, Em - Erythromycin, Amo - amoxycillin, Nit - nitrofurantoin, Gen - gentamicin, Sul - sulphamethoxazole

Table 7. Antibiotic resistance to staphylococci by agar diffusion and Agar dilution methods

Sl.No.	Strain No.	Resistance*	
		Agar diffusion method (14 antibiotics)	Agar dilution method (8 antibiotics)
1	2	3	4
1	M2	Tet; Amp; Nit; Str; Amo	Pen; Tet; Str
2	M5	Tet; Pen	Nil
3	M6	Tet; Str; Sul	Str
4	M7	Amp; Sul; Met; Str; Amo	Pen
5	M9	Str; Sul	Pen; Str; Tet
6	M10	Nil	Nil
7	M11	Str	Str
8	M12	Str; Neo; Amo	Pen; Str
9	M13	Sul	Nil
10	M14	Str; Sul; Amp; Amo	Str
11	M17	Em; Pen; Str; Amp; Chl	Chl
12	M18	Pen; Str; Sul; Amp; Amo	Pen; Str; Amp
13	M19	Nil	Nil
14	M22	Em; Tet	Em; Tet
15	M25	Tet; Sul	Nil
16	M26	Amp; Amo; Sul	Nil
17	M29	Nil	Nil
18	M31	Nil	Nil
19	M34	Sul; Str; Amp; Amo	Str; Pen
20	M35	Tet; Str; Amp; Sul; Amo	Str; Tet; Pen; Amp
21	M36	Nil	Nil
22	M37	Tet	Tet
23	M38	Str; Sul	Str; Pen
24	M39	Sul	Nil
25	M41	Pen; Tet; Sul; Em; Amp	Tet; Sul; Pen
26	M43	Nil	Nil
27	M44	Pen; Sul; Str; Amp	Pen

1	2	3	4
28	M45	Sul	Nil
29	M46	Tet	Tet
30	M48	Tet; Em; Str	Tet; Str
31	M49	Tet; Pen; Str; Amp	Tet; Pen; Str
32	M50	Str; Sul	Nil
33	M51	Pen; Tet; Str; Sul; Amp; Amo	Pen; Str; Tet
34	M52	Pen; Sul; Tet; Amp	Pen; Str; Tet
35	M56	Nil	Nil
36	M57	Pen;Tet; Amp; Str	Pen; Str; Tet
37	M64	Nil	Nil
38	M65	Nil	Nil
39	M66	Chl; Sul	Nil
40	M67	Em; Sul	Em; Tet
41	M68	Em	Em
42	M69	Em; Pen; Amp; Chl; Str; Clo; Sul; Amo	Pen
43	M70	Amp; Em; Tet	Em; Tet
44	M78	Str	Str
45	M79	Nil	Nil
46	M80	Em; Tet; Str; Sul; Amp; Gm; Amo	Pen; Str; Tet
47	M81	Tet	Nil
48	M82	Pen; Sul	Pen
49	M84	Em; Chl	Nil
50	M85	Sul	Nil
51	M87	Tet; Sul	Tet
52	M89	Tet; Em; Bac; Sul	Tet; Em

Abbreviations used: Pen - Penicillin; Str - Streptomycin; Chl - Chloramphenicol; Tet - Tetracycline; Em - Erythromycin; Sul - Sulphamethoxazole Amp - Ampicillin; Clo - Cloxacillin; Gm - Gentamicin; Bac - Bacitracin; Nit - Nitrofurantoin; Amo - Amoxicillin; Neo - Neomycin; Met - Methicillin.

* In the agar diffusion method, intermediate category was also grouped under resistant category.

(concl.)

Table 8. Results of drug sensitivity of staphylococci to various antibiotics by agar diffusion method

Sl.No.	Antibiotic	Number of the strains tested	No. sensitive	No. intermediate	No. resistant	% of resistance*
1	2	3	4	5	6	7
1	Amoxycillin	a) S. aureus-17	8(47.06)	7(41.18)	2(11.76)	52.94
		b) Coagulase-negative staph-35	31(38.58)	2(5.71)	2(5.71)	11.43
2	Ampicillin	a) S. aureus-17	8(47.06)	Nil	9(52.94)	52.94
		b) Coagulase-negative staph-35	27(77.14)	4(11.43)	4(11.43)	22.86
3	Bacitracin	a) S. aureus-17	17(100)	Nil	Nil	Nil
		b) Coagulase negative staph-35	34(97.14)	Nil	1(2.86)	2.86
4	Chloramphenicol	a) S. aureus-17	15(88.24)	Nil	2(11.76)	11.76
		b) Coagulase negative-staph-35	33(94.29)	1(2.857)	1(2.857)	2.86
5	Cloxacillin	a) S. aureus-17	16(94.12)	1(5.88)	Nil	5.88
		b) Coagulase negative staph-35	35	Nil	Nil	Nil
6	Erythromycin	a) S. aureus-17	13(76.47)	Nil	4(23.53)	23.53
		b) Coagulase negative staph-35	29(82.86)	2(5.71)	4(11.43)	17.14

(Contd.)

1	2	3	4	5	6	7
7	Gentamicin	a) S. aureus-17	16(94.12)	Nil	1(5.88)	5.88
		b) Coagulase negative staph-35	35(100)	Nil	Nil	Nil
8	Methicillin	a) S. aureus-17	16(94.12)	1(5.88)	Nil	5.88
		b) Coagulase negative staph-35	35(100)	Nil	Nil	Nil
9	Neomycin	a) S. aureus-17	16(94.12)	1(5.88)	Nil	5.88
		b) Coagulase negative staph-35	35(100)	Nil	Nil	Nil
10	Nitrofurantoin	a) S. aureus-17	16(94.12)	Nil	1(5.88)	5.88
		b) Coagulase negative staph-35	35(100)	Nil	Nil	Nil
11	Penicillin	a) S. aureus-17	13(76.47)	Nil	4(23.53)	23.53
		b) Coagulase negative staph-35	27(77.14)	2(5.71)	6(17.14)	22.86
12	Streptomycin	a) S. aureus-17	6(35.29)	2(11.76)	9(52.94)	64.70
		b) Coagulase negative staph-35	20(57.14)	6(17.14)	9(25.71)	42.86
13	Sulphamethoxazole	a) S. aureus-17	7(41.18)	2(11.76)	8(47.05)	58.82
		b) Coagulase negative staph-35	20(57.14)	Nil	15(42.86)	42.86
14	Tetracycline	a) S. aureus-17	10(58.82)	Nil	7(41.18)	41.18
		b) Coagulase negative staph-35	22(62.86)	Nil	13(37.14)	37.14

*To find out the percentage of resistance, intermediate category was also grouped under resistant strains

(Concl.)

Table 9. Results of drug sensitivity of staphylococci to various antibiotics by agar dilution method

Sl.No.	Antibiotic	Number of strains tested	No. and % of sensitivity	No. and % of resistance
1	Ampicillin	a) S. aureus-17	15(88.23)	2(11.77)
		b) Coagulase negative staph-35	35(100)	Nil
2	Chloramphenicol	a) S. aureus-17	16(94.12)	1(5.88)
		b) Coagulase negative staph-35	35(100)	Nil
3	Erythromycin	a) S. aureus-17	17(100)	Nil
		b) Coagulase negative staph-35	30(85.71)	5(14.29)
4	Gentamicin	a) S. aureus-17	17(100)	Nil
		b) Coagulase negative staph-35	35(100)	Nil
5	Benzyl penicillin	a) S. aureus-17	9(52.94)	8(47.06)
		b) Coagulase negative staph-35	27(77.14)	8(22.86)
6	Streptomycin	a) S. aureus-17	9(52.94)	8(47.06)
		b) Coagulase negative staph-35	25(71.43)	10(28.57)
7	Tetracycline	a) S. aureus-17	11(64.71)	6(35.29)
		b) Coagulase negative staph-35	24(68.57)	11(31.43)
8	Rifampicin	a) S. aureus-17	17(100)	Nil
		b) Coagulase negative staph-35	35(100)	Nil

Table 10. Details of the donors, recipient, selective donor markers and transcipients obtained

Sl.No.	Donors	Recipient	Selective donor marker(s)	Resistance of the transcipients
1	2	3	4	5
	a) *S.aureus* strains	*S.aureus* RN 450RF		
1	M2	Phage-free	Str, Tet	Nil
2	M12	Plasmid-free Rif^r/Fus^r	Str	Str
3	M17	"	Chl	Nil
4	M18	"	Pen; Str; Amp	Str
5	M34	"	Str; Amp	Nil
6	M35	"	Str; Tet; Amp; Pen	Str; Pen
7	M48	"	Str; Tet	Str; Tet
8	M51	"	Str; Tet; Pen	Tet
9	M80	"	Str; Tet; Pen; Em	Pen; Str
10	M87	"	Tet	Nil
	b) Coagulase-negative staphylococci			
11	M9	"	Str	Str
12	M22	"	Em; Tet	Nil
13	M37	"	Tet	Nil
14	M38	"	Str	Str

(Contd.)

1	2	3	4	5
15	M41	"	Str Pen; Tet	Str
16	M49	"	Str; Pen; Tet	Str
17	M67	"	Em; Tet	Nil
18	M68	"	Em	Nil
19	M70	"	Em; Tet	Nil
20	M89	"	Em; Tet	Nil

Abbreviations used: Str - Streptomycin; Pen - Penicillin; Tet - Tetracycline; Em - Erythromycin; Amp - Ampicillin; Chl - Chloramphenicol; Rif - Rifampicin; Fus - Fusidicacid

(Concl.)

Table 11. Details of the donors, recipient, selective donor markers and transcipients

Sl.No.	Donors	Recipient	Selective donor marker(s)	Resistance of the transcipient
	a) *S. aureus* strains	*S. epidermidis* 1315		
1	M87	Plasmid-free, Str^r	Tet	Nil
	b) Coagulase-negative staphylococci			
2	M22	"	Tet; Em	Nil
3	M37	"	Tet	Nil
4	M67	"	Tet; Em	Nil
5	M68	"	Em	Nil
6	M70	"	Tet; Em	Nil
7	M89	"	Tet; Em	Nil

Abbreviations used: Tet - Tetracycline; Em - Erythromycin

(Concl.)

DISCUSSION

DISCUSSION

Association of a wide variety of bacterial organisms with bovine mastitis have been reported by several authors. The most frequently encountered organisms are staphylococci, streptococci, E. Coli, corynebacterium and pseudomonas (Verma and Mishra, 1977; Singh and Baxi, 1982). Eventhough incidence due to coliforms has been reported considerably high, the most frequently encountered organism in case of mastitis is staphylococci (Misra et al., 1973; Jhala, 1976). The results of the present study indicated staphylococci as one of the important organisms causing mastitis in cattle.

I. Isolation and identification

Of the 360 milk samples screened, 195 were culturally positive in which 52 were identified as staphylococci based on the following tests. An important criteria for distinguishing staphylococci from micrococci was reported to be glucose fermentation, where staphylococci could anaerobically ferment glucose while micrococci could not do so (Baird-Parker, 1966). In the present study all the coccal isolates could ferment glucose and none showed the character of micrococci. This agreed with the observation made by Hodges et al. (1984).

As reported by Kapur et al. (1979) no correlation was obtained with coagulase production and mannitol fermentation. It was observed that some of the coagulase-negative strains also fermented mannitol like the S. aureus. All the isolates were catalase positive and this was in complete agreement with the observations of McDonald et al. (1981).

Only 76.48% of the coagulase-positive strains could produce hemolysis of sheep red blood cells. The result suggested that the absence of hemolysis of sheep RBC cannot be considered as an indication of lack of pathogenicity. Similarly 25.70% of the coagulase-negative staphylococci was found to produce hemolysis which could be considered as an indication of the role of coagulase-negative staphylococci in producing mastitis in animals. Similar observations were made by Hodges et al. (1984).

In the present work 32.69% of coagulase-positive and 67.31% coagulase-negative strains were obtained which completely agreed with the results of Kapur et al. (1979). The time taken for a positive coagulase reaction varied from one hour to overnight which might be due to the differences between the strains in their capacity to produce the enzyme staphylocoagulase.

Majority of the isolates (90.38%) was sensitive to the lytic action of lysostaphin. All the coagulase-positive

strains tested were sensitive to its action. Dwarakanath and Murthy (1984) reported that lysostaphin, produced by certain strains of staphylococci was not effective neither on viable nor on heat killed cells of its own types. The resistance to the lytic activity of lysostaphin to the five coagulase-negative strains in the present study could be due to the fact that these strains might be similar or closely related ones as those from which the lysostaphin was manufactured. It was also observed that all the lysostaphin resistant coagulase-negative staphylococci were unable to produce hemolysis of sheep red blood cells. The exact reasons for some of the coagulase-negative staphylococci to resist the lytic activity of lysostaphin require further study. The lysostaphin sensitivity could not be used to differentiate between coagulase-positive and coagulase-negative strains since both were lysed by lysostaphin. This finding agreed with the observations of Gutierrez et al. (1981) who had used lysostaphin in the characterization of ovine strains of staphylococci. From the available literature it is seen that this formed the first study in which lysostaphin sensitivity was used in the characterization of staphylococci isolated from cases of bovine mastitis.

Bacteriophage typing

The percentage of coagulase-positive strains that could be typed with the international basic set of 23 phages was

found to be low when compared to the reports of other investigators. Bonin and Blobel (1967) reported that 73% of the staphylococcal strains isolated from bovine milk samples could be typed with the international basic set. Sengupta et al. (1982) reported that 64.5% of the coagulase-positive staphylococci was typable with these set of phages. In the present study the percentage of strains that could be typed was only 41.17%. The results also signified the role of human strains of staphylococci in producing mastitis in animals. A similar type of observation was reported by Rahman and Baxi (1983a) who commented on the role of human carriers in the spread of this disease in bovine. The predominance of group III phages noticed in the present investigation was similar to that reported by Rahman and Baxi (1983b); whereas Loken and Harvey (1952) and Frost (1970) reported a predominance of group IV phages. Among the typable strains all except strain No.M56 were resistant to one or more antibiotics, while among the non-typable strains all except strains M19 and M79 were resistant to one or more antibiotics. One out of the seven typable strains and two out of the ten non-typable strains of S.aureus were sensitive to all antibiotics tested. No correlation was observed between the phage patterns and antibiogram of the typable strains. Hence no conclusion could be made based on this data regarding the phage pattern and antibiotic resistance.

II. Antibiotic sensitivity

Of the 52 staphylococcal isolates 10 (19.23%) were sensitive to the 14 antibiotics tested; 10 (19.23%) were resistant to single antibiotic and 32 (61.54%) were resistant to two or more antibiotics. Van and Devriese (1971) reported that only 3% of their - 132 strains from bovine mastitis possessed multiple drug resistance. A higher percentage of multiple drug resistance in the present study points out the indiscriminate use of various chemotherapeutic agents. Multiple drug resistance in staphylococci causing mastitis requires special attention and this necessitates assessing the sensitivity prior to administration of chemotherapeutic agents.

A. Agar diffusion method

From the results it was observed that all the S.aureus isolates were sensitive to bacitracin and hence this drug could be effectively used in the treatment of bovine mastitis caused by this organism. The reason for the complete sensitivity to this antibiotic might be due to the fact that this drug is seldom used in treating animal diseases in this area. Resistance to the commonly used antibiotics such as streptomycin, tetracycline, ampicillin and sulphamethoxazole was found to be high. The percentage of strains resistant to these antibiotics were higher than that reported by other investigators like Van and Devriese (1971) and

Nag and Ghosh (1982). The constant use of these antibiotics in the livestock farms and veterinary hospitals from where these samples were collected could be considered as the probable reason for the higher percentage of resistance to these antibiotics.

Only 5.88% of the S. aureus strains was resistant to cloxacillin, nitrofurantoin, neomycin and gentamicin and 11.76% to chloramphenicol which is almost in agreement with the findings of Mc Donald and Anderson (1981). Only 23.53% of S. aureus strains was resistant to penicillin and erythromycin. The percentage of resistance to penicillin was much lower when compared to the observations of other investigators like Nag and Ghosh (1982) and Kapur et al. (1984) who have reported 76% and 63.64% resistance respectively. The low percentage of resistance to penicillin and erythromycin in the present study agreed with the findings of McDonald and Anderson (1981) who have reported that more than 90% of the staphylococcal isolates were sensitive to penicillin and erythromycin.

All the coagulase-negative staphylococci examined were sensitive to cloxacillin, gentamicin, methicillin, neomycin and nitrofurantoin. This finding is almost in consistent with the observations of Van and Devriese (1971) and McDonald and Anderson (1981). A higher percentage of coagulase-negative

staphylococci was resistant to streptomycin (42.86%), Sulphamethoxazole (42.86%) and tetracycline (37.15%) and this amply explained the role of these bacteria as a reservoir in the infectious drug resistance of staphylococci. Cohen et al. (1982) have commented the reservoir role of S. epidermidis in spreading antibiotic resistance to S. aureus in hospitals. The percentage of resistances to other antibiotics like ampicillin, penicillin, amoxycillin, erythromycin, bacitracin and chloramphenicol ranged from 22.86% to 2.86% only. None of the strains was resistant to all the 14 antibiotics tested which almost agreed with the findings of Sudharma et al. (1985). On the other hand 19.23% of the staphylococci was sensitive to all the 14 antibiotics tested.

B. Agar dilution method

Using single concentration of the antibiotics all the S. aureus strains were sensitive to rifampicin, gentamicin and erythromycin. The reason for this could be the minimal use of these drugs in veterinary practice in this area. All the coagulase-negative staphylococci were sensitive to ampicillin, gentamicin, chloramphenicol and rifampicin, while a good percentage were resistant to tetracycline, streptomycin and penicillin which are commonly used in the treatment of mastitis which might have resulted the selection of drug resistant coagulase-negative staphylococci.

From the results it could be concluded that rifampicin, bacitracin, neomycin, methicillin, gentamicin, cloxacillin, nitrofurantoin and chloramphenicol could be used in the treatment of bovine mastitis caused by staphylococci.

III. Mercuric resistance

Koiranen (1969) reported that 5.6% of the staphylococcal isolates from bovine mastitis were resistant to mercuric chloride. In the present study all the isolates were found to be sensitive to mercuric chloride. This might be due to the non-exposure of mercurical antiseptics to the microorganism.

IV. Conjugation experiments

1) Using S. aureus NW 450RF as recipient:

3) S. aureus as donors

Of the ten strains of S. aureus used as donors, six were found to transfer either one or more drug resistance markers to the recipient. Transduction, transformation and conjugation were considered as the means of transfer of resistance. No transfer of resistance markers was observed when the cell-free filtrate of each donors was mixed with recipient, plated on to appropriate selective media and incubated. Hence the possibility of transduction and transformation was excluded even though, that could not be completely ruled out, since both the recipient and donor

strains were S. aureus. The possibility of phage-mediated conjugation also should be considered and the reason for the exact mechanisms of transfer requires further studies. Another interesting finding was that streptomycin resistance was most readily transferred, since five out of the eight streptomycin resistant donors were able to transfer the resistance to the recipient. No chloramphenicol, ampicillin and erythromycin resistance have been found transferred in the study, even though the resistance to these drugs are reported to be encoded on R plasmids (Lacey, 1975). Novick (1980) reported that staphylococcal R plasmids could not apparently, promote their own transfer by conjugation. The failure of some of the drug resistance markers to transfer the resistance in the present study also might be due to the inability of these R plasmids to promote their own transfer by conjugation.

b) Coagulase-negative staphylococci as donors

All the four streptomycin resistant donors were able to transfer their resistance to the recipient. But two multiple resistant strains - (M41 and M49) could transfer only the streptomycin resistance and not the penicillin and tetracycline resistances. The reason for the non-transfer requires further studies. Fawcett et al. (1981) failed to transfer antibiotic resistance from coagulase-negative staphylococci to S. aureus 1030 in mixed cultures. Eventhough

there are reports that, in general, phages of S.epidermidis do not adsorb to S. aureus and virtually all cultures of staphylococci contain high levels of nucleases which could be expected to prevent transformation under natural conditions (Lacey, 1975); tests were conducted to exclude the possibility of transduction and transformation. From the results it could be concluded that the mode of transfer of streptomycin resistance was through conjugation. Mathew and Punnoose (1986) made similar conclusions in the conjugal transfer of streptomycin resistance plasmids from coagulase-negative strains of staphylococci to S. aureus RN 450RF in mixed cultures.

The exact mechanism of conjugation in bacteria is still a controversy. The precise manner by which the donor and recipient cells conjugate is not known, though Brinton (1971) has proposed DNA transfer between conjugating cells through extended F pili which connected donor cells with recipient cells. However, Achtman et al. (1978) was of the opinion that extended F pili played no role at all in DNA transfer but were only needed for the cell-cell interactions.

According to Willets and Wilkins (1984) the transfer mechanism of conjugative plasmids in gram-positive bacteria were likely to be biochemically as well as genetically distinct from those in gram-negative species since these plasmids

did not encode pili for recognition of potential recipient cells. In the present study the streptomycin resistance plasmids were found transferred through conjugation. The mechanism involved in conjugation might be by a local fusion at a point of contact of the donor and recipient and the formation of a transmembrane "pore" through which DNA is transferred, and the evidence favouring this concept is provided by the report that phage lambda receptor sites are exchanged bidirectionally between Hfr and F⁻ cells as a consequence of conjugation (Willets and Wilkins, 1984). Panicker and Minkley (1985) also was of the opinion that the F pilus do not play a direct role in DNA transfer and a comparison could be made between the function of the sex pilus found in gram negative bacteria and cell clumping induced by the action of 'sex pheromone' in gram positive bacteria like S. faecalis. Whether such sex pheromones are produced by staphylococci for conjugation requires further work. The mechanism of conjugation observed in the present study might be by a local fusion at a point of contact of the donor and recipient as reported by Willets and Wilkins (1984). Whether the bacteria has produced any 'sex pheromone' for the initiation of conjugation of the staphylococci in the study, requires further investigations.

ii) Using S. epidermidis 1318 as recipient

No resistance markers were found transferred from either

the S. aureus or the coagulase-negative staphylococci to the recipient in the study. The reasons for the failure of all the strains to transfer drug resistance to the recipient requires further investigations.

SUMMARY

SUMMARY

A study was undertaken to characterize the staphylococci isolated from cases of bovine mastitis and to study the R plasmid transfer through conjugation, *in vitro*. Milk samples from 360 cases of bovine mastitis were screened for the presence of bacterial organisms and 195 organisms were isolated. From these 52 strains of staphylococci were identified based on Gram staining, anaerobic fermentation of glucose, aerobic and anaerobic utilization of mannitol, catalase test, haemolysin production using sheep blood, coagulase test using rabbit plasma and lysostaphin sensitivity. The strains were grouped into *Staphylococcus aureus* and coagulase-negative staphylococci. Phage-typing was done using the international basic set of 23 phages.

Antibiogram of all the strains was studied by agar diffusion and agar dilution methods. Mercuric resistance of the strains was done using a peptone agar base containing mercuric chloride at a concentration of 1 in 27,500. Drug resistance transfer from selected *S. aureus* and coagulase-negative strains was done in separate experiments using *S. aureus* RN 450RF, phage-free, plasmid-free, Rif^r/Fus^r and *S. epidermidis* 1318, plasmid-free, str^r as recipients.

From the study the following observations were made.

1. From the 360 samples tested, 195 (54.17%) were culturally positive for various bacterial organisms, from which 52 strains of staphylococci were isolated.

2. All the staphylococcal strains were able to ferment glucose; 39 strains were able to ferment mannitol while 13 were unable to do so. Mannitol was utilized aerobically by 40 strains while 12 strains failed to utilise mannitol aerobically. All the strains were positive for catalase test.

3. Twentyone (40.38%) out of the 52 staphylococci produced haemolysis on 5% sheep blood agar. Of the S. aureus strains 23.52% were unable to produce hemolysis. Haemolysis was produced by 25.71% of the coagulase-negative strains.

4. Out of the 52 strains of staphylococci 17 (32.69%) were coagulase-positive while 35 (67.31%) were coagulase-negative.

5. Lysostaphin could produce lysis of 47 (90.38%) strains.

6. Only 7 (41.17%) of the 17 coagulase-positive staphylococci could be typed with the international basic set of 23 phages.

7. Using agar diffusion method, all the S. aureus strains were found sensitive to bacitracin while the percentage of strains resistant to streptomycin, sulphamethoxazole,

amoxycillin, ampicillin, tetracycline, penicillin, erythromycin, cloxacillin, nitrofurantoin, neomycin, methicillin and gentamicin varied from 64.70% to 5.88%.

All the 35 coagulase-negative strains were sensitive to cloxacillin, gentamicin, neomycin, methicillin and nitrofurantoin. The percentage of strains resistant to streptomycin, sulphamethoxazole, tetracycline, penicillin, ampicillin, erythromycin, amoxycillin, bacitracin and chloramphenicol varied from 42.86% to 2.86%.

8. By agar dilution method all the S. aureus strains were found sensitive to gentamicin, erythromycin and rifampicin; while all the coagulase-negative strains were sensitive to ampicillin, gentamicin, chloramphenicol and rifampicin. The percentage of resistance of S.aureus strains resistant to streptomycin, penicillin, tetracycline, ampicillin and chloramphenicol varied from 47.06% to 5.88% and that of the coagulase-negative strains resistant to tetracycline, streptomycin, penicillin and erythromycin varied from 31.43% to 14.29%.

9. All the staphylococcal isolates were sensitive to mercuric chloride.

10. Tetracycline, streptomycin and penicillin resistances were transferred either singly or jointly while using S. aureus RN 450RF as recipient and the selected S.aureus

strains as donors, whereas, no chloramphenicol, ampicillin or erythromycin resistances were found transferred from the donors to the recipient.

All the four streptomycin resistant coagulase-negative donor strains studied were able to transfer the resistance to the recipient, but the strains failed to transfer erythromycin, tetracycline and penicillin resistances.

11. No resistance markers could be transferred from either S. aureus strains or from the coagulase-negative strains when S. epidermidis 1313 was used as the recipient.

12. None of the recipients could pick up the R plasmid DNA either through transduction or through transformation when the filtrates of the donors were mixed with the recipients.

From the studies it was concluded that:

1. The anaerobic fermentation of glucose was found to be a method for distinguishing staphylococci from micrococci.

2. Mannitol fermentation could not be correlated with coagulase-production.

3. Coagulase-negative staphylococci also could produce haemolysis of sheep red blood cells, and this organism also was capable of producing mastitis since majority of the isolates were coagulase-negative.

4. Though lysostaphin could lyse most of the staphylococcal strains, it could not be used to differentiate between coagulase-positive and coagulase-negative strains.

5. Group III phages were predominant among the coagulase-positive staphylococci typed and the result indicates the possibility of human strains of staphylococci in producing mastitis in animals.

6. Rifampicin, bacitracin, neomycin, methicillin, gentamicin, cloxacillin, nitrofurantoin and chloramphenicol are the drugs of choice in the treatment of bovine mastitis caused by staphylococci.

7. Mercuric resistance was not prevalent among the staphylococci isolated during the study.

8. There was transfer of drug resistance between strains of staphylococci; but conjugation was confirmed only between S. aureus RN 450RF and coagulase-negative staphylococci.

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CHARACTERIZATION OF STAPHYLOCOCCI ISOLATED FROM CASES OF MASTITIS AND STUDY OF THEIR R PLASMIDS

By

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ABSTRACT OF A THESIS

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ABSTRACT

The emergence of drug resistant staphylococci causing mastitis deserves serious investigation. The work was intended to characterize staphylococci isolated from cases of bovine mastitis and to study their R plasmid transfer *in vitro*. The characterization was done using glucose and mannitol fermentation, catalase production, coagulase-production, lysostaphin sensitivity and bacteriophage typing. The antibiogram of the isolates was done by agar diffusion method using 14 chemotherapeutic agents (amoxycillin, ampicillin, bacitracin, chloramphenicol, cloxacillin, erythromycin, gentamicin, methicillin, neomycin, nitrofurantoin, penicillin, streptomycin, sulphamethoxazole, and tetracycline) and by agar dilution method using eight antibiotics (ampicillin, chloramphenicol, rifampicin, streptomycin, tetracycline, gentamicin, erythromycin and penicillin). The *in vitro* transfer of R plasmids was tried using selected *S. aureus* and coagulase-negative staphylococcal isolates as donors and *S. aureus* RN 450RF and *S. epidermidis* 131S as recipients.

From 360 milk samples collected from cases of bovine mastitis 17 strains of *S. aureus* and 35 strains of coagulase-negative staphylococci were isolated. Lysostaphin sensitivity test was positive for 90.38% of the isolates, but

this could not be used to differentiate between coagulase-positive and negative staphylococci. The results of phage-typing revealed a predominance of group III phages over the other groups and the possible role of human strains of staphylococci in producing mastitis in animals. None of the strains were resistant to mercuric chloride. From the antibiogram rifampicin, bacitracin, neomycin, methicillin, gentamicin, cloxacillin, nitrofurantoin and chloramphenicol were found to be the drugs of choice in the treatment of bovine mastitis caused by staphylococci.

When S. aureus RN 450RF was used as recipient six of the ten selected S. aureus isolates could transfer either one or more drug resistance markers and the mode of transfer was suspected to be by conjugation. All the four selected streptomycin resistant coagulase-negative staphylococcal donors were found to transfer the R plasmid DNA to the recipient, S. aureus RN 450RF, which was established to be through conjugation.

The drug resistant S. aureus as well as coagulase-negative staphylococcal strains failed to transfer their resistances to S. epidermidis 131S