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CHARACTERIZATION OF PLASMIDS OF *Escherichia coli* ISOLATED FROM MASTITIS

By

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THESIS

Submitted in partial fulfilment of the
requirement for the degree

Doctor of Philosophy

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

Department of Microbiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
Mannuthy, Dist. Thrissur

1993

DECLARATION

I hereby declare that this thesis entitled **Characterization of plasmids of *Escherichia coli* isolated from mastitis** is a bonafide record of research work done by me during the course of research and 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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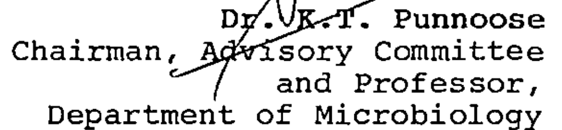
Certified that this thesis entitled **Characterization of plasmids of *Escherichia coli* isolated from mastitis** is a record of research work done independently by **Dr. Avinash Ganpatrao Karpe** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.



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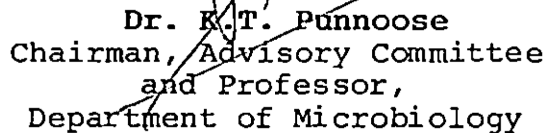

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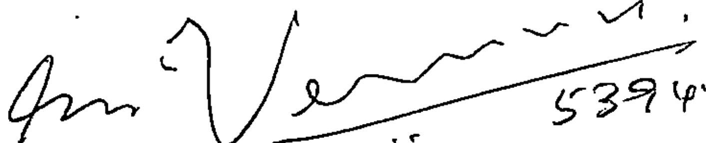

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TO MY PARENTS

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ABBREVIATIONS

A	-	Ampicillin
Ag	-	Silver
AO	-	Acridine orange
C	-	Chloramphenicol
CCC	-	Covalently closed circular
Cd	-	Cadmium
Cl	-	Cloxacillin
Cn	-	Carbenicillin
Cu	-	Copper
DI	-	Dilatation index
DNA	-	Deoxyribonucleic acid
E	-	Erythromycin
EB	-	Ethidium bromide
EDTA	-	Ethylene diamine tetraacetate
Ent	-	Enterotoxin producing plasmid
ETEC	-	Enterotoxigenic *E. coli*
F	-	Furazolidone
$\bar{F}$	-	A bacterium without F plasmid
FC	-	Frequency of curing
FH	-	Frequency of hemolysis
F'*lac*	-	Lactose fermenting plasmid

Hg	-	Mercury
Hly	-	Hemolysin producing plasmid
J	-	Gentamicin
K	-	Kanamycin
Kb	-	Kilobase
LB	-	Luria Bertani
MDa	-	Megadalton
µg	-	Microgram
MDR	-	Multiple drug resistance
Mz	-	Metronidazole
N	-	Neomycin
Na	-	Nalidixic acid
Na^r	-	Nalidixic acid resistant
Nf	-	Norfloxacin
O	-	Oxytetracycline
P	-	Penicillin
PB	-	Polymyxin B
Pb	-	Lead
Q	-	Co-trimoxazole
QAC	-	Quaternary ammonium compound
R	-	Rifampicin
R-factor	-	Resistance factor
RLIL	-	Rabbit ligated ileal loop
R-plasmid	-	Resistance plasmid

S	-	Streptomycin
Sb	-	Antimony
SDS	-	Sodium dodecyl sulphate
SDS-NaOH	-	Sodium dodecyl sulphate - sodium hydroxide
SLR	-	Single lens reflex
Sp	-	Spectinomycin
Su	-	Sulphadiazine
T	-	Tetracycline
TBE	-	Tris borate electrophoresis
TE	-	Tris EDTA
TEG	-	Tris EDTA glucose
UV	-	Ultra Violet

Introduction

INTRODUCTION

Mastitis is one of the important problems confronting the farmer in the economic management of dairy farming. Among the various organisms causing mastitis, *Escherichia coli* is an important organism. The mastitis caused by *E. coli* is also called the environmental mastitis as these bacteria are usually transferred from the environment where the cow is living, rather than from infected udder (Howell, 1972). This type of mastitis is more resistant to hygienic control measures and hence a serious problem. Apart from the economic loss due to reduction in milk yield, contamination of milk by bacteria may lead to milk borne infections in human being. In addition the residues of antibiotics seen in milk of treated animals may precipitate health hazards in man.

Antibiotic resistant bacterial population arises in animals due to the indiscriminate use of antibiotics in therapy, prophylaxis and growth promotion (Siegel *et al*., 1974). *Escherichia coli* is the predominant organism found in the intestinal tract of animals. Cattle suffer from various disease conditions due to pathogenic strains of *E. coli* even from calfhood and indiscriminate use of antibiotics results in the emergence of multiple drug resistant (MDR) pathogenic as

well as non-pathogenic strains of *E. coli* due to selection pressure. Emergence of MDR *E. coli* is a limiting factor in the control of mastitis due to *E. coli*.

The genes responsible for drug resistance are located either on chromosome or plasmids (Watanabe and Fukasawa, 1961b). Plasmids also harbour genes responsible for phenotypic characters exhibited by bacteria, such as hemolysin production, enterotoxin production, colicin production, antibiotic production, colonization factors, and fermentation of sugars (Datta, 1962; Falkow *et al*., 1964; and Smith and Linggood, 1971).

Smith and Halls (1967a, 1968) observed that hemolysin and enterotoxin production are governed by plasmids designated as *Hly* and *Ent* respectively. It has been observed that normal *E. coli* could assume pathogenic characters on conjugation with *E. coli* harbouring virulent plasmids such as *Ent*, *Hly*, R-factor, adhesion and colicin factors.

Disease surveillance and epidemiological data are very essential for efficient control of disease process and this is applicable in the case of mastitis also. Study of the antibiogram, resistogram, hemolysin production, enterotoxin production and analysis of plasmid profile of wild type of *E. coli* isolated from bovine mastitis cases will help to study

the epidemiology of mastitis. The routine methods used in epidemiological studies like, serotyping, biotyping, phage typing and colicin typing are cumbersome and time consuming. The study of origin and spread of bacteria requires appropriate typing methods. With the increased spread of multiple antibiotic resistant E. coli strains, a typing system has to be sought out which could differentiate the epidemic strain from the endemic ones (Wasteson et al., 1992). The study of plasmid profile was used as a method of identifying different strains of species (Premkumar David et al., 1991; Wasteson et al.,1992; Manohar Paul, 1992).

A search of literature did not reveal any comprehensive study encompassing antibiogram, resistogram, hemolysin and enterotoxin production characters of E. coli isolated from mastitis; their transfer by conjugation, curing of these characters by physical and chemical methods and plasmid profiles, of wild strains, transconjugants and cured strains. Hence this project was undertaken to study the above characters of E. coli strains isolated from bovine mastitis and establish a correlation between antibiotic resistance and other plasmid borne characters responsible for virulence.

This project envisages the study of:

1. isolation of *E. coli* from cases of mastitis,

2. the antibiogram of the isolates and suggest suitable antibiotic/chemotherapeutic agents for the treatment of mastitis due to *E. coli*,

3. the MDR, resistogram, hemolysin and enterotoxin production of *E. coli*,

4. transfer of drug resistance, hemolysin and enterotoxin producing character to recipient *E. coli*,

5. curing of drug resistance, hemolysin and enterotoxin producing characters by chemical and physical agents,

6. the plasmid profiles and molecular sizes of the plasmids of wild strains of *E. coli*, the transconjugants and cured isolates,

7. if there is co-transfer of antibiotic resistance and virulence characters,

8. the application of antibiogram and resistogram typing in discriminating the isolates,

9. to identify whether there is any direct correlation between MDR and plasmid mediated virulence factor of *Escherichia. coli*.

REVIEW OF LITERATURE

The use of antibiotics, chemotherapeutic agents and heavy metals had an enormous impact on the pattern of infectious diseases throughout the world. Chemotherapy while helping to solve the problems of infectious disease, has created some problems of its own. One of these problems has been the appearance of organisms resistant to certain antibiotics and heavy metals. Apart from its obvious medical importance, the study of the genetic and biochemical aspects of microbial drug resistance has provided an important avenue to several aspects of fundamental biology.

It was Lederberg who in 1952 proposed the term 'plasmid' to extrachromosomal autonomous DNA molecules (replicons) found in many bacterial cells (Lederberg, 1952). The antibiotic resistance determined by plasmids was first discovered in Japan in 1959, when transmissible drug resistance was discovered (Ochiai _et al_., 1959; Akiba _et al_., 1960; Watanabe, 1963).

Although plasmids represented only small fraction of the prokaryotic gene pool, they have attracted a disproportionate degree of research interest, because the genes they carry determine many of the more interesting

features displayed by bacteria; such as transmissible multiple drug resistance (Datta, 1962; Watanabe, 1963), resistance to heavy metals (Richmond and John, 1964; Novick and Roth, 1968), lactose fermentation (Falkow et al., 1964), virulence i.e. enterotoxigenicity, hemolysin production and colonization factor (Smith and Linggood, 1971).

2.1 Escherichia coli isolated from mastitis

Theobald Escherich in 1885 isolated Escherichia coli from faeces of new-born babies. Escherichia coli was first recorded in bovine milk in 1896 (Kitt, 1896). Kitt (1903) reported the disease by "lightly sticking" E. coli, on teat orifice. In 1942, only two per cent of 2000 cases of mastitis in United Kingdom were found to be due to E. coli, compared to 11.4 per cent of 3500 cases in 1961 (Wilson, 1962) but by late 1970's E. coli was the most common organism causing acute mastitis (Marr et al., 1979). Wilesmith et al., 1986 noticed E. coli as the most common cause of mastitis in 45,000 cases from 378 herds in UK. Anderson (1987) studied the incidence and agents responsible for acute clinical mastitis in 35 Danish dairy herds comprising of 989 cows and reported 23.3 per cent of incidence due to E. coli.

Prabhakar et al. (1988) observed that E. coli was second most common causative agent of mastitis in cows and

buffaloes. Pal *et al*. (1988) observed 20 per cent incidence of mastitis due to *E. coli* when milk samples from 81 cows and 56 buffaloes were analysed. Katholam (1989) also reported similar percentage of *E. coli* mastitis in cows, whereas Jepsen *et al*. (1989) reported 67.3 per cent of mastitis due to *E. coli* from 104 cases of per acute mastitis from two herds. Barrow and Hill (1989) identified 422 *E. coli* (89.78%) out of 470 Gram-negative facultative anaerobes isolated from bovine mastitis in England and Wales.

Rahman and Boro (1990) isolated only nine *E. coli* (9.57%) out of 94 bacterial isolates from 115 milk samples of cows. Bansal *et al*. (1990) reported that *Staphylococcus aureus* and *E. coli* were the most common agents causing mastitis in cows. Bergmann and Alkaff (1990) differentiated Gram-negative bacteria isolated from cows having mastitis and identified biochemically 117 isolates as *E. coli* (72.22%), out of 162 Gram-negative bacteria

Messadi *et al*. (1991) identified 30 *E. coli* (11.58%) out of 259 bacteria isolated from 337 cases of clinical mastitis in Tunisia during 1986-89. Todhunter *et al*. (1991) isolated 46 *E. coli* strains (28.75%) during two year period from 160 quarters of 99 cows with Gram-negative bacterial intramammary infections.

2.2 Identification of *Escherichia coli*

The Enterobacteriaceae sub-committee of the International Association of Microbiological Societies described *Escherichia* on the basis of biochemical tests (Sojka, 1965). *Escherichia coli* was further identified by a battery of biochemical tests (Edwards and Ewing, 1972; Cowan, 1974; Cruickshank *et al*., 1975).

2.3 Antibiogram testing

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. The contagious transfer of drug resistance in enterobacteriaceae was first demonstrated in Japan (Ochiai *et al*., 1959; Akiba, *et al*., 1960). They reported that the multiple drug resistance could be transferred from *Shigella flexneri* to *E. coli* through R-factor. Resistance factors (R-factor or R-plasmid) are group of genes containing resistance transfer factor (RTF) and R-determinants which render their host resistance to a number of antimicrobial agents and at the same time, give their hosts the ability to transfer the resistance to other bacteria (Falkow, 1975).

Different species of bacteria were found to harbour different characteristic types of plasmids, some of which could mediate their own transfer by conjugation. The resistance genes are often incorporated into discrete genetic units called transposons, which have the capacity to transpose from one DNA molecule to another resulting in the rapid dissemination of antibiotic resistance (Kleckner, 1981).

Antibiotic resistance could be assessed by using various antibiotics in critical concentrations by the agar diffusion test (Bauer et al., 1966). The various patterns of antibiogram could be capable of distinguishing between strains of bacteria.

Elek et al. (1973) studied the antibiogram of 273 isolates of Shigella sonnei from 30 sources using sulphonamide (Su), streptomycin (S), tetracycline (T), ampicillin (A) and neomycin (N) discs to differentiate various strains epidemiologically. It was reported that the isolates from a particular location revealed similar pattern of antibiogram with few exceptions, which showed two to three antibiogram patterns from a single location.

Gianelli et al. (1979 b) reported that among E. coli strains isolated from fowl and turkey 48 per cent were resistant to S, 29 per cent to T, 23 per cent to C and 23 per

cent to Su. Adetosoye (1980 b) reported that 31.15 per cent of *E. coli* isolated from faeces of kids, chickens, piglets, calves and lambs, transferred part or whole of their resistance determinants to a sensitive recipient. Of the nineteen resistance patterns seen, OTSuS was the most common (25%). However C resistance was not common.

Arunkumar *et al.* (1981) observed that among the isolates of *E. coli* from day-old chicks, week-old chicks and hens, the week-old had higher percentage of R factors (68.77%) than day-old chicks (40%) and hens (39.2%). Khanna *et al.* (1981) evaluated the antibiotic sensitivity of donor and recipient strains by single antibiotic disc diffusion method to study the R-factor of *E. coli* from faeces.

Saida *et al.* (1981) examined the drug resistance of 1120 strains of *E. coli* from swine, butchers, breeders and urban residents. Fifty-eight per cent strains from swine, 50 per cent from butchers, 42 per cent from pig breeders and 12 per cent from urban residents were resistant to TCSSu and R-plasmids were obtained from half of the resistant strains. Kanamycin resistant strains were isolated from pigs (40.3%), butchers (22.6%) and urban residents (3.4%). The R-plasmids for K and A resistances were observed in 24 per cent strains from pigs, 23.6 per cent from butchers and 21.1 per cent from breeders but none from urban residents. They opined that

swine being the reservoirs of bacteria possessing antibiotic resistance plasmids, could transfer their resistance to human beings.

Jackson (1981) recorded during 1971-77 the following percentage of antibiotic resistance in UK in 306 isolates of E. coli from cases of mastitis, C, 9.5; S, 17.3; T, 21.1; N, 4.2; A, 11.4 and F, 2.3.

Punnoose (1982) studied the resistance patterns of E. coli isolated from poultry. Among eleven patterns of multiple drug resistance (MDR) the SSuTF was the most common and multiple resistance against seven antibiotics (ASSuTFCnC) was observed.

Rangnekar et al. (1982) reported that all Salmonella wein isolated at Bombay during 1978-80 from various clinical cases were resistant to A, C, K, S, Su and T and five of these were resistant to Q. It was noticed that each isolate harboured a plasmid encoding ASSu resistance and Inc Fme, irrespective of its source and time of isolation. They concluded, based on antibiogram and similarity of plasmid, that an endemic focus was established in Bombay with clonal origin.

Panhotra *et al*. (1982) studied the drug resistance of *E. coli* isolated from food and observed the following patterns of resistance, C, 5.8 per cent; F, 7.8 per cent; T, 29.4 per cent; S, 15.6 per cent; Su, 52.9 per cent and Q, 47.1 per cent. The presence of R plasmids was established in 86.6 per cent strains by conjugation.

Tewari and Agarwal (1983) reported that 70.14 per cent of *E. coli* strains isolated from faeces were resistant to one or more drugs, and only 29.86 per cent strains were sensitive to all the drugs used. Multiple resistance pattern of SSuTAC was most common (24.75%). The most effective drug was J, 96.04 per cent, followed by N, 79.75 per cent; F, 74.45 per cent and Q, 59.90 per cent.

Prabhakar and Sud (1984) reported 66 per cent of resistant *E. coli* in normal individuals, 76 per cent in diarrhoeic patients and 80 per cent in hospitalized patients. The ACST was the common pattern of resistance. Transmissible plasmids were observed in all the cases. Harnett and Gyles (1984) reported that multiple resistance was common in *E. coli* isolated from bovines and resistance to C and K was less common than resistance to other drugs. Most of the strains were resistant to four or more drugs (SSuTA).

Gonzalez and Blanco (1986) reported an enterotoxin - producing gentamicin - resistant E. coli belonging to serogroup O141:K88 ab and possessing P987 antigen which had an additional plasmid of molecular weight 16 x 10^6 daltons. This additional plasmid was absent in gentamicin-sensitive E. coli.

Chaslus-Dancla et al. (1987) observed transmission of aminoglycosides resistant plasmids in E. coli between calves and lambs. Anderson (1987) while studying mastitis cases in Danish cows observed that all the E. coli strains isolated were resistant to P but sensitive to S, T and N.

Mackie et al. (1988) recorded an increase in A and N resistance in coliforms isolated from udders in Northern Ireland during 1984-88. Prabhakar et al. (1988) studied the antibiogram of E. coli and reported that the organisms were sensitive in descending order to C, N, F, T, A, E, S, O and Cl. They found that a combination of Cl and A was more effective than A alone in treating cases of mastitis.

Mahamoud (1988) reported that E. coli isolated from subclinical cases of mastitis in dairy cattle were sensitive in descending order to J, N, P, K and S. Jarp et al. (1988) investigated the efficiency of different sulfonamides against E. coli isolated from mastitis cases and observed that

sulphamethoxypyridazine was the most effective drug followed by sulfonamide and sulphadimedine.

Barrow and Hill (1989) determined antibiotic resistance using multidiscs in *E. coli* isolated from bovine mastitis and reported that 22 per cent strains were resistant to one or more antibiotics, (T,15%; A, 14%; Su, 15%; Sp, 5%; N, 4%; Q, 2%; C, 5% and F, 2%) and 78 per cent were sensitive. Kovlalov *et al*. (1989) studied the antibiogram of 5762 *E. coli* isolated from disease conditions in cattle and pigs during the period from 1980 to 1984. A comparison of results with similar tests done between 1976 and 1979, showed that there was a considerable increase in resistance to T and C, but little change was noticed in resistance to N and K.

Hosoda *et al*. (1990) examined 119 *E. coli* isolates from calves for drug resistance and R plasmids. The percentage resistance to each drug was as follows: T, 59.7; C, 21.0; S, 52.9; Su, 37.8; K, 45.4; P, 43.7 and Na, 28.6. R-plasmids were carried by 53.7 per cent of the strains. Rahman and Boro (1990) observed that *E. coli* isolated from cows with mastitis were highly susceptible to J, N, K and C.

Bergmann and Alkaff (1990) observed that the most effective antibiotics against *E. coli* isolated from cows with mastitis were N and O. Hartmann (1990) studied the

antibiogram of E. coli isolated from cases of mastitis in cows and found that gentamicin alone or in combination with cloxacillin was effective against E. coli.

Premkumar David et al. (1991) assessed the resistance and susceptibility of 20 avian E. coli against different antimicrobial agents. All the strains were sensitive to PB, Na, and J, but cent per cent resistance was noticed to Cl, Mz and R. Nineteen different drug resistance patterns (95% variability) were noticed, indicating a reliability of 90% (18 distinct individual patterns) in differentiating 20 E. coli isolates. Only two isolates exhibited identical antibiotic resistance pattern. They opined that the antibiogram testing along with plasmid profile analysis was a valuable epidemiological tool.

In a study conducted by Kaura et al. (1991) on E. coli isolates of diarrhoeic cattle, calves and buffalo-calves, it was observed that the organisms were highly resistant to T but only marginally resistant to Na, F, C and K.

Messadi et al. (1991) observed that E. coli isolated from clinical mastitis in Tunisia were resistant to most of the antibiotics in common use, but the lowest resistance was seen in case of cefaperazone.

It was reported by Mahipal Singh et al. (1992) that out of 302 E. coli isolates from man and animals 63.2 per cent (37.7% from man and 25.5% from animals) were resistant to one or more drugs (A, 43; 0, 36.4% and Q, 9.3%) and out of this, 41 per cent were multiresistant. The multiresistant strains were present at high frequency among human and animal isolates.

2.4 Resistogram typing

Plasmids were found to carry a number of genetic determinants like resistance to antibiotics (Watanabe, 1963) and to mercuric ions (Richmond and John, 1964). Novick and Roth (1968) reported the existence of determinants for resistance in Staphylococcus aureus to various inorganic ions including arsenate, arsenite, antimony, lead, cadmium, zinc, bismuth and mercury.

Many antibacterial substances when tested in critical concentrations and in very fine steps could be capable of distinguishing between strains of species. This approach called "resistogram typing" or resistotyping was successfully applied to E. coli, initially by using various chemicals like sodium arsenate, phenyl mercuric nitrate, 4:4 diamidino diphenylamine dihydrochloride, boric acid, acriflavine, 4-chloresorsinol, copper sulphate and malachite green. It was

noticed that in few instances different strains fell into a single resistogram typing pattern, but in other cases strains of the same serotype from different patients could be distinguished by means of the resistogram (Elek and Higney, 1970). Hedges and Baumberg (1973) reported for the first time the existence of plasmid borne resistance to arsenic compounds which was transmissible between strains of the E. coli.

Elek et al. (1973) applied the principle of selective toxicity of randomly chosen chemicals. They postulated that by using suitable range of selective substances at a particular concentrations a pattern of resistance could be obtained that would be characteristic of the strain. Eleven chemicals were chosen as the basis of resistogram system for Shigella sonnei. They found that the resistogram markers were independent of the colicin type and antibiotic resistance markers and hence present on different episomes. They recommended the resistotyping as a useful adjunct for detailed epidemiology of Shigella sonnei.

Summer and Jacoby (1977) reported that the resistance to heavy metals like mercury, cadmium, lead, antimony, arsenic, copper, silver, tellurium, and quaternary ammonium compounds (QAC) like cetrimide was plasmid determined.

Smith *et al*. (1978) observed that the enterobacteria with plasmid borne resistance to chloramphenicol were also bearing resistance to mercury, arsenite, and tellurite. All these organisms were isolated from the unrelated sources such as sewage, river water etc.

Smith (1978) examined the strains of *E. coli*, *Shigella*, *Proteus*, *Klebsiella pneumoniae* and *Salmonella*, which were resistant to sodium arsenite. The incidence of arsenite resistance was higher in animal than human strains of *E. coli*. The resistance was observed to be transmissible and plasmid borne. Resistogram typing was applied to assess the efficacy of discrimination of urinary strains of *E. coli*.

Wilson *et al*. (1981) reported that the resistotyping combined with biotyping was sufficient for identification of (96.33%) pairs of urinary isolates of *E. coli* stored for twelve to fifteen years. It was concluded that multiple typing approach was useful for retrospective analysis of stored cultures of *E. coli*.

Foster (1983) observed that the resistance to heavy metals and quaternary ammonium compounds like cetrimide were found to be plasmid determined.

In South Africa, Sommerville *et al*. (1983) investigated the potential value of resistotyping in

differentiating Salmonella typhi. The method although demanding and bit difficult technically, was found to be capable of characterizing strains. They noticed that S. typhi resistotypes obtained from the same patient at different times or from different sites were similar. They chose tannic acid, proflavine hemisulphate, P-Amino-N-N-diethyl aniline sulphate, acriflavine and rosaline hydrochloride for testing. The resistotyping grouped all the eighty-seven isolates into sixteen resistotypes. The resistotyping traced the source of infection to drinking water supply. Tetaz and Luke (1983) reported that E. coli was frequently resistant to heavy-metal ions and the plasmid mediated resistance could often be transferred by conjugation.

Harnett and Gyles (1984) reported that the resistance of bovine and porcine strains of E. coli to sodium arsenate, mercury and tellurium was to the extent of 90 per cent, 16 per cent and 5 per cent respectively. Other chemicals used were cadmium chloride, cobalt chloride, copper sulphate, lead nitrate and zinc sulphate.

Robinson and Tuovinen (1984) observed that resistance to mercury and organomercurials was determined by plasmid which also encoded resistance to other heavy metals and antibiotics. This was confirmed by conjugation, isolation of covalently closed circular (CCC) DNA and ability to transfer

mercury resistance to the recipient. Moreover, strong correlation between antibiotic resistance and resistance to Hg and other metals was reported.

Chaslus-Dancla and Lafont (1985) isolated E. coli having plasmid borne tellurite resistance from broiler chicken carcasses. Emslie et al. (1985) noticed that isolates of S. aureus from Australia and several other countries revealed low levels of resistance to cetyl trimethyl ammonium bromide.

Kaur et al. (1985) undertook a study to determine the extent of development of silver ion resistance in E. coli. The silver resistance was developed by passaging in silver nitrate ($AgNO_3$) solution and no concomitant development of resistance to other heavy metal ions or antibiotics was observed.

Barrow and Hill (1989) studied the resistogram of E. coli isolated from bovine mastitis by incorporating metal salts such as sodium arsenate, sodium arsenite, cadmium chloride, copper sulphate, and mercuric chloride. The strains resistant to sodium arsenate and sodium arsenite were encountered.

Grewal and Tiwari (1990) reported that all the E. coli strains isolated from food stuffs were resistant to at least one or a panel of four metallic ions tested. The multiple

resistance to three or four metals was noticed. The degree of resistance was 94.9 per cent against cadmium, 76.9 per cent against arsenate, 71.8 per cent against silver and 61.5 per cent against mercury. It was also observed that the resistance to kanamycin was correlated with silver and cadmium ions and ampicillin and cephalothin resistance was associated with cadmium ions.

Premkumar David et al. (1991) reported that resistotyping of *E. coli* in conjunction with plasmid profile analysis was a valuable epidemiological tool. Grover et al. (1992) noticed that all the *Pseudomonas* and *Alcaligenes* were sensitive to mercury but some were multiple resistant to two or more metal ions when tested against mercury, zinc, copper, nickel and lead. Nickel resistance was reported to be plasmid mediated in *Pseudomonas putida*.

Manohar Paul (1992) studied the resistogram typing of fifty *S. aureus* isolates, and observed thirty-five different patterns with reliability of 70 per cent in differentiation of strains. The organisms evinced different degrees of resistance to chemicals such as lead nitrate 50 per cent, cadmium acetate 48 per cent, and cetyl trimethyl ammonium bromide 30 per cent. It was observed that resistogram typing in conjunction with plasmid profile analysis could differentiate and identify the isolates.

2.5 Hemolysins of Escherichia coli

Many organisms produce substances that dissolve or lyse red blood cells. These substances are called hemolysins and liberation of hemoglobulin is hemolysis.

Kayser (1903) reported that some E. coli cultures lysed erythrocytes. This was confirmed later (Dudgeon and Pulvertaft, 1927). Smith (1963) was first to differentiate cell-bound hemolysisn (β -hemolysin) and the cell free hemolysin (α -hemolysin). Walton and Smith (1969) found a third hemolysin (γ -hemoysin) produced by E. coli mutants resistant to nalidixic acid. Unlike the α-and β -hemolysins, theγ-hemolysin did not hemolyze human or rabbit erythrocytes but lysed erythrocytes of other species.

Transmissible plasmids bearing hemolysin determinants were demonstrated by various workers (Smith and Halls, 1967a; Goebel and Schremps, 1971). The locations and functions of the genes required for hemolysin production and secretion have been investigated. The mechanism whereby hemolysin is produced and excreted appeared to be under complex genetic control (Goebel and Schremps, 1971; Springer and Goebel, 1980).

Gyles et al. (1971) reported that 86 per cent of faecal E. coli were hemolytic when the production was observed

on 5 per cent bovine blood agar. But other workers could not find any hemolysin producing E. coli in cattle and pigs (Smith et al., 1978 and Harnett and Gyles, 1984).

Noegel et al. (1981) opined that α -hemolytic activity of E. coli was plasmid borne and they confirmed that genetic determinant required for hemolysin was located entirely on plasmid pHly 152. They opined that there were at least three clustered cistrons responsible for hemolysin production.

A significantly higher association of hemolytic E. coli with extraintestinal infections than with normal flora led to the consensus that hemolytic E. coli were more likely to cause disease than non-hemolytic E. coli and it led to the study evaluating the importance of the hemolysin as a virulence factor. Cytotoxicity and stimulation of growth effects (by making iron available after RBC lysis) appeared to be the most likely mechanisms by which the hemolysin could function to increase the virulence of E. coli (Linggood and Ingram, 1982; Cavalieri et al., 1984).

Waalwijk et al. (1983) confirmed that a plasmid was responsible for hemolysin production and thus enhancement of the virulence. Cavalieri et al. (1984) reported that virulence in E. coli was multifactorial and certain properties were associated with virulence like ability to produce

hemolysin. They noticed that a high percentage of extraintestinal isolates produced hemolysis and hence could be proposed as a virulence factor.

Neeraja et al. (1984) reported that more number of strains of E. coli isolated from extraintestinal sources like lochia and cervicovaginal discharges produced hemolysin (60%) than the strains isolated from intestinal source (12%). This property of the strains attributed them virulence.

Klimuszko et al. (1987) studied the transfer of hemolysin producing plasmid Hly from E. coli, recovered from Polish pigs, to recipient E. coli. They noticed the transfer of Hly in 30 per cent of cases only.

Barrow and Hill (1989) studied the virulence characteristics of E. coli isolated from cases of bovine mastitis and reported that the hemolysin production on sheep blood agar in 5 per cent of strains and enterotoxin in 1 per cent strains. They opined that these strains did not possess the virulence markers normally associated with invasive or enteropathogenic types of E. coli. Thus hemolysin, enterotoxin, verotoxin producing characters were possessed by few strains. The possession of these characters at least by a few strains reflected their occasional occurrence among intestinal strains. Klimuszko et al. (1989) confirmed the

role of plasmid in the production of hemolysin by *in vivo* transfer of *Hly* plasmid to autochthonous *E. coli* organisms in pigs.

Kaura *et al*. (1991) reported α-hemolysin production by *E. coli* strains of bovine origin showing mannose resistant haemagglutination and multiple antibiotic resistance. Prada *et al*. (1991) observed that all *E. coli* isolated from dogs suffering from gastroenteritis were α -hemolysin producing. The hemolysin production in eight isolates was cured of their *Hly* plasmid with ethidium bromide, thus confirming their plasmid borne nature. Bertin (1992) reported that enterotoxigenic *E. coli* (ETEC) of porcine origin possessed plasmid determined Hly^+ character among other characters and he could transfer the Hly^+ character to other *E. coli* by mating experiment.

Sharma *et al*. (1992) reported α -hemolysin production by 43 per cent and β - hemolysin production by 12 per cent of *E. coli* strains isolated from meat. Wasteson *et al*. (1992) noticed that 78 per cent of *E. coli* from pigs with edema disease were hemolytic and this character was used for the detection of toxigenic genes.

Emery *et al*. (1992) observed that none of the chickens and turkey isolates of *E. coli* from colisepticemic birds

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produced hemolysin. Hirsh *et al.* (1993) reported that 18 per cent of *E. coli* isolates from septic foals were hemolytic but none from the faecal isolates.

2.6 Enterotoxins of *Escherichia coli*

Some Strains of *E. coli* produce exotoxins that are under genetic control of transmissible plasmids. *Escherichia coli* causes pathological effects by the action of its toxins, classed as exotoxins and endotoxins. Various exotoxins are elaborated by living organisms and they are termed 'enterotoxins' when produced in the intestines.

It was found that some strains of *E. coli* that were associated with enteritis in pigs caused dilatation of the ligated rabbit gut segments in a manner similar to that observed in case of cholera toxin (De *et al.*, 1956; McNaught and Roberts, 1958).

Bovine mastitis due to *E. coli* was thought to be the result of endotoxin action. This endotoxin could be called *E. coli* endotoxin or simply 'endotoxin' or lipopolysaccharide (LPS) (Sojka, 1965). The *Escherichia coli* enterotoxin was recognized for the first time and the term 'Ent' was used to describe enterotoxin encoding plasmids (Smith and Halls, 1967b, 1968).

The enterotoxigenic E. coli (ETEC) were the strains of E. coli capable of producing enterotoxins. In ETEC, two distinct types of toxins were characterized, the heat-labile enterotoxin (LT) and the heat-stable enterotoxin ST (Gyles and Barnum, 1969).

Smith and Linggood (1971) reported that in many of the enterotoxigenic E. coli the genetic determinants for toxigenicity and adherence were considered to be present in plasmids. The transmissible nature of these plasmids was utilized to study the contribution of virulence factors to the pathogenesis of the disease in piglets. Gyles et al. (1974) reported that at least one transmissible plasmid was noticed to be present in 90 per cent of toxigenic E. coli strains.

Sack (1975) observed that Ent plasmids could encode either LT or ST or both. They found that the toxins were descernible by several characteristics such as biological activity, immunogenicity and relative heat stability. So et al. (1975) opined that plasmid coding for LT + ST constituted a homogeneous group of extrachromosomal elements that had similar mole fraction, guanine + cytosine molecular mass (60 Mdal) and DNA sequence homology.

Franklin et al. (1981) observed that genes coding for enterotoxin production, K88 antigen, colicin and antibiotic

resistance are located on different plasmids which could be transferred independently of one another.

Klimuszko *et al*. (1987) studied the transfer of *Ent* plasmids along with *Hly* markers from *E. coli* strains isolated from pigs to the recipient *E. coli*.

The genes responsible for ST and LT toxins were found to be plasmid borne (Woodward *et al*., 1990; Baloda and Mansson, 1991).

Alkaff *et al*. (1991) recorded 5.98 per cent (7 of 117) enterotoxin producing *E. coli* from cases of bovine mastitis. They noticed that endotoxins were more important than enterotoxins in mastitis. Neneth *et al*. (1991) identified 36 (25.35 %) enterotoxigenic (ETEC) *E. coli* out of the 95 *E. coli* isolates from bovine mastitis and 47 from milking machine fitters. Prada *et al*. (1991) could find ST toxin only in *E. coli* isolated from dogs with gastroenteritis while Emery *et al.* (1992) could detect LT toxin only in *E. coli* isolated from turkeys and chickens with colisepticemia.

2.6.1 Detection of toxins

Detection of enterotoxins (ST and LT) could be done using various animal models. *Escherichia coli* isolated from different sources and capable of producing enterotoxins were

found to produce dilatation of ligated intestine of rabbit or piglets (De *et al.*, 1956; MacNaught and Roberts, 1958; Smith and Halls, 1967; Gyles *et al.*, 1971 and Baldev Raj, 1991).

Agarwal and Tewari (1983) observed that the toxic changes produced in ligated loop were due to lipid carbohydrate components of LT.

Only one per cent of *E. coli* strains isolated from bovine mastitis were enterotoxigenic and presence of LT toxin was confirmed by elongation of Chinese hamster ovary cells (CHO) (Barrow and Hill, 1989. Emery *et al.* (1992) detected the presence of LT in the culture supernatants and sonic extracts from turkey and chicken *E. coli* isolates, by cytotoxicity assay using tissue cultures of Vero and Y-I cells.

2.6.2 Heat-labile enterotoxin (LT)

Heat-labile toxin (LT) is a high molecular weight protein, acting intracellularly. The toxin is a polypeptide composed of subunits that are synthesized separately and then associated to form the A-B structure; the A chain possessed the biological activity, whereas the B chain mediated receptor binding. The production of LT was found to be controlled by plasmids. The LT genes were flanked by inverted repeat DNA

sequences suggesting that the genetic determinant for LT might be transposable (Yamamoto and Yokota, 1981, Ronnberg, 1986).

Identification of LT producing ETEC was originally carried out using the rabbit ileal loop model (De *et al*., 1956), the skin permeability factor assay (Evans *et al*., 1973) and the tissue culture methods as adrenal cell and Chinese Hamster Ovary (CHO) cells (Donta *et al*., 1974; Gurrent *et al*., 1974).

2.7 *In vitro* transfer of resistance by conjugation

Genetic material was found to be transferred to *E. coli* by direct cell to cell contact (Lederberg and Tatum, 1946). Contagious transfer of drug resistance in enterobacteriaceae was first demonstrated in Japan (Ochiai *et al*., 1959; Akiba *et al*., 1960). Discovery of multiple resistance was the result of careful epidemiological observation of multiple resistant *Salmonella* and this led to the discovery of R-factors. R-factor could be transferred to many other genera of enterobacteriaceae (Watanabe, 1963).

The conjugation encoded by conjugative plasmids was observed in a diverse range of Gram-negative bacteria (Bukhari *et al*., 1977; Datta, 1979). All of these plasmids encoded conjugative pili necessary for establishing cell to cell

contact and their molecular mechanisms of conjugation would be similar (Ou and Anderson, 1970).

The processing of plasmid DNA during conjugative transfer and sequence of events in conjugation were studied in detail in E. coli (Willets and Skurray, 1980; Bradley, 1981; Willets and Wilkins, 1984).

The ability to transfer drug resistance indicated the presence of plasmid borne resistance (Davies and Stewart, 1978; Barrow and Hill, 1989; Purushothaman and Venkatesan, 1993).

2.7.1 In vitro transfer of plasmids

Watanabe and Fukasawa (1961a) reported the intergeneric transfer of resistances from naturally occurring multiple drug resistant Shigella to E. coli, Salmonella typhimurium and S. enteritidis. The resistances were further transferred to sensitive recipients and replicted autonomously in cytoplasm. The frequency of transfer and degree of resistance differed greatly among recipients.

Anderson and Lewis (1965) observed that the organisms having same resistance pattern could act differently, one transferring all the resistance en-bloc while other transferred resistances independently. They further noticed

that the resistance to the same antibiotic was found to be determined by a number of R-factors, each presumably with its separate resistance transfer factor (RTF).

Smith (1971) reported that in recently inoculated bacterial cultures the plasmids were found to multiply rapidly and conjugation and plasmid transfer took place before cell division and later the plasmid multiplication was synchronised with the chromosomal multiplication due to repression genes which inhibited pili formation and conjugation.

Gyles et al. (1971) reported the intergeneric transfer of Ent plasmids. The hemolysin (Hly) transfer was reported from hemolysin producing E. coli to non-hemolytic recipient E. coli (Goebel and Schremps, 1971; Prada et al., 1991).

Davies (1979) observed that plasmid mediated resistances were not observed in case of nitrofurans, nalidixic acid and polymyxins.

It was noticed that the infective drug resistance (R plasmids) of faecal E. coli isolated from calves, poultry and infants could be transferred to E. coli K12 by conjugation (Ishiguro et al., 1980; Taku et al., 1990).

In his study Adetosoye (1980a) observed that all the seven E. coli strains isolated from diarrhoeic lambs

transferred their resistance to E. coli K 12 through conjugation, whereas Adetosoye (1980b) in a separate study found that 129 (31.5%) of 414 E. coli isolates transferred part or whole of their resistance determinants to a sensitive recipient E. coli J5 K 12.

Hirsh et al. (1981) studied in vitro transfer of resistance from Pasteurella multocida to E. coli, and it was noticed that the genes responsible for resistance and transfer of resistance were independent.

Khanna et al. (1981) observed the transfer of R-factor from donor E. coli to recipient E. coli K 12 and from the transconjugant of E. coli K 12 to Salmonella typhi and S. typhimurium.

Punnoose (1982) studied the in vitro transfer of resistance of E. coli isolated from poultry. He observed an increase in R-factor of E. coli isolated from chicks after low level feeding of bamberamycin and furazolidone. The furazolidone resistance either alone or with other resistances was transferred to recipient E. coli K 12 Ml 1410 indicating the existence of plasmid borne resistance to furazolidone.

Pai and Joshi (1984) studied the intergeneric transfer of R-factors from Pseudomonas aeruginosa to E. coli J53-1 and observed no change of molecular weight of plasmid on transfer.

Kaur *et al*. (1985) observed the transfer of resistance to silver ions from *E. coli* to *Salmonella typhimurium*.

Klimuszko *et al*. (1987) investigated the ability of *E. coli* strains isolated from Polish pigs to transfer *Hly*, *Ent* and K88 (F4) markers. They observed that *Hly* and *Ent* markers were transferred at low frequency than K88 F4 markers. They opined that strains need to pass through several consecutive conjugations before they become fully pathogenic.

Barrow and Hill (1989) observed the transfer of T, C and S drug resistance of *E. coli* isolated from bovine mastitis to recipient *E. coli*. Abdul Rashid and Misra (1989) noticed *in vitro* transfer of enterotoxin and R-factor from donor *E. coli* to recipient *E. coli* at very low level. Baldev Raj (1991) observed transfer of enterotoxin genes only in one out of 18 (7.6%) enterotoxigenic *E. coli* along with streptomycin and tetracycline resistance. But in rest of the *E. coli* only drug resistance markers were transferred.

Prada *et al*. (1991) noticed the *in vitro* transfer of hemolysin producing genes alongwith streptomycin resistance in *E. coli* on blood agar. Similarly Bertin (1992) observed the transfer of hemolysin producing character (*Hly*) along with antibiotic resistance to S, Su and T from donor *E. coli* to recipient *E. coli*.

Verma and Gupta (1992) reported very high frequency (82.3 %) transfer of tetracycline resistance from Salmonella virchow to E. coli K12 (F Lac Nx^r) in primary cross, which indicated infectious nature of R-factor. But the reduction in the frequency of transfer was noticed during secondary transfer from S. virchow - E. coli K 12 transconjugant to S. typhimurium and E. coli (E-200:C600). Furadantin resistance could not be transferred in any of the strains. Purushothaman and Venkatesan (1993) also studied the in vitro transfer of antibiotic resistance from different species of Salmonella to E. coli K12 27662 (F Na^r).

2.8 Plasmid profile analysis

The plasmid profile analysis, is a sensitive, rapid, reproducible, relatively inexpensive and suitable for the examination of large number of bacterial cultures and it also detects the interference of chromosomal DNA fragments. The plasmid content of several bacterial species was examined by plasmid profile analysis by agarose gel electrophoresis 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. Plasmid profile analysis was found to be useful for identifying and characterizing the organisms for the epidemiological studies.

2.8.1 Plasmid profile analysis technique

The techniques used for isolation of plasmid DNA involved lysing bacterial cells so that the plasmid DNA is preserved intact and could be physically separated from the more massive chromosonal DNA (Sambrook et al., 1989). Any method applied for plasmid isolation involves three basic steps.

1. Growth of the bacterial culture
2. Harvesting and lysis of the bacteria
3. Purification of plasmid DNA

2.8.1.1 Isolation of plasmid DNA

2.8.1.1.1 Alkaline extraction method

Most of the techniques employed for the isolation of plasmid deoxyribonucleic acid (DNA) are based on their supercoiled, covalently closed circular (CCC) configuration within the bacterial cell. The "clear lysate method" with the incorporation of Brij, a detergent was widely used (Clewell and Helinski, 1969; Macrina et al., 1978).

Guerry et al. (1973) reported that the plasmid DNA could be isolated from chromosonal 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).

Birnboim 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. The lysozyme treated cells were acted upon by SDS and sodium hydroxide (NaOH) for obtaining alkaline pH. The chromosomal DNA was selectively denatured and the lysate was neutralized by acidic sodium acetate pH 4.8. The chromosomal DNA gets renatured and aggregated to form an insoluble network. The high concentration of sodium acetate precipitated the protein-SDS complex and high molecular weight RNA. The major contaminating macromolecules could be co-precipitated and removed by centrifugation and plasmid DNA could be recovered from supernatant by ethanol precipitation.

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

Barrow and Hill (1989) separated the plasmids from E. coli isolated from cases of bovine mastitis in England and Wales by alkaline denaturation of the bacterial chromosome.

The plasmids (CCC-DNA) were separated for studying virulence factors of E. coli by alkaline sodium dodecyl sulphate method of Kado and Liu (Vidotto et al., 1990).

2.8.1.1.2 **Boiling method**

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 was equally applicable for rapid small scale isolation as well as large scale isolation of plasmid DNA.

Gomez-Marquez et al. (1987) developed a procedure, for large scale purification of plasmid DNA. It was a modification of boiling method of Holmes and Quigley (1981). Recovered plasmids were free of RNA and chromosomal DNA and were supercoiled and suitable for restriction analysis.

2.8.1.1.3 Other procedures

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).

2.8.2 Agarose gel electrophoresis 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 has 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. A variety of large plasmids ranging from 70-300 MDa were readily visualised with AGE (Hansen and Olsen, 1978).

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 the chromosomal DNA fragment in the crude preparations. It was noticed that the AGE provided information on the distribution of particular plasmids.

2.8.3 Molecular size estimation of plasmid DNA

Plasmid size may be estimated conveniently by agarose gel electrophoresis (AGE) using either purified plasmid DNA or plasmid containing cell lysates that have been cleared of chromosomal DNA by high speed centrifugation. Such size estimations are made possible by the inclusion of plasmid molecules of known molecular size in the same electrophoretic run.

Aaij and Borst (1972) reported that the migration rates of purified bacteriophage 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 covalently closed circular (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 weights of CCC (DNA) were calculated from the contour lengths by using the conversion factor of 1 um = 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 ranging from 1.36×10^6 to 35.8×10^6 daltons. It was noticed that the strain would serve as a single source of plasmid reference molecules (Macrina *et al*., 1978).

The molecular sizes of the plasmid DNA were estimated by comparison with the plasmids of *E. coli* V 517. Using the least squares technique for fitting the regression line of the logarithms of the molecular weights to the distance migrated in cms, the molecular weights were computed for the measured distances of migration (Premkumar David *et al*., 1991).

2.8.4 Plasmid profile analysis of different organisms

Pai and Joshi (1984) examined the plasmid profile of drug resistant *Pseudomonas aeruginosa* strains and the

transconjugants obtained after conjugation with _E. coli_ J53-1. The molecular weights of plasmid DNA ranged from 1.5×10^6 to 65×10^6 daltons. Plasmids with molecular size less than 20×10^6 daltons were found to be non-conjugative. There was no change of molecular weight when the plasmid determining resistance was transferred to the recipient _E. coli_ J-53.1. The transfer of particular molecular weight plasmid and the resistance pattern revealed the association between a plasmid species and the specific phenotypic trait. It was suggested that comparison of molecular properties of resistant plasmids during an outbreak could help in locating the source.

Gonzalez and Blanco (1986) investigated sixteen heat stable enterotoxin STa^+ strains from piglets for plasmid content. Of these, fifteen belonged to serotype O 141:K 85 ab with fimbrial antigen P 987, and remaining one belonged to serotype O 101:K 30. All fifteen STa^+ P 987 possessed similar plasmid pattern with three plasmids ranging from 33×10^6 to 74×10^6 daltons. Gentamicin resistant strains possessed an additional plasmid of mol. wt. 16×10^6 daltons which was absent in the J-sensitive strains. It was suggested that plasmid analysis was useful in differentiating the strains based on various characters.

Towner _et al_. (1986) reported that the plasmid analysis helped in detecting the close similarities within

human and animal E. coli and was useful for obtaining information about the epidemiology of R-plasmids, as they could notice the plasmids belonging to incompatibility groups B, Fm and Id in both human and animal isolates.

Shimizu et al. (1988) noticed the linkage of K 99 and enterotoxin producing activity in a plasmid of a porcine E. coli strain ZP 118. The strain harboured a 68 (Md) plasmid coding for colonization factor K 99 and heat-stable enteroxin (STa) apart from a self transmissible 51 MDa plasmid for drug resistance.

Watlhuber et al. (1988) investigated E. coli strains isolated from faecal samples of diarrhoeic dogs, healthy dogs and persons from household with diarrhoeic dogs. Enterotoxigenic E. coli (ETEC) strains were isolated from sick dogs only and these strains contained plasmids. In one family, a strain with identical plasmid was noticed from dog and child. In four other families the dog and family members had similar plasmid containing faecal E. coli.

Barrow and Hill (1989) studied the plasmid profiles of E. coli isolated from the cases of bovine mastitis in England and Wales. It was observed that E. coli strains possessed the plasmids of the sizes ranging from 3 Kb to greater than 40 Kb. Several strains possessing single virulence

characteristic harboured one or even more large molecular weight plasmids.

Premkumar David et al. (1991) investigated the plasmid profile of twenty E. coli isolates comprised of one to eight plasmids with molecular size ranging from 1.54 to 34.67 Kb. Altogether sixteen different profiles were obtained (80%) variability) comprising thirteen distinct individual profiles. It was observed that the plasmid profile analysis has 65 per cent reliability in identifying individual isolates.

Wasteson et al. (1992) reported that all the E. coli strains isolated from pigs with oedema disease contained plasmids and fourteen different plasmid profile groups were defined. With reference to molecular weight, no plasmids common to all strains could be observed, and only two plasmid profile groups contained more than one strain (two strains each). The similarity between plasmid profiles was confirmed by EcoRI restriction endonuclease digestion of plasmid DNA.

2.9 Curing of E. coli plasmids

A variety of chemical agents, such as acridine dyes, ethidium bromide, sodium dodecyl sulphate and novobiocin, as well as growth at elevated temperatures were found to free or "cure" bacterial cells of plasmid DNA molecules (Novick, 1969). Plasmid molecules, which occur as autonomously

existing circular DNA duplexes, could be eliminated by these agents either due to interference with their replication (acridines, ethidium bromide, novobiocin) or by alterations of their membrane attachment sites (Sodium dodecyl sulphate, elevated temperatures). The intercalative compounds eliminated resistance determinants, at concentrations that permit growth and replication of R-factor carrying bacteria. In view of the importance of plasmid DNA as carriers of various genetic determinants for antibiotic resistance, heavy metal resistance, production of antibiotics and complex metabolic functions, the curing methods have invited attention (Carlton and Brown, 1981; Crosa and Falkow, 1981).

Mitsuhashi et al. (1961) observed that the drug resistance of both Escherichia and Shigella was eliminated with acriflavin. It was also noticed that all markers for drug resistance in Escherichia and Shigella were lost after treatment with acriflavin and the cells became sensitive to all the drugs used i.e. S, C, T and su.

Watanabe and Fukasawa (1961b) reported the elimination of drug resistance in strains of Shigella flexneri and E. coli by treatment with acridines. The resistance factors against S, C and T were eliminated together. It was not possible to transfer sulphonamide resistance as it was found to be located on chromosome. They further observed that ultraviolet

irradiation of the resistant cells increased the frequency of elimination of resistance factors when treated with acridenes.

May et al. (1964) reported that tetracycline resistant and penicillinase positive strain of S. aureus grown at 44°C gave rise to progressively increasing proportions of tetracycline sensitive and penicillinase negative cocci. The loss appeared after quite a number of generations at the elevated temperature and then apparently proceeded independently. The tetracycline resistance was lost more rapidly than the ability to produce penicillinase.

Terawaki et al. (1967) found that the elimination of resistance factor was possible at higher temperature (44°C) and not at 25°C, although the transfer of resistance was possible at 25°C in E. coli. It was postulated that thermosensitive transfer and the spontaneous elimination of the kanamycin resistance factor at higher temperature was due to thermosensitive replication of kanamycin resistance factor. Tomoeda et al. (1968) observed that the detergent, sodium dodecyl sulphate (SDS) was effective in eliminating F'lac and the R-factor in E. coli.

Bounachaud et al. (1969) observed that ethidium bromide (EB) eliminated antibiotic resistance in E. coli, Salmonella and S.aureus. The antibiotic resistances of two

strains of *E. coli* with same antibiogram were eliminated at different rates. They also noticed that F'*lac* factor was also eliminated in *E. coli* K 12. Elimination of antibiotic resistance by EB was at high frequency and easily reproducible than with acridine dyes.

Salisbury *et al*. (1972) noticed that acridine orange efficiently eliminated F'*lac* from *E. coli* K 12. However, R-factors could not be eliminated by it. Whereas sodium dodecyl sulphate known to eliminate plasmid determined F pili, had no effect on cultures with wild type (repressed) R-factors. Bacteria with derepressed synthesis of pili showed increased sensitivity to SDS. Thus they found acridine orange as a true 'curing' agent for F, whilst SDS acted only by selection of spontaneous variants.

Hahn and Ciak (1976) examined eighteen deoxyribonucleic (DNA) - complexing compounds. Among them fifteen intercalative compounds and nalidixic acid eliminated four antibiotic resistance determinants in *Salmonella typhimurium* with different frequencies. Eliminating concentrations did not inhibit growth of the bacteria. Nitroacridine II proved to be most active compound. All the fourteen compounds were tested at the standard concentration of 10^{-4}M. They noticed that the eliminations resulted from

selective toxicity for plasmid template DNA and inhibitions of R-factor replication.

Robinson et al. (1980) studied the temperature sensitive resistance factor in E. coli which was stable at 30°C but was rapidly lost during growth at 42°C, thereby curing the drug resistance to ampicillin and kanamycin. The loss of the drug resistance was due to the failure of plasmid DNA replication.

All the strains of E. coli isolated from diarrhoeic lambs retained their R-factor plasmids, in spite of exposure to acriflavine, mepacrine and 3-6 diamino-10-methyl acridium chloride, and all of them transferred their resistance to E. coli K 12 (Adetosoye, 1980a).

Carlton and Brown (1981) evolved methods for curing by incorporating the chemicals like acridines, ethidium bromide and sodium dodecyl sulphate and elevated temperatures.

Subramanyam and Agarwal (1982) opined that the elevated temperature (44°C) and ethidium bromide eliminated the drug resistance markers in S. aureus. It was noticed that the drug resistance markers were lost in combinations of two to four antibiotics simultaneously.

Kaur et al. (1985) reported the curing of silver ion resistance by the classical curing agents like acridine orange, ethidium bromide and sodium dodecyl sulphate at the rate of 40-70 per cent in E. coli.

Poppe and Gyles (1988) cultured the bacteria at elevated temperature and applied chemical agents like acridine, ethidium bromide, novobiocin and SDS for curing of virulence plasmids in Salmonella. They concluded that the plasmids were more readily eliminated by incubation at 45.5°C than by the chemical curing. No plasmid was cured by acridine orange, novobiocin or SDS although ethidium bromide was proved to be of some value. Sekazaki et al. (1989) observed that the virulence of E. coli to chickens was lost by curing, and they concluded that the virulence was plasmid associated.

Reddy et al. (1990) examined Klebsiella sp and E. coli for their susceptibility to curing agents like AO, EB, SDS, plumbagin (PB) - a plant derived phenolic compound and hexamine ruthenium III chloride (HRC). The SDS and PB treatment cured 8 and 12 isolates respectively (out of 49 isolates) with a frequency of 6-100 per cent. Whereas AO, EB and HRC sensitized only one isolate each. Simultaneous loss of drug resistance was also observed.

Prada et al. (1991) observed that hemolysin production in E. coli isolated from dogs suffering from gastroenteritis, was lost in the presence of ethidium bromide at a concentration of 100-200 ug/ml. The hemolytic character was confirmed to be plasmid borne by plasmid profiling. Singh (1992) reported the loss of toxigenicity in Clostridium perfringens type D by cultivating the organisms in medium with ethidium bromide or growing the cells at elevated temperature (45°C). He also observed that the synthesis of epsilon toxin was plasmid mediated.

Anjanappa et al. (1993) noticed that the curing of R-plasmids in Salmonella gallinarum was less frequent with chemical agents like ethidium bromide, novobiocin, but could be easily accomplished by growing the culture at 45°C for a period of fifty days.

Materials and Methods

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MATERIALS AND METHODS

3.1 Collection of milk samples

The 300 milk samples from cases of bovine mastitis were collected aseptically from various government and university hospitals, private and university dairy farms in and around Trichur.

3.2 Isolation and identification of *E. coli*

The organisms were isolated using the standard procedures and were identified by various tests (Sojka, 1965; Cowan, 1974; Cruickshank *et al.*, 1975).

3.3 Antibiotic sensitivity test

3.3.1 Agar diffusion method

Sensitivity of 46 *E. coli* isolates to various antibiotics and chemotherapeutic agents was studied by agar diffusion method, using single discs of antibiotics as described by Bauer *et al.* (1966).

3.3.1.1 Medium

Mueller and Hinton Agar (M.H agar) (Hi Media) was used throughout the study.

3.3.1.2 Antibiotic discs

Antibiotic sensitivity discs (Dispens-0-Disc) manufactured by Difco Laboratories, USA, and Span Diagnostics, India were used. The following antibiotic discs were used with their potency in micrograms (µg) per disc.

1. Ampicillin (A)	10 µg
2. Chloramphenicol (C)	30 µg
3. Co-trimoxazole (Q)	25 µg
4. Furazolidone (F)	100 µg
5. Gentamicin (J)	10 µg
6. Kanamicin (K)	30 µg
7. Nalidixic Acid (Na)	30 µg
8. Norfloxacin (Nf)	10 µg
9. Oxytetracycline (O)	30 µg
10. Rifampicin (R)	5 µg
11. Streptomycin (S)	10 µg
12. Sulphadiazine (Su)	300 µg
13. Tetracycline (T)	30 µg

The discs were stored at 4°C.

3.4 Resistogram typing

The resistance to metal ions and quaternary ammonium compound was detected, using the method described by Novick and Roth (1968) by incorporating the following compounds.

Compound	Symbol	Concentration (per cent)	Strength (µg/disc)
1. Copper sulphate ($CuSO_4$)	A	10	2000 µg
2. Silver nitrate ($AgNO_3$)	B	1.7	340 µg
3. Cadmium chloride ($CdCl_3$)	C	10	2000 µg
4. Mercuric chloride ($HgCl_2$)	D	0.1	20 µg
5. Lead oxide (Pb_3O_4)	E	6.86	1372 µg
6. Antimony chloride ($SbCl_3$)	F	10	2000 µg
7. Cetyltrimethyl ammonium bromide (Cetrimide) (C19H42NBr)	G	3.64	728 µg

The solutions of the metal salts were made in sterile distilled water and kept at 4°C and used within a week.

3.4.1 Preparation of discs

The blank discs of 6 mm diameter were prepared from Whatman No.1 filter paper. The discs were sterilized at 140°C for 1 h and impregnated with 20 µl of a salt solution of stipulated concentration. The discs were dried in the incubator at 37°C for 18 h.

3.4.2 Susceptibility testing

The *E. coli* isolates were screened for resistance to copper, silver, cadmium, mercury, lead, antimony and cetrimide

by filter paper disc method. The resistance was assayed by placing 7 discs on Mueller and Hinton agar plates previously seeded with 4-6 h old bacterial culture. The plates were incubated at 37°C for 18 h. The zones of inhibition were measured.

3.5 Hemolysin production

Hemolysin production was detected on 5 per cent sheep blood agar with nutrient agar base as described by Cruickshank et al. (1975).

3.6 Detection of heat-labile enterotoxin

3.6.1 Preparation of culture filtrates

The cell free filtrate of each E. coli isolate was prepared as per the method of Giannella (1976). Escherichia coli isolates were inoculated in Brain Heart Infusion (BHI) broth (Hi Media) and incubated for 18 h at 37°C. The cultures were centrifuged in refrigerated centrifuge at 18000 x g for 45 min. The supernatant was collected and filtered through a membrane filter of 0.45 µm average pore diameter to obtain bacteria-free filtrate. These cell free filtrates were stored at -20°C until used.

3.6.2 Rabbit ligated ileal loop assay

The rabbit ligated ileal loop (RLIL) assay was conducted as per the method described by Bergdoll (1988). Adult New Zealand white rabbits weighing about 1.5 to 2 kg were kept off feed for 24 h prior to the test, but water was provided liberally. The abdomen was shaved and prepared for surgery. The rabbits were anaesthetized with sub-cutaneous infiltration of 1-2 ml of lignocaine solution (2 per cent).

The animals were fixed in a proper position and the abdomen was opened with a 2 cm vertical incision on flank. The proximal end of small intestine was located and after leaving the first 20-30 cm of intestine, five to six loops, each of approximately 10 cm length were made by ligating the intestine tightly. About 3 cm long segments were left in between the test segments which served as blank loop. Two ml of cell free filtrate was inoculated in to the test loop with a sterile 26 G needle. The last loop was injected with BHI broth, which served as media control. The intestine was placed back, and the abdomen was sutured. After 24 h the rabbits were euthanatised by intravenous injection of saturated solution of magnesium sulphate ($MgSO_4$) and the inoculated gut loops were observed for distension and, the volume of the fluid inside the loops was measured with syringe.

Dilation index (D.I.) was calculated

$$D.I. = \frac{\text{Volume of fluid accumulated (ml)}}{\text{Length of loop (cm)}}$$

3.7 *In Vitro* transfer of plasmids by conjugation

3.7.1 Selection of donor

The *E. coli* isolates (24) from bovine mastitis, which were sensitive to nalidixic acid but resistant to other drugs were used as donors.

3.7.2 Recipient strain

Escherichia coli K-12 ML 1410 (F^-,Na^r) sensitive to antibiotics was used as recipient in the present study.

3.7.3 Antibiotic solutions

Standard antibiotic/antimicrobials were obtained from the Director, Central Drug Research Laboratory, Calcutta and Glaxo Laboratories (India) Ltd., Bombay. The antibiotic solutions were prepared as per Maniatis *et al.* (1982). The concentration of antibiotics incorporated for the selection of resistant organisms was similar to that used in drug sensitivity discs. All the antibiotic solutions were stored in 1 ml aliquots at -20°C.

3.7.4 Procedure for In Vitro transfer of drug resistance

The conjugation was carried out as described by Punnoose (1982). Donor and recipient organisms were grown separately in 3 ml of Luria Bertani (LB) broth for eight hours at 37°C. To 10 ml of LB broth 0.1 ml of donor and 0.2 ml of recipient organisms were added and incubated at 37°C for 18-24 h. After incubation, the bacterial growth was inoculated on MacConkey's agar containing 50 µg/ml of nalidixic acid and any one of the antibiotics for which the donor was resistant (Selective medium). The plates were incubated at 37°C for 24 h. The colonies coming up on the selective media-transconjugants-were further purified by subculturing on selective media. The resistance markers of transcongugants were identified phenotypically by disc diffusion method.

The donor, recipient and donor plus recipient mixture were inoculated on selective media containing Na only and Na + antibiotic and on non-selective media for control studies.

3.8 In vitro transfer of hemolysin producing plasmids

3.8.1 Donor

Three hemolysin producing *E. coli* isolates sensitive to nalidixic acid were used as donors.

3.8.2 Recipient

Escherichia coli K12 ML1410 (F^-, Na^r), non-hemolytic, sensitive to common antibiotics but resistant to nalidixic acid was used as recipient.

3.8.3 Procedure

The procedure of Prada *et al*. (1991) was followed.

To 10 ml of LB medium 0.1 ml of donor and 0.2 ml of recipient organisms were added and incubated at 37°C for 18-24 h. After incubation, the bacterial growth was inoculated on 5 per cent sheep blood agar containing 50 µg/ml of nalidixic acid. The plates were incubated at 37°C for 24 h, and, later examined for the presence of hemolysin producing colonies. The frequency of hemolysin production (FH) was calculated as

$$F.H. = \frac{\text{No. of hemolytic colonies on the plate}}{\text{Total no. (hemolytic and non-hemolytic) of colonies}} \times 100$$

3.9 *In vitro* transfer of enterotoxigenic plasmids

3.9.1 Donor

Eleven enterotoxigenic *E. coli* isolates resistant to various antibiotics except nalidixic acid were used as donors.

3.9.2 Recipient

Escherichia coli K 12 ML 1410 (F^-,Na^r), non-enterotoxigenic, sensitive to common antibiotics was used as recipient.

3.9.3 Procedure

The technique used for transfer of *Ent* plasmids was as described by Rashid and Misra (1989) with some modification. The heat labile enterotoxin producing *E. coli* strain (donor) was conjugated with *E. coli* K 12 ML 1410 (F^-Na^r). The mixture of donor and recipient was inoculated on MacConkey's agar containing 50 µg/ml of nalidixic acid. The plates were incubated at 37°C for 24 h. The transconjugants so obtained were tested for the presence of enterotoxigenicity by rabbit ligated ileal loop assay. The antibiogram of the transconjugants were studied.

3.10 Plasmid profile analysis

3.10.1 Buffers and reagents

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

3.10.1.1 Luria Bertani (LB) medium

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
Sodium chloride	10 g
Distilled water	1000 ml
pH	7.5

Autoclaved at 121°C for 15 min at 15 lb/Sq. in

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

Glucose	50 mM
Tris. Cl (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM

Autoclaved for 15 min at 10 lb/sq in and stored at 4°C

3.10.1.3 SDS-NaOH (Solution II)

Sodium hydroxide	0.2 N (freshly diluted from 10 N stock)
Sodium dodecyl sulphate	1%

Taken in equal volumes and mixed together

3.10.1.4 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

3.10.1.5 Tris EDTA Buffer (TE)

Tris-Cl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

Sterilized by autoclaving at 121°C for 15 min at 10 lb/sq in

3.10.1.6 RNAase solution

Pancreatic RNAase (RNAase A) was dissolved at a concentration of 10 mg/ml in 10 mM Tris.Cl (pH 7.5), 15 mM NaCl. The solution was heated to 100°C for 15 min and allowed to cool at room temperature. It was then dispensed into aliquots and stored at -20°C.

3.10.1.7 Tris Borate Electrophoresis Buffer TBE (5x)

Tris base	54.0 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
Distilled water	1000 ml

The concentrated stock solution (5x) was diluted to 1x before use

3.10.1.8 Gel-loading Buffer

Bromophenol blue	0.25%
Sucrose in water	40% (w/v)

Stored at 4°C

3.10.1.9 Ethidium Bromide solution (10 mg/ml) of H_2O

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.

3.10.1.10 Sodium Dodecyl Sulphate (10%)

Hundred grams of electrophoresis grade SDS was dissolved in 900 ml of H_2O 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 aliquots.

3.11 Plasmid isolation and characterization

3.11.1 Plasmid isolation

Plasmid isolation was done by the alkaline lysis method of Birnboim and Doly (1979) modified by Sambrook *et al.* (1989).

3.11.1.1 Harvesting and lysis of bacteria

3.11.1.1.1 Harvesting

A single bacterial colony of *E. coli* isolate was inoculated in 5 ml of LB broth containing the appropriate antibiotic and incubated at 37°C overnight with vigorous shaking.

The culture was transferred to 1.5 ml microfuge tube and centrifuged at 7500 x g for 10 min at 4°C.

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

3.11.1.1.2 Lysis by alkali

The lysozyme (2 mg/ml) was added to ice cold solution I of TEG buffer and the pellet was resuspended in 100 µl of Sol.I by vigorous vortexing.

Later 200 µl 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 rapidly 3-4 times. The tubes were stored in ice for 15 min.

Then 150 µl of ice cold solution III of 5 M potassium acetate was added. The tubes were closed and vortexed gently in an inverted position and stored in ice for 15 min.

The tubes were then centrifuged at 15000 x g for 10 min at 4°C. The measured volume of supernatant was transferred to a fresh tube.

The supernatant was precipitated with double volume of ethanol and mixed by vortexing. The mixture was kept on ice for 15 min.

After centrifuging at 18000 x g for 10 min at 4°C the supernatant was removed.

The pellet was resuspended in 1 ml of 70 per cent enthanol and recentrifuged.

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

The pellet was dissolved in 20 µl of TE pH 8.0 containing DNAase free RNAase (20 µg/ml) and mixed gently.

The plasmid DNA was stored at -20°C till further used.

3.12 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 (13.0 x 6.5 cm) was sealed with adhesive cell-tape on either sides and a comb with 8 teeth (5 x 1.5 mm/tooth) was clamped on it, 0.5 to 1.0 mm above the tray surface so as to form completely sealed wells. To prepare 0.8 per cent of agarose gel, 280 mg of agarose (Sigma) was dissolved in 35 ml of Tris-borate buffer (TBE, pH 8.0).

The slurry was heated in a boiling water bath until the agarose was dissolved.

After cooling to 50°C the agarose solution was poured in a gel tray to form 4 mm thick gel 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 8.0) in the electrophoresis tank.

To 20 µl each of plasmid DNA preparations, 2 µl of bromophenol gel-loading buffer was mixed and the mixture was loaded in the eight wells of the submerged gel by a micropipettor.

The electrophoresis was run at 120 V for 3 h or until the dye (bromophenol blue) reached the appropriate distance through the gel.

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

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

3.13 Plasmid DNA photography

The photographs of plasmid DNA containing gels were taken under UV illumination using 100 ASA (Konica) 135/36 colour film at f/16 aperture with prolonged exposure of 90 sec, using ordinary 35 mm single lense reflex (SLR) photographic camera. Developing of the film and printing of the photographs were done on film and print processors.

3.14 Molecular size determination of plasmids

Molecular sizes of the plasmids were examined by employing *E. coli* V 517 strain of Macrina *et al*. (1978). The plasmid DNA separated from *E. coli* V 517 containing 8 plasmid species ranging from 2.1 to 53.70 Kb size was run simultaneously in each gel. The distance migrated by each

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

3.15 Curing of plasmids

The method of Carlton and Brown (1981) was applied for curing of plasmids by all the following methods.

3.15.1 Curing with ethidium bromide (EB)

On comparison of different concentrations of EB ranging from 20-200 µg/ml, a concentration of 100 µg/ml was found most suitable for curing, as the cultures showed just detectable increase in turbidity at this concentration. From a 6 h old culture, 20 µl was inoculated in to 5 ml of LB broth containing 100 µg/ml of EB.

The cultures were incubated at 37°C for 24 h. A master plate was then prepared by streaking culture on a MacConkey agar plate separately to obtain single isolated colonies.

The master plate was replica plated to a MacConkey agar plate with one of the antibiotics to which the host strain was resistant and incubated at 37°C for 24 h. A colony was considered to be cured when it failed to grow on antibiotic containing MacConkey's medium, but grew on antibiotic free MacConkey's medium.

Similarly the experiment was repeated after 7 days of incubation at 37°C.

3.15.2 Curing with acridine orange AO

The curing with acridine orange was carried out by treating the organisms at the concentration of 100 µg/ml of AO in 5 ml of LB broth at 37°C for 24 h. The curing effect was examined by replica plating.

3.15.3 Curing with sodium dodecyl sulphate (SDS)

The curing with sodium dodecyl sulphate was carried out by culturing the organisms in 5 ml of LB broth containing 10 per cent (100 mg/ml) SDS. The cultures were incubated at 37°C for 3 days. The curing effect was examined by replica plating.

3.15.4 Curing at elevated temperature

Escherichia coli isolates were incubated in 5 ml of LB broth at 45°C for 24 h. The cultures were daily transferred to fresh LB broth and the colonies were examined by replica plating on MacConkey's medium containing antibiotic at an interval of five days. The bacterial isolates which were not cured and those with low percentage of curing were further observed for development of curing.

The frequency of curing (FC) was calculated as

$$FC = \frac{\text{Number of cured colonies}}{\text{Total colonies on master plate}} \times 100$$

3.16 Curing of hemolysin producing plasmids

The curing of hemolysin producing plasmids of *E. coli* in isolates (3) was carried out by different methods described before and the effect of curing was tested on 5 per cent sheep blood agar.

3.17 Curing of enterotoxin producing plasmids

The curing of enterotoxin producing plasmids of *E. coli* isolates (4) was carried out by different methods described before, and the effect of curing was tested by rabbit ligated ileal loop assay.

The cured strains obtained by above methods were further verified for the loss of plasmid(s) by applying plasmid profile studies.

All the "plasmid cured" isolates were checked for the transferability of drug resistance, and hemolysin production by conjugal transfer.

Results

RESULTS

Out of the 130 Gram-negative bacilli isolated from 300 bovine mastitis milk samples, 46 were identified as *E. coli*, based on morphological, cultural and biochemical tests.

4.1 Antibiogram of *E. coli*

The results of antibiotic sensitivity tests of 46 *E. coli* isolates by agar diffusion method, using 13 antibiotic (discs) are presented in Table 1. The percentage of resistance to various antibiotics are furnished in Table 2. The maximum resistance (78.26%) was observed in case of rifampicin followed by oxytetracycline (50.00%), sulphadiazine (36.95%), tetracycline (34.78%), ampicillin (21.73%) chloramphenicol (21.73%) streptomycin (23.91%), nalidixic acid (19.56%), furazolidone (13.04%), co-trimoxazole (8.69%), and gentamicin (6.52%). The organisms were sensitive to kanamycin and norfloxacin.

Among the forty-six *E. coli*, nine (19.56%) were resistant to single antibiotic and 34 (73.91%) were multiple drug resistant (MDR). Among the multiple drug resistant isolates, 11 (23.91%) isolates were double antibiotic resistant, 7 (15.21%) were triple resistant, 2 (4.34%) were

Table 1. Antibiograms of *E. coli* isolated from bovine mastitis

Sl.No.	*E. coli* isolates	Antibiogram*
1.	F_2C48H	ORT
2.	LFB 5192	R
3.	M-11-3	CORST
4.	PM 6	OR
5.	GNI 25491	OR
6.	MSP 7392	OR
7.	FD 22	OR
8.	Nil 24991	R
9.	LFB 4	OR
10.	MSP 7	AORT
11.	E 1-8	R
12.	I-43	ORSuT
13.	LFB 24991	R
14.	G-Ve 2711	R
15.	R 205	RSu
16.	LF 024	OSuT
17.	G-Ve 2811	CRT
18.	LF 524	AFJORSSu
19.	3	R
20.	LFB 623	AORSSu
21.	EC 1	ACSu
22.	320	AT
23.	X	R
24.	H 1	R
25.	HSGNR	ACRSSu
26.	E 3-25	RSu
27.	980	ACORSu

Contd.

Table 1 (Contd.)

Sl.No.	*E. coli* isolates	Antibiogram*
28.	MS-Aloor	RSSu
29.	2	Sensitive to all the drugs used
30.	RLFB	OGRSSuT
31.	LF 5	Sensitive to all the drugs used
32.	MD	R
33.	LFC-24	ACJQRSSuT
34.	LFB5	RSuT
35.	Nil 30892	Sensitive to all the drugs used
36.	GNR 13192	NaO
37.	V6 III	CFNaQRT
38.	G-Ve 301191	NaRT
39.	LFI-25	NaOSSuT
40.	V8	CFNaORT
41.	GNR 131192	ACFNaOQRSuT
42.	E 182	CFNaQS
43.	F 444	NaORSSuT
44.	979	NaO
45.	LF 82	AFJORSSu
46.	GNR 20	OR

* A = ampicillin, C = chloramphenicol, Q = co-trimoxazole, F = furazolidone, J = gentamicin, Na= nalidixic acid, Nf= norfloxacin, O = oxytetracycline, R = rifampicin, S = streptomycin, Su= sulphadiazine, T = tetracycline

Table 2. Percentage of antibiotic resistance of *E. coli*

Sl. No.	Antibacterial agent (µg/disc)		No. of strains	Per cent
1.	Ampicillin	(A,10)	10	(21.73)
2.	Chloramphenicol	(C,30)	10	(21.73)
3.	Co-trimoxazole	(Q,25)	4	(8.69)
4.	Furazolidone	(F,100)	6	(13.04)
5.	Gentamicin	(J,10)	3	(6.52)
6.	Kanamycin	(K,30)	0	(0)
7.	Nalidixic Acid	(Na,30)	9	(19.56)
8.	Norfloxacin	(Nf,10)	0	(0)
9.	Oxytetracycline	(O,30)	23	(50.00)
10.	Rifampicin	(R,5)	36	(78.26)
11.	Streptomycin	(S,10)	11	(23.91)
12.	Sulphadiazine	(Su,300)	17	(36.95)
13.	Tetracycline	(T,30)	16	(34.78)

quadruple resistant, 6 (13.04%) were quintuple resistant, 4 (8.69%) were sextuple resistant and 2 (4.34%) were septuple, resistant, and 1 (2.17%) each was octuple and nonuple resistant. The drug wise patterns of antibiotic resistance are furnished in Table 3. Three isolates (6.52%) were sensitive to all the antibiotics tested whereas 43 (93.47%) isolates were resistant to one or more antibiotics. Twenty-six different patterns of antibiotic resistances ranging from one to nine antibiotics were observed among the 43 isolates.

4.2 Resistogram of *E. coli*

The resistograms of 46 *E. coli* isolates are presented in Table 4. The maximum degree of resistance (100%) was noticed against lead ions, followed by antimony (32.60%), copper (30.43%) and silver (19.56%). Only one (2.17%) out of forty-six isolates was found to be resistant to cetyl trimethyl ammonium bromide (cetrimide). None of the isolates was resistant to cadmium and mercury ions. The details of percentage of heavy metal resistance are furnished in Table 5.

Nine different patterns of heavy metal resistance were recognized among 46 *E. coli* isolates. The details are presented in Table 6. The most common resistance was noticed against lead oxide alone (E-41.3%), followed by multiple resistance against lead and antimony (EF-15.21%); copper and

Table 3. Antibiotic resistance patterns of *E. coli*

Sl. No.	Antibiotic resistance pattern	Number of isolates (%) (n = 46)
1.	R	9 (19.56)
2.	OR	6 (13.04)
3.	RSu	2 (4.34)
4.	NaO	2 (4.34)
5.	AFJORSSu	2 (4.34)
6.	CFNaORT	2 (4.34)
7.	ORT	1 (2.17)
8.	CORST	1 (2.17)
9.	AORT	1 (2.17)
10.	ORSuT	1 (2.17)
11.	OSuT	1 (2.17)
12.	CRT	1 (2.17)
13.	AORSSu	1 (2.17)
14.	ACSu	1 (2.17)
15.	AT	1 (2.17)
16.	ACRSSu	1 (2.17)
17.	ACORSu	1 (2.17)
18.	RSSu	1 (2.17)
19.	OQRSSuT	1 (2.17)
20.	ACJQRSSuT	1 (2.17)
21.	RSuT	1 (2.17)
22.	NaRT	1 (2.17)
23.	NaOSSuT	1 (2.17)
24.	ACFNaOQRSuT	1 (2.17)
25.	CFNaQS	1 (2.17)
26.	NaORSSuT	1 (2.17)
27.	Sensitive*	3 (6.52)

* Sensitive to all the antibiotics used

Table 4. Resistograms of E. coli isolated from bovine mastitis

Sl.No.	E. coli isolates	Resistogram
1.	F_2C48H	ABD
2.	LFB 5192	AEF
3.	M-11-3	AEFST
4.	PM 6	AEF
5.	GNI 25491	AEF
6.	MSP 7392	E
7.	FD 22	E
8.	Nil 24991	BE
9.	LFB 4	EF
10.	MSP 7	E
11.	E 1-8	AE
12.	I-43	AEF
13.	LFB 24991	E
14.	G-Ve 2711	EF
15.	R 205	ABE
16.	LF 024	AE
17.	G-Ve 2811	E
18.	LF 524	AE
19.	3	E
20.	LFB 623	E
21.	EC 1	E
22.	320	E
23.	X	EF
24.	H 1	E
25.	HSGNR	AE
26.	E 3-25	E
27.	980	BE

Contd.

Table 4 (Contd.)

Sl.No.	*E. coli* isolates	Resistogram
28.	MS-Aloor	E
29.	2	E
30.	RLFB	AE
31.	LF 5	E
32.	MD	E
33.	LFC-24	BEF
34.	LFB5	EF
35.	Nil 30892	BEF
36.	GNR 13192	ANEF
37.	V6 III	EF
38.	G-Ve 301191	E
39.	LFI-25	E
40.	V8	ABE
41.	GNR 131192	EFG
42.	E 182	EF
43.	F 444	E
44.	979	BE
45.	LF 82	AE
46.	GNR 20	E

* A = copper sulphate, B = silver nitrate

C = cadmium chloride D = mercuric chloride

E = lead oxide F = antimony chloride

G = cetyl trimethyl ammonium bromide (cetrimide)

Table 5. Percentage of heavy metal resistance of *E. coli*

Sl. No.	Heavy metal salts	Symbol	No. of strains	Per cent
1.	Copper sulphate ($CuSO_4$)	A	14	(30.43)
2.	Silver nitrate ($AgNO_3$)	B	9	(19.56)
3.	Cadmium chloride ($CdCl_3$)	C	0	(0)
4.	Mercuric chloride ($HgCl_2$)	D	0	(0)
5.	Lead oxide (Pb_3O_4)	E	46	(100)
6.	Antimony chloride ($SbCl_3$)	F	15	(32.60)
7.	Cetyl trimethyl ammonium bromide (cetrimide) (C19H42NBr)	G	1	(2.17)

Table 6. Heavy metal resistance patterns of *E. coli*

Sl. No.	Heavy metal resistance patterns	No.of isolates (n = 46)	Per cent
1.	E	19	(41.30)
2.	EF	7	(15.21)
3.	AE	6	(13.04)
4.	AEF	4	(8.69)
5.	ABE	3	(6.52)
6.	BE	3	(6.52)
7.	BEF	2	(4.34)
8.	ABEF	1	(2.17)
9.	EFG	1	(2.17)

Fig.1 Dilatation of rabbit ligated ileal loop noticed 24 h after inoculation of cell-free filtrate of *E. coli*. Loops, 1, 2, 3, 4 positive; 5-negative and 6-broth control

lead (AE-13.04%), copper, lead and antimony (AEF-8.69%), copper, silver and lead (ABE-4.14%), silver and lead (BE-6.52%), silver, lead and antimony (BEF-4.34%), copper, silver, lead and antimony (ABEF-2.17%) and lead, antimony and cetrimide (EFG-2.17%).

4.3 Correlation between antibiotic and heavy metal resistance

Of the 43 antibiotic resistant isolates, 36, 23, 17, 16, 11, 10, 10, 9, 6, 4 and 3 isolates were resistant to R, O, Su, T, S, A, C, Na, F, Q and J respectively making 145 individual resistances (Table 7). Among these lead revealed (145/145) 100 per cent resistance, copper (54/145) 37.24 per cent, antimony (50/145) 34.48 per cent, silver (30/145) 20.6 per cent and cetrimide (9/145) 6.2 per cent.

The rifampicin resistant isolates revealed 33.33 per cent correlation between copper (Cu) and antimony (Sb). The percentage of correlation between other antibiotics and heavy metal ions was as follows: O, resistance, 43.77 and 30.43 with Cu and Sb, respectively; Su resistance, 41.17 and 23.52 with Cu and Sb respectively; T resistance 37.5 each with Cu and Sb; St resistance 45.45 with Cu and 27.27 with Sb; A resistance, 30 with Cu and 20 with Sb and Silver (Ag); C resistance 50 with Sb and 30 each with Cu and Ag; Na resistance 44.44 with Sb and 33.33 with Ag; F resistance 50

Table 7. Correlation between antibiotic and metal ion resistance in *E. coli*

Antibiotic	Number of resistant isolates	Number resistant to metal ions						
		Cu	Ag	Cd	Hg	Pb	Sb	$C_{19}H_{42}NBr$
	(43)	(14)	(9)	(0)	(0)	(46)	(15)	(1)
Ampicillin	10	3	2	0	0	10	2	1
Chloromphenical	10	3	3	0	0	10	5	1
Co-trimoxazole	4	1	1	0	0	4	3	1
Furazolidone	6	3	1	0	0	6	3	1
Gentamicin	3	2	1	0	0	3	1	0
Kanamicin	0	0	0	0	0	0	0	0
Nalidixic acid	9	2	3	0	0	9	4	1
Norfloxacin	0	0	0	0	0	0	0	0
Oxytetracycline	23	10	6	0	0	23	7	1
Rifampicin	36	12	6	0	0	36	12	1
Streptomycin	11	5	1	0	0	11	3	0
Sulphadiazine	17	7	3	0	0	17	4	1
Tetracycline	16	6	3	0	0	16	6	1
Total number of resistances	145	54	30	0	0	145	50	9

with Sb and 25 with Cu, Ag and cetrimide; Q resistance, 25 with Cu and Ag and 75 with Sb; and J resistance 66.66 with Cu and 33.33 with Sb and Ag.

4.4 Hemolysin production

Out of the 46 isolates of E. coli. tested for hemolysin production, 3 (6.52 %) were found to produce α-hemolysin on 5 per cent sheep blood agar. Of these three (MSP-7, 980, MS-Aloor) two (MS-Aloor and 980) were enterotoxin producers (Table 8).

4.5 Enterotoxin production

All the 46 E. coli isolates were tested for the production of heat-labile enterotoxin (LT), using rabbit ligated ileal loop assay. In rabbits inoculated with cell-free filtrates of E. coli isolates, in ligated intestinal loops, petechial haemorrhages congestion and dilatation were observed in some of the ligated loops, due to the accumulation of fluid, whereas other loops did not show such changes (Fig.1). The E. coli isolates which caused dilatation reactions were considered as enterotoxin producers whereas those which did not cause any dilatation reaction were considered negative for enterotoxin production.

Fig.1 Dilatation of rabbit ligated ileal loop noticed 24 h after inoculation of cell-free filtrate of *E. coli*. Loops, 1, 2, 3, 4 positive; 5-negative and 6-broth control

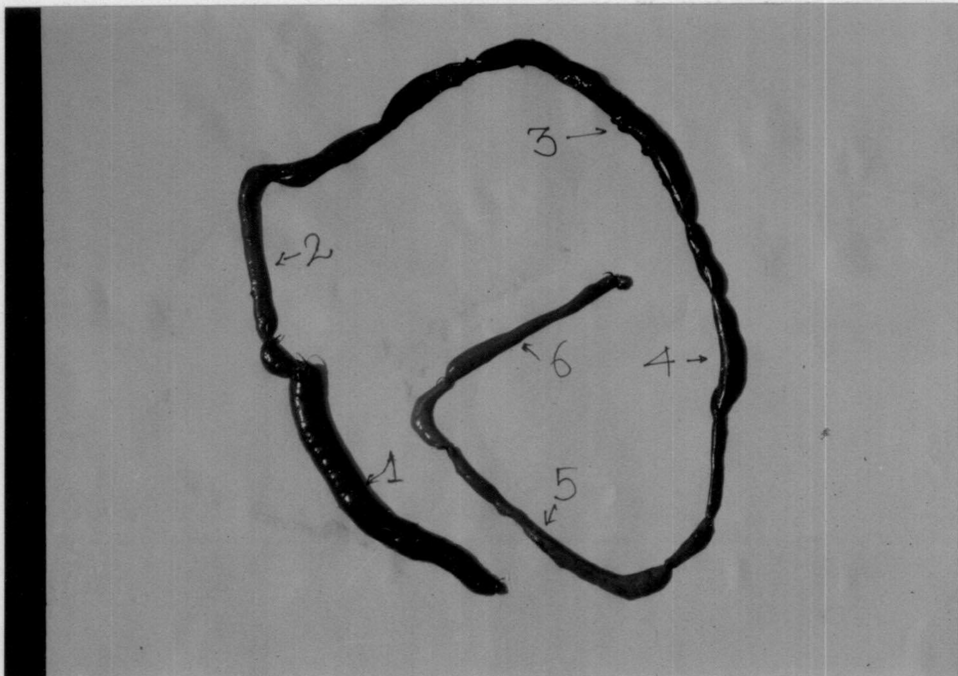

3
2
6
4
1
5

Table 8. Hemolytic and enterotoxigenic *E. coli* from bovine mastitis

Sl.No.	*E. coli* isolates	Enterotoxin	Hemolysin
1.	F_2C48H	-	-
2.	LFB 5192	-	-
3.	M-11-3	-	-
4.	PM 6	-	-
5.	GNI 25491	+	-
6.	MSP 7392	-	-
7.	FD 22	+	-
8.	Nil 24991	+	-
9.	LFB 4	-	-
10.	MSP 7	-	+
11.	E 1-8	-	-
12.	I-43	-	-
13.	LFB 24991	-	-
14.	G-Ve 2711	-	-
15.	R 205	-	-
16.	LF 024	-	-
17.	G-Ve 2811	+	-
18.	LF 524	-	-
19.	3	+	-
20.	LFB 623	-	-
21.	EC 1	-	-
22.	320	-	-
23.	X	-	-
24.	H 1	-	-
25.	HSGNR	-	-
26.	E 3-25	+	-
27.	980	+	+

Contd.

Table 8 (Contd.)

Sl.No.	*E. coli* isolates	Enterotoxin	Hemolysin
28.	MS-Aloor	+	+
29.	2	+	-
30.	RLFB	+	-
31.	LF 5	-	-
32.	MD	-	-
33.	LFC-24	-	-
34.	LFB5	-	-
35.	Nil 30892	-	-
36.	GNR 13192	-	-
37.	V6 III	-	-
38.	G-Ve 301191	-	-
39.	LFI-25	-	-
40.	V8	-	-
41.	GNR 131192	+	-
42.	E 182	+	-
43.	F 444	-	-
44.	979	-	-
45.	LF 82	-	-
46.	GNR 20	+	-

Of the 46 *E. coli* isolates tested 13 (28.26%) were found to be enterotoxigenic (Table 8). A dilatation index of 0.4 and above was taken as positive for enterotoxigenicity. The dilatation indices of various enterotoxigenic (*Ent*) isolates are furnished in Table 9. Out of the 13 *Ent E. coli* 2 (15.38%) were hemolysin producing. The antibiogram, resistogram, hemolysin producing character of 13 enterotoxigenic *E. coli* are presented in Table 10.

4.6 *In vitro* transfer of plasmids by conjugation

4.6.1 Transfer of antibiotic resistance (R-plasmids)

Out of the 46 *E. coli* isolates 9 (19.56%) were nalidixic acid resistant, hence these isolates were excluded from the transfer studies of antibiotic resistance, because the recipient *E. coli* K 12 ML1410 had nalidixic acid resistance as a marker. Three *E. coli* isolates (6.52%) were sensitive to all the thirteen drugs used in the study.

The conjugal transfer of antibiotic resistance by broth mating was attempted with 25 *E. coli* isolates. Of these, 24 isolates were resistant to one or more antibiotics and one was sensitive to all the drugs used but postive for enterotoxin production. Fourteen out of 24 (58.33%) isolates could transfer the resistance for one or more drugs to the recipient *E. coli* K 12 ML1410. The isolates transferred the

Table 9. Dilatation indices of enterotoxigenic *E. coli*

Sl. No.	*E. coli* isolates	Length of loop (cm)	fluid accumulated (ml)	Dilatation index
1.	GNI-25491	10	26	2.6
2.	FD-22	10	35	3.5
3.	Nil 24991	10	10	1.0
4.	G-ve 2811	10	8	0.8
5.	3	10	15	1.5
6.	E3-25	10	15	1.5
7.	980	10	28	2.8
8.	MS-Aloor	10	28	2.8
9.	2	10	30	3.0
10.	RLFB	10	12	1.2
11.	GNR-13112	10	12	1.2
12.	E 182	10	35	3.5
13.	GNR 20	10	23	2.3

Table 10. Various characters of enterotoxigenic *E. coli*

Sl. No.	*E. coli* isolates	Antibiogram	Resisto gram	Hemo lysin	Entero-toxin
1.	GNI 25491	OR	E	-	+
2.	FD 22	OR	BE	-	+
3.	Nil 24991	R	EF	-	+
4.	G-ve 2811	CRT	E	-	+
5.	3	R	E	-	+
6.	E 3-25	RSu	E	-	+
7.	980	ACORSu	BE	+	+
8.	MS-Aloor	RSSu	E	+	+
9.	2	Sensitive to all drugs used	E	-	+
10.	RLFB	OQRSSuT	AE	-	+
11.	GNR 131192	ACFNaOQRSuT	EFG	-	+
12.	E 182	CFNaQS	EF	-	+
13.	GNR 20	OR	E	-	+

A, C, O, Q, S and Su resistance, either singly or in combination. The details of antibiotic resistance and their transfer are furnished in Table 11.

4.6.2 Transfer of enterotoxigenic (Ent) plasmids

Two of the 13 enterotoxigenic isolates were nalidixic acid resistant, hence could not be tried for the conjugal transfer of the enterotoxigenicity. None of the remaining 11 E. coli isolates could transfer the enterotoxin producing plasmids (Ent) by conjugation (Table 11, Fig.2).

4.6.3 Transfer of hemolysin producing (Hly) plasmids

All the three hemolytic E. coli isolates were tried for the conjugal transfer of hemolysin producing plasmid (Hly). The transconjugants obtained after mating of donor (hemolytic isolates) and recipient E. coli K 12 ML 1410 (F,Na^r), when cultured on 5 per cent sheep blood agar revealed the presence of α-hemolysin producing colonies, after incubation at 37°C for 24 h. The details of transfer of hemolytic character are presented in Table 12.

4.7 Plasmid profile analysis

Forty E. coli isolates were processed for studying their plasmid profiles. The plasmids separated by alkali lysis method were subjected to electrophoresis in agarose gel.

Fig.2 Rabbit ligated ileal loops (I-IV) showing no dilatation even after 24 h of inoculation of cell-free filtrate of transconjugants, obtained from enterotoxigenic *E. coli*. Loop V-broth control

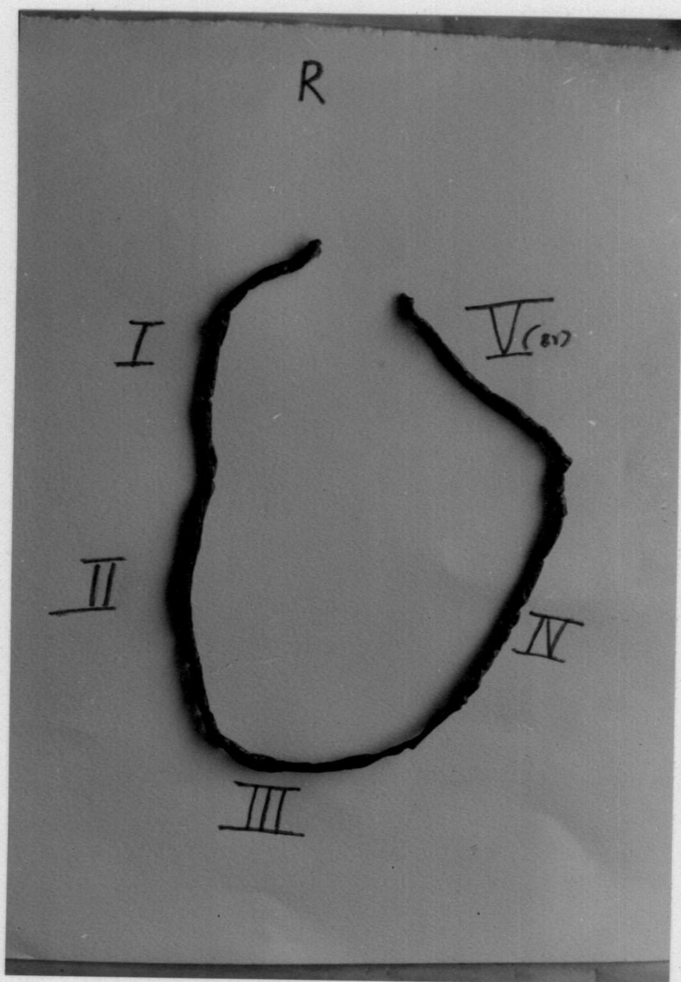

R
I
II
III
IV
V

Table 11. *In vitro* transfer of R-factor, *Hly* and *Ent* by conjugation

Sl. No.	*E. coli* isolates	Resistance tried by conjugation	Resistance transferred to *E. coli* K-12 ML1410	Enterotoxigenicity (*Ent*)	Enterotoxigenicity transferred	Hemolysin (*Hly*)	Hemolysin transferred
1	2	3	4	5	6	7	8
1.	E3-25	Su	Su	+	–	–	–
2.	3	Su	Su	+	–	–	–
3.	FD-22	O	O	+	–	–	–
4.	GNI 25491	O	–	+	–	–	–
5.	G-ve (28-11)	C	–	+	–	–	–
6.	RLFB	CQRSSu	SSu	+	–	–	–
7.	Nil 24991	R	–	+	–	–	–
8.	2	Sensitive*	–	+	–	–	–
9.	GNR 20	O	–	+	–	–	–
10.	980	COSu	O	+	–	+	+
11.	MS-Aloor	SSu	Su	+	–	+	+
12.	MSP-7	AO	–	–	–	+	+

Contd.

Table 11. *In vitro* transfer of R-factor, *Hly* and *Ent* by conjugation

Sl. No.	*E. coli* isolates	Resistance tried by conjugation	Resistance transferred to *E. coli* K-12 ML1410	Enterotoxigenicity (*Ent*)	Enterotoxigenicity transferred	Hemolysin (*Hly*)	Hemolysin transferred
1	2	3	4	5	6	7	8
1.	E3-25	Su	Su	+	-	-	-
2.	3	Su	Su	+	-	-	-
3.	FD-22	O	O	+	-	-	-
4.	GNI 25491	O	-	+	-	-	-
5.	G-ve (28-11)	C	-	+	-	-	-
6.	RLFB	OQRSSu	SSu	+	-	-	-
7.	Nil 24991	R	-	+	-	-	-
8.	2	Sensitive*	-	+	-	-	-
9.	GNR 20	O	-	+	-	-	-
10.	980	COSu	O	+	-	+	+
11.	MS-Aloor	SSu	Su	+	-	+	+
12.	MSP-7	AO	-	-	-	+	+

Contd.

Table 11 (Contd.)

1	2	3	4	5	6	7	8
13.	LFB 24991	R	–	–	–	–	–
14.	I-43	OSu	Su	–	–	–	–
15.	HSGNR	ASu	Su	–	–	–	–
16.	M-11-3	COS	COS	–	–	–	–
17.	LFB-524	FSSu	–	–	–	–	–
18.	320	A	A	–	–	–	–
19.	LF 623	AQSSu	–	–	–	–	–
20.	PM 6	OR	O	–	–	–	–
21.	LF024	OSu	OSu	–	–	–	–
22.	LFB 5192	R	–	–	–	–	–
23.	LFB 5	SuT	–	–	–	–	–
24.	LF C-24	ACJQSuT	AQSu	–	–	–	–
25.	R205	Su	Su	–	–	–	–

* Sensitive to all the drugs used

Table 12. Conjugal transfer of hemolysin producing plasmids

Sl. No.	*E. coli* isolates	Character possessed	Character transferred	Frequency of *Hly* transfer	Per cent
1.	980	*Hly*	*Hly*	182/274*	66.42
2.	MS-Aloor	*Hly*	*Hly*	136/258	52.71
3.	MSP-7	*Hly*	*Hly*	240/290	82.75

* Number of hemolytic colonies by total number (hemolytic and nonhemolytic) of colonies

The agarose gel electrophoresis revealed the presence of plasmids ranging from 1 to 5, in various isolates.

The molecular sizes in kilobase (Kb) were calculated by plotting the values of distance migrated in cm by plasmid DNA fragments, versus the log 10 Kb values of the molecular sizes of corresponding plasmids obtained from E. coli V517, the molecular weight reference plasmid strain. The standard linear curve was obtained on the graph paper (Fig.3). The molecular sizes of the plasmids of E. coli isolates under study were determined by interpolation of standard linear curve from the values of distance migrated in centimetre, by the plasmid DNA fragments. The log value so obtained after interpolation on Y axis (log 10 Kb) was converted into antilogarithmic value, so as to estimate the molecular size in kilobase, of the particular plasmid DNAs. The details are given in Table 13 and Fig.4.

The molecular sizes of the plasmids from the forty E. coli isolates and their plasmid profiles are listed in Table 14. Thirty-eight of the strains were carrying one to five plasmids of different molecular sizes ranging from 1.47 to 128.8 Kb (Fig.5). Two E. coli isolates were without any plasmid and these two isolates were negative for enterotoxin and hemolysin production. Altogether 35 different patterns of plasmid profiles were obtained. With reference to molecular

Table 13. Estimation of molecular size of plasmid DNA of *E. coli*

Plasmid DNA sample of *E. coli*	Number of plasmids	Distance migrated in cm X	Log 10 Kb Y	Antilog 10 Kb (Mol size in Kb)
HSGNR	1	6.2	0.73	5.370
MSP-7392	1	4.6	0.83	6.761
	2	6.2	0.73	5.370
V 8	1	2.3	2.05	112.200
	2	4.8	0.81	6.457
	3	7.2	0.70	5.010
	4	9.2	0.51	3.236
E. coli	1	2.8	1.7300	53.7
	2	4.2	0.8573	7.2
	3	5.8	0.7482	5.6
	4	7.3	0.7076	5.1
	5	8.2	0.5911	3.9
	6	9.6	0.4771	3.0
	7	10.5	0.4314	2.7
	8	11.2	0.3222	2.1

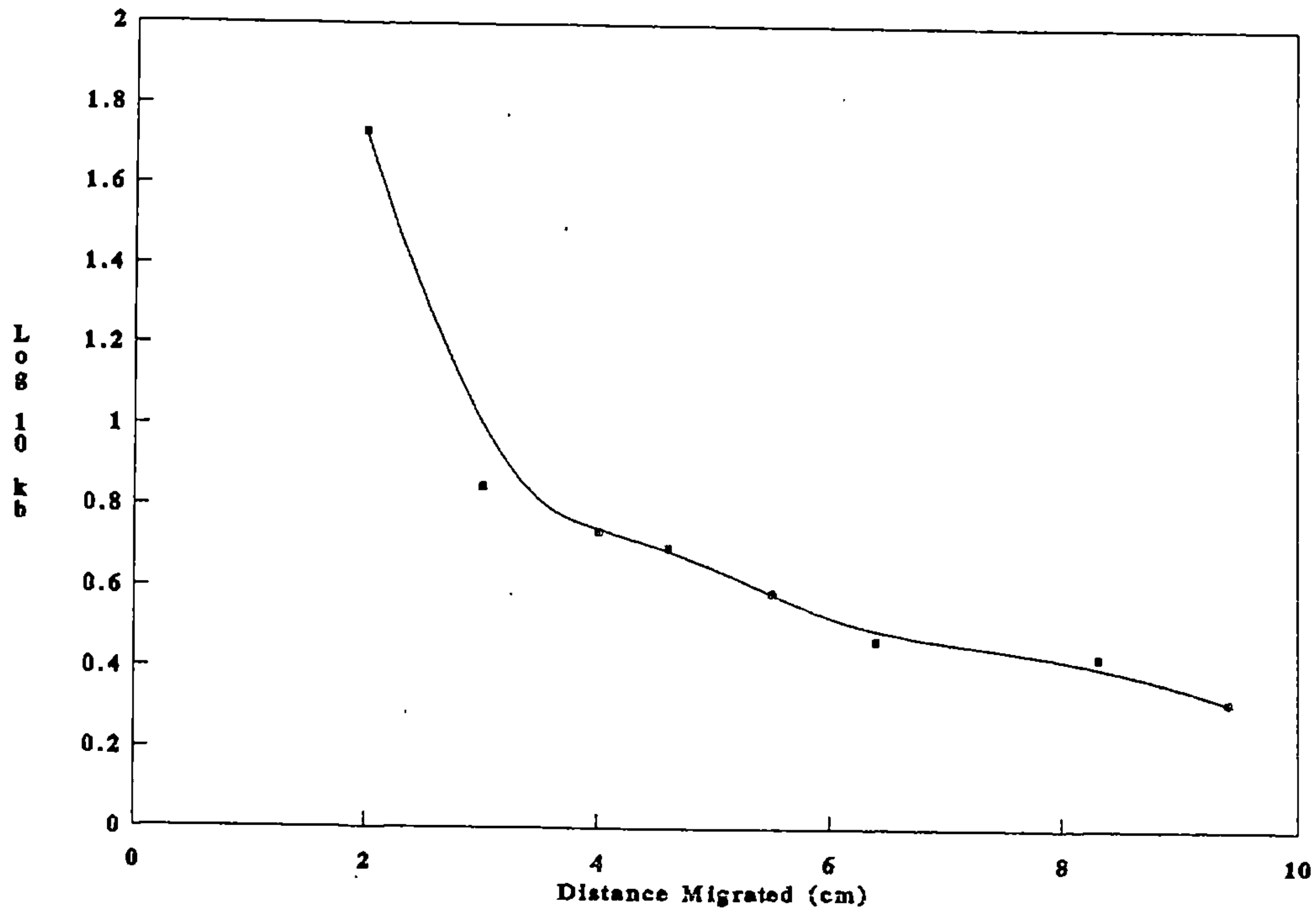


FIG 3 MOLECULAR SIZE vs MIGRATION
OF E.COLI V517 PLASMID DNA
Log 10 kb
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Distance Migrated (cm)

FIG.4 ESTIMATION OF MOLECULAR SIZE OF E.COLI PLASMID DNA

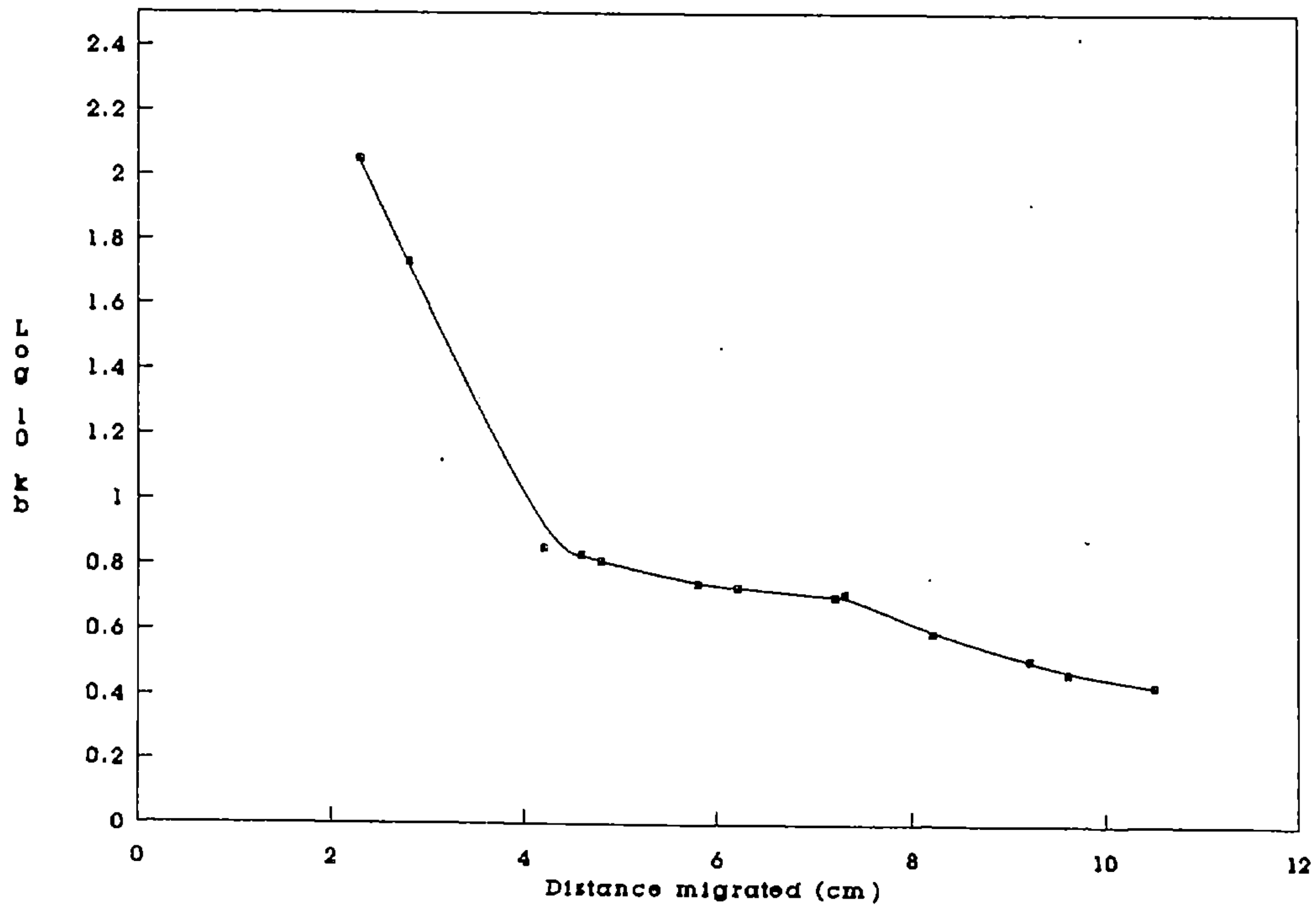
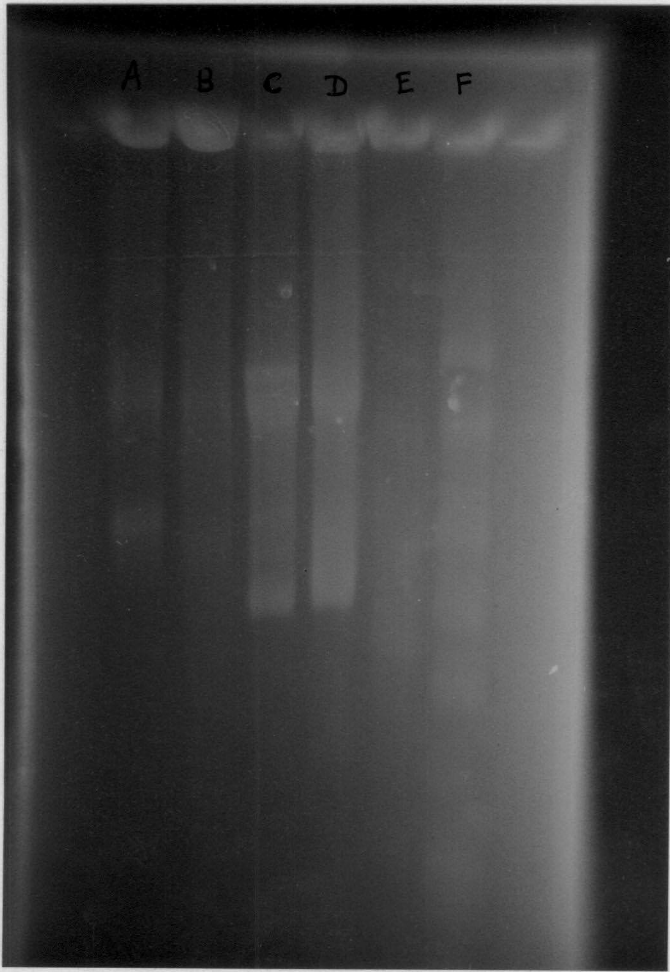


Fig.5. Plasmid profiles of wild *E. coli* strains

A-B *E. coli* EC 1 with 2 plasmids 7.24 and 5.62 Kb

C-D *E. coli* 320 with 2 plasmids 7.24 and 5.24 Kb

E-F *E. coli* V517 (molecular size marker) with 53.7, 7.2, 5.6, 5.1, 3.9, 3.0 and 2.7 Kb


A B C D E F

size, no plasmids common to all the isolates could be observed. Only three plasmid profile patterns contained two isolates each, such as plasmid profile 6.02 Kb was common for isolates GNI 25491 and GNR 20, while H1 and MD isolates had 5.60 and 5.70 Kb plasmids and LFB 524 and LF 82 had 10.00 Kb plasmid in common (Table 14). Out of the 38 profiles 20 had small plasmids <30 Kb, seven had only large and 11 had large and small plasmids.

Association between the virulence factors and the plasmids harboured by enterotoxigenic and hemolytic isolates is depicted in Table 15. Plasmid profiles of 3 hemolytic strains, of which 2 (980 and MS Aloor) were also enterotoxigenic, comprised of plasmid DNAs of various molecular sizes. E. coli 980 possessed a plasmid profile containing 5 plasmids of 107.2, 14.13, 5.37, 3.89 and 3.54 Kb; and isolate MS-Aloor, harboured a plasmid profile containing 3 plasmids of 67.61, 5.49 and 4.07 Kb. Another isolate MSP-7 which was hemolytic only, possessed a plasmid profile containing 3 plasmids of 63.10, 17.78 and 6.78 Kb. All the hemolytic E. coli were multiple resistant.

Most of the enterotoxigenic (Ent) E. coli isolates possessed low molecular size plasmids. Seven of the 13 isolates harboured only small plasmids of molecular size less than 41 Kb, whereas remaining six isolates possessed large as

Table 14. Plasmid profiles of *E. coli* isolated from bovine mastitis

Sl. No.	*E. coli* isolate number	Antibiogram	Resistogram	Molecular size of plasmids in Kb
1.	F2C48H	ORT	ABE	None
2.	LFB 5192	R	AEF	15.14
3.	M-11-3	CORST	AEF	128.8, 45.71
4.	PM 6	OR	AEF	107.2, 12.59
5.	GNI 25491	OR	E	6.02
6.	MSP 7392	OR	E	6.76, 5.37
7.	FD 22	OR	BE	6.31, 5.01
8.	Nil 24991	R	EF	7.08
9.	LFBH	OR	EF	None
10.	MSP-7	AORT	E	63.10, 17.78, 6.76
11.	I-43	ORSuT	AEF	53.70
12.	R 205	RSu	ABE	5.37
13.	LF 024	OSuT	AE	120.2, 93.33, 45.71
14.	G-ve 2811	CRT	E	6.17, 2.82
15.	LFB 524	AFJORSSu	AE	10.00
16.	3	R	E	40.74
17.	LFB 623	AORSSu	E	5.89
18.	EC 1	ACSu	E	7.24, 5.62
19.	320	AT	E	7.24, 5.25
20.	X	R	EF	50.12
21.	H 1	R	E	5.60, 5.10
22.	HSGNR	ACRSSu	AE	5.37
23.	E 3-25	RSu	E	51.85, 13.18
24.	980	ACORSu	BE	107.2, 14.13, 5.37, 3.89, 3.55

Contd.

Table 14 (Contd.)

Sl. No.	*E. coli* isolate number	Antibiogram	Resistogram	Molecular size of plasmids in Kb
25.	MS-Aloor	RSSu	E	67.61, 5.50, 4.07
26.	2	Sensitive to all drugs used	E	5.75
27.	RLFB	OQRSSuT	AE	79.43, 5.01, 1,95, 1.48
28.	MD	R	E	5.60, 5.10
29.	LFC 24	ACJQRSSuT	BEF	45, 5.37
30.	LFB 5	RSuT	EF	65, 5.01
31.	GNR 13192	NaO	ABEF	70.79, 26.30
32.	V 6 III	CFNaORT	EF	14.13
33.	LFI 25	NaOSSuT	E	53.70, 6.31
34.	V 8	CFNaORT	ABE	112.20, 6.46, 5.01, 3.24
35.	GNR 131192	ACFNaOQRSuT	EFG	114.80, 72.44
36.	E 182	CF NaQS	EF	87.10, 46.77
37.	F 444	NaORSSuT	E	7.08, 5.89
38.	979	NaO	BE	6.77
39.	LF 82	AFJORSSu	AE	10.00
40.	GNR 20	OR	E	6.03

Table 15. Plasmid profiles of virulent *E. coli*

Sl. No.	*E. coli* isolates	Antibiogram	Resistogram	Hemolysin	Enterotoxin	Mol. size (Kb)
1.	GNI 25491	OR	E	-	+	6.02
2.	FD 22	OR	BE	-	+	6.31, 5.01
3.	Nil 24991	R	EF	-	+	7.07
4.	G-ve 2811	CRT	E	-	+	6.16, 28
5.	3	R	E	-	+	40.74
6.	E3-25	RSu	E	-	+	51.85, 13.18
7.	980	ACORSu	BE	+	+	107.2,14.13, 5.37, 3.89, 3.54
8.	MS-Aloor	RSSu	E	+	+	67.61, 5.49, 4.07
9.	2	Sensitive to all drugs	E	-	+	5.75
10.	RLFB	OQRSSuT	AE	-	+	79.43, 5.01, 1.95, 1.47
11.	GNR131192	ACFNaOQRSuT	EFG	-	+	114.8, 72.44
12.	E 182	CFNaQS	EF	-	+	87.10, 46.77
13.	GNR 20	OR	E	-	+	6.02
14.	MSP 7	AORT	E	+	-	63.10, 17.78 6.76

well as small plasmids. One isolate, E. coli 2, which was sensitive to all the drugs tested and non-hemolytic but enterotoxigenic harboured only a small plasmid of 5.7 Kb. Two (980 and MS-Aloor) Hly^+, and Ent^+ E. coli strains used in the present study were multiple resistant and possessed more number of plasmids including high molecular size plasmids (Table 15).

4.7.1 Plasmid profiles of transconjugants

Transfer of antibiotic resistance was observed in 14 out of 24 (58.33%) E. coli isolates. The resistances transferred were A, C, O, Q, S, and Su, either singly or in combination.

None of the 11 enterotoxin producing strains of E. coli tested could transfer the property to recipient organisms, although the transfer of antibiotic resistance was noticed in these isolates. The transfer of hemolytic character (Hly) was noticed in all the three isolates (Table 12).

The study of the plasmid profiles of nine transconjugants was carried out, the results are furnished in Table 16. The E. coli M-11-3 harboured two plasmids of 128.8 and 45.71 Kb and resistance to COS was found transferred by conjugation. The analysis of the plasmid profile of the

transconjugant indicated that both the plasmids 128.8 and 45.77 Kb were transferred. Escherichia coli PM-6 possessed two plasmids (107.2 and 12.59 Kb), of which a plasmid of 107.2 Kb alongwith oxytetracycline resistance was transferred to the transconjugant. The isolate I-43 had only one plasmid of 53.7 Kb which was transferred to transconjugant alongwith sulphadiazine resistance.

Isolate 980 carried five plasmids ranging from 107.2 Kb to 3.54 Kb. However, only one large plasmid of 107.2 Kb was transferred along with the expression of hemolytic character and oxytetracycline resistance. Similarly E. coli MS-Aloor transferred 67.61 Kb plasmid along with hemolytic activity and Su resistance, whereas E. coli MSP-7 transferred 63.10 Kb plasmid along with Hly character. E. coli RLFB-transferred a 79.43 Kb plasmid along with S and Su resistance. A large plasmid of 51.85 Kb was found to be transferred with Su resistance in case of isolate E3-25. In E. coli LF024 having three plasmids of 120.2, 93.33 and 45.75 Kb, the O and Su resistances were transferred along with 93.33 and 45.71 Kb plasmids (Table 16, Fig.6).

The recipient E. coli K 12 ML1410 did not show any plasmid.

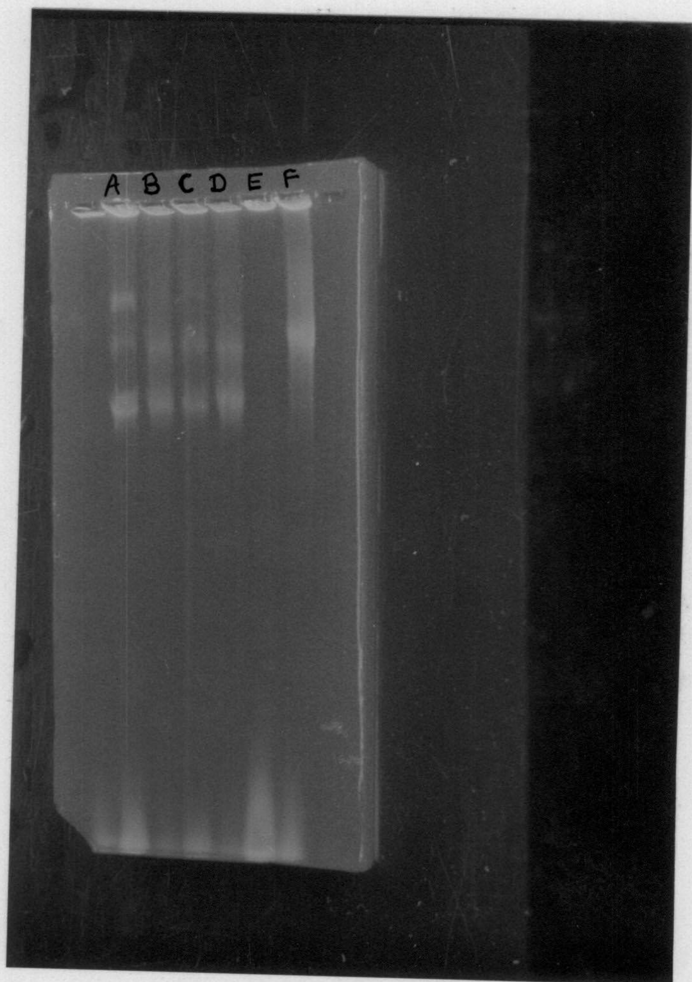
Fig.6. *E. coli* LF024, with

A Three plasmids 120.2, 93.33 and 45.71 Kb

BCD Transconjugant of LF024 with 2 plasmids, 93.33 and 45.71 Kb

E Recipient strain *E. coli* K 12 ML 1410 without any plasmid

F *E. coli* V517


A B C D E F

170411

Table 16. Plasmid profiles of wild strains and their transconjugants

Sl. No.	*E. coli* isolate	Plasmid Mol.size of Donor *E. coli* (Kb)	Plasmid Mol.size of transconjugant (Kb)
1.	M 11-3	128.8, 45.71	128.8, 45.71
2.	PM-6	107.2, 12.59	107.2
3.	I-43	53.7	53.7
4.	980	107.2, 14.13, 5.37 3.89, 3.54	107.2
5.	MS-Aloor	67.61, 5.49, 4.07	67.61
6.	MSP-7	63.10, 17.78, 6.76	63.10
7.	RLFB	79.43, 5.01, 1.95, 1.47	79.43
8.	E 3-25	51.85, 13.18	51.85
9.	LFO-24	120.2, 93.33, 45.71	93.33, 45.71

4.8 Curing of plasmids

4.8.1 Curing of antibiotic resistance

4.8.1.1 Curing with ethidium bromide (EB)

Eleven E. coli isolates were inoculated into LB broth containing 100 µg/ml EB and incubated at 37°C for 1-7 days. The observations were taken at 24 h and at 168 h. The colonies on master plate were replicated on the MacConkey's agar containing the antibiotic against which the curing effect was to be studied (Fig.7 and 8). Ethidium bromide did not cure the plasmids of E. coli FD-22 and M-11-3 as they continued to possess resistance even after curing.

The effect of curing by EB is furnished in Table 17. There was substantial increase in the frequency of curing due to 7 days exposure to EB as compared to 1 day. It was noticed that in E. coli 980, ACOSu; MSP-7, AO; RLFB, OS, LFB 5, Su; LFC-24, J; 979, Na and V6 III, Na resistance were cured at higher frequency after seven days than one day incubation with EB. In certain cases there was no curing after one day exposure but showed significant curing after 7 days exposure as in E. coli RLFB, Q and Su; MS-Aloor, SSu; and LFB 524, J resistance. In certain cases there was no curing even after 7 days exposure to EB (FD 22, O; M-11-3, COS, LFB-524, F and V6 III, F resistance).

Fig.7 A replica plate (R) showing curing of ampicillin resistance of *E. coli*. Note the elimination of colonies on replica plate in comparison with master plate (L)

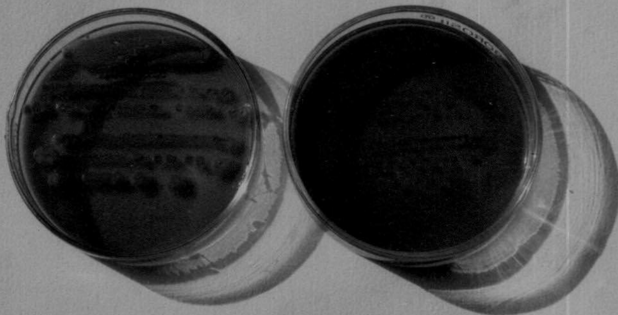

L
R

Fig.8 A replica plate (R) showing curing of oxytetracycline resistance of *E. coli*. Note the elimination of colonies on replica plate in comparison with master plate (L).

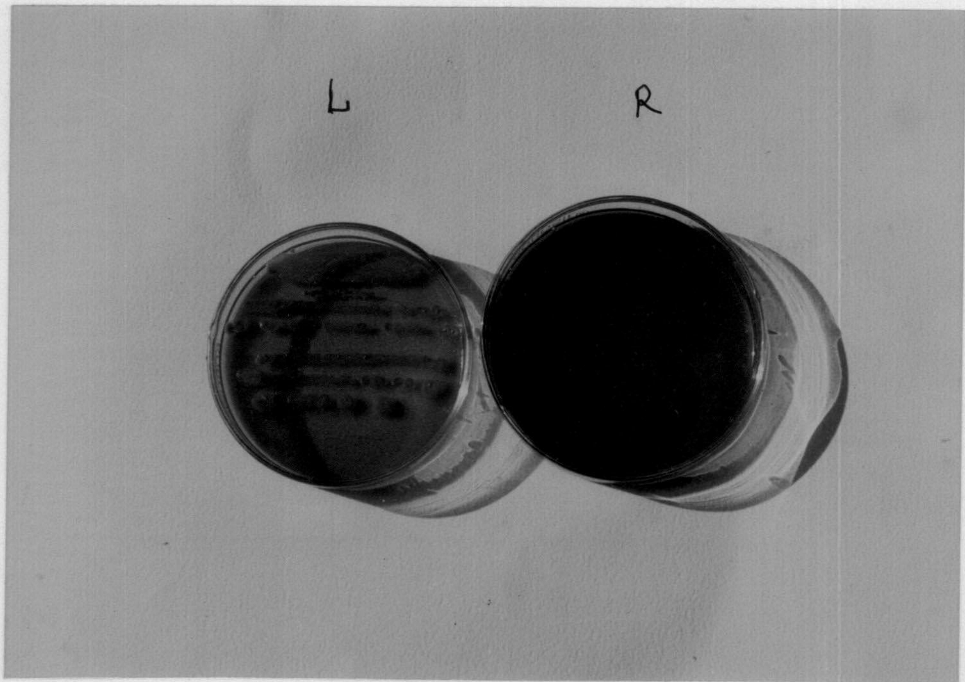

L
R

Table 17. Frequency of curing of plasmids in _E. coli_ by chemical agents

Sl. No.	_E. coli_ isolates	Drug resistance cured	Frequency of curing by different agents at 37°C			
			Ethidium bromide (1 day)	Ethidium bromide (7 days)	Acridine orange (1 day)	Sodium dodecyl sulphate (3 days)
1	2	3	4		5	6
1.	980	A	22.85	26.4	0	91.34
		C	20.00	46.4	81.65	89.42
		O	10.47	20.4	20.18	76.92
		Su	18.09	24.0	0	0
2.	MSP-7	A	34.56	62.35	93.84	78.82
		O	48.14	52.94	92.30	95.29
3.	RLFB	O	6.31	30.00	0	0
		Q	0	32.85	0	0
		S	5.26	34.28	0	0
		Su	0	22.85	0	0

Table 17 (Contd.)

1	2	3	4		5	6
4.	MS-Aloor	S	0	4.54	0	0
		Su	0	20.54	0	0
5.	LFB-5	Su	70.0	72.69	0	0
6.	FD-22	O	0	0	0	0
7.	M-11-3	C	0	0	0	0
		O	0	0	0	0
		S	0	0	0	0
8.	LFB 524	F	0	0	0	0
		J	0	90.56	93.10	90.52
9.	LFC 24	J	97.88	98.33	91.33	95.45
10.	979	Na	50.00	95.06	0	0
11.	V6 III	F	0	0	ND	ND
		Na	97.89	100	ND	ND

4.8.1.2 Curing with acridine orange (AO)

The acridine orange was used at the concentration of 100 µg/ml and the organisms were exposed to AO at 37°C for 24 h. Acridine orange eliminated the COSu resistance of *E. coli* 980; the AO resistance of MSP-7, J in LFB-524 and J resistance of LFC-24, significantly. Whereas it was found to be ineffective in curing the resistance of other isolates. In *E. coli* isolates RLFB, MS-Aloor, LFB-5, FD-22, M-11-3 and 979 the resistance to different antibiotics was not eliminated. Acridine orange was noticed to be less effective in curing as compared to ethidium bromide. The details are listed in Table 17.

4.8.1.3 Curing with sodium dodecyl sulphate (SDS)

Different isolates when exposed to 10 per cent SDS in LB broth at 37°C for 3 days (72 h), it was observed that ACO resistance of isolate 980; AO of isolate MSP-7 and J resistance of LFB-524 and LFC-24 were cured markedly. Whereas SDS had no effect in curing the resistance in other isolates. The frequencies of curing by EB, AO and SDS are furnished in Table 17.

4.8.1.4 Curing at elevated temperature

Seven *E. coli* isolates were cultured in LB broth and

Table 18. Frequency of curing of plasmids in *E. coli* at elevated temperature

Sl. No.	*E. coli* isolates	Drug resistance cured	Frequency of curing on day		
			1	5	10
1.	980	A	0	25	27
		C	0	55	76
		O	0	20	21
		Su	0	55	69.5
2.	MSP-7	A	0	50	67.33
		O	0	33.33	47.33
3.	RLFB	O	0	8	14.80
		Q	0	4.0	33.87
		S	0	6	29.03
		Su	0	18	18.18
4.	MS-Aloor	S	0	18.75	23
		Su	0	22.50	25
5.	LFB-5	Su	0	4.22	22.70
6.	FD-22	O	0	28	35
7.	M-11-3	C	0	26	30.
		O	0	40	45
		S	0	44	49

incubated at 45°C. The isolates were subcultured daily. The master plates were prepared after first, 5th and 10th day of incubation and replica plated on antibiotic agar against which the curing effect was to be studied. The results are presented in Table 18. There was no curing of resistance after 24 h incubation at 45°C in all the isolates, but a steady increase in the curing frequencies was noticed from 5th to 10th day in all the isolates.

4.8.2 Plasmid profiles of *E. coli* cured by various agents

Nine different *E. coli* isolates which were cured of their drug resistance were tested for their plasmid profiles. The plasmid profiles of untreated *E. coli* (control) were compared with that of the cured *E. coli* isolate. It was observed that the curing agents eliminated different plasmid fragments along with the elimination of drug resistance. The loss of plasmid fragments was found to be variable from isolate to isolate. In some cases more than one plasmids were lost when only one antibiotic character was cured. It was also noticed that one plasmid was bearing the resistance for two drugs and some virulence character such as hemolysin production. The details of the cured plasmids in relation to loss of character(s) are furnished in Tables 19 and 20. Figures 9 and 10 depict the plasmid profiles of cured and control (wild) *E. coli*.

Fig.9 Well-A : *E. coli* LFB-5 (control) with 2 plasmids of 65 and 5.01 Kb, B : LFB-5, cured of both the plasmids by ethidium bromide, C and D : *E. coli* V517.

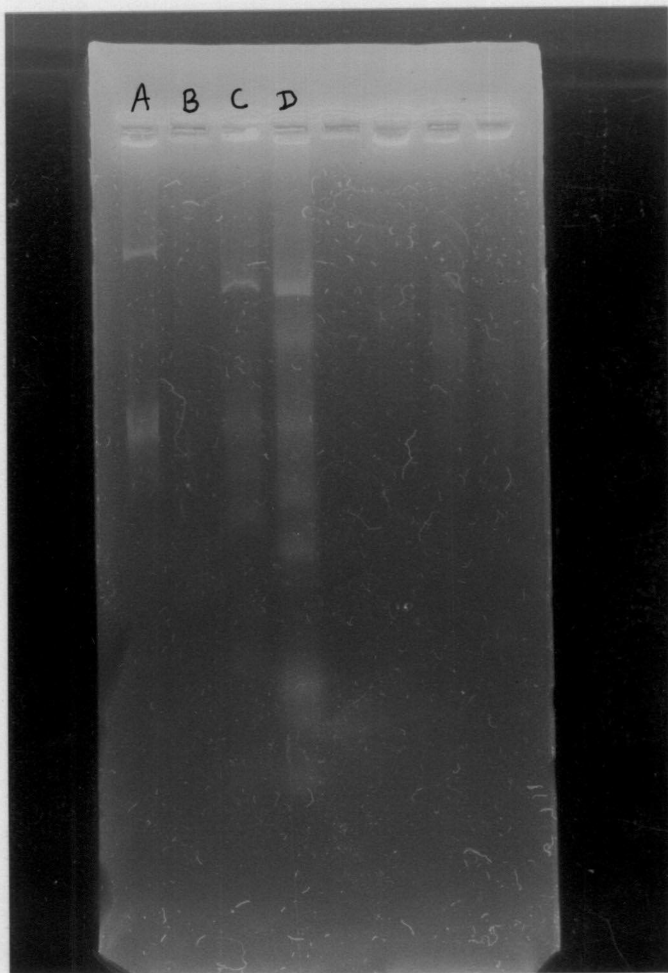
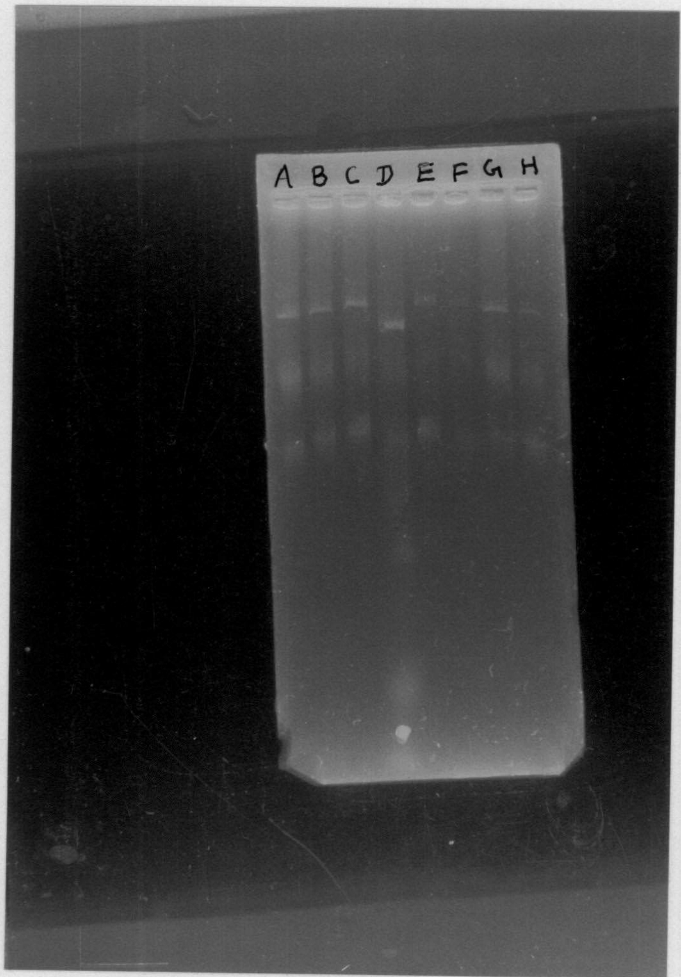

A B C D

Fig.10 Plasmid profiles of *E. coli* MSP-7 (A-C and E-H) obtained from different lots of plasmid lysates, showing the reproducibility of plasmid fingerprinting technique. *E. coli* MSP-7 with three plasmids of 63.1, 17.78 and 6.76 Kb. D - *E. coli* V517.


A B C D E F G H

4.8.2.1 Curing of hemolysin producing (Hly) plasmids

All the *Hly* plasmid carrying isolates such as MSP-7, 980 and MS-Aloor were exposed to EB, AO, SDS and elevated temperature, for elimination (curing) of plasmids. In all the three isolates, *Hly* plasmids were noticed to have cured. On comparison of the plasmid profiles of hemolytic isolates before and after elimination, it was observed that they lost some plasmids. In *E. coli* MSP-7, EB treatment resulted in loss of 63.10 and 17.78 Kb plasmids whereas SDS, AO and elevated temperature cured only one, the 63.1 Kb plasmid. In case of isolate 980, three plasmids of 107.2 Kb, 5.37 Kb and 3.54 Kb were eliminated by various treatments, whereas all the five plasmids were lost by EB and elevated temperature. Plasmid 3.89 Kb was cured by AO, EB and elevated temperature. In case of *E. coli* MS-Aloor EB treatment resulted in curing of all the three plasmids of 67.61, 5.49, and 4.07 Kb molecular size (Table 19).

4.8.2.2 Curing of lactose fermenting (F'*lac*) plasmids

While studying the curing of antibiotic resistance of *E. coli* isolates, it was observed that a few of them lost the lactose fermentation character, when the cultures were exposed to EB, AO, SDS at 37°C and elevated temperature at 45°C. Three isolates (980, MS-Aloor and 979) revealed the F'*lac*

Table 19. Plasmid profiles of *E. coli* cured by various agents

E. coli isolates	Character cured	Curing agent	Plasmid profile (Kb)	
1	2	3	4	
LFB-5 control	--	--	65.00	5.01
LFB-5	Su	EB	cured	cured
V 6 III-control	--	--	14.13	
V 6 III	Na	EB	cured	
979 control	--	--	6.76	
979	NA	EB	cured	
LFB 524 control	--	--	10.00	
LFB 524	J	SDS	cured	
LFB 524	J	AO	cured	
LFC-24 control	--	--	45.00	5.37
LFC-24	J	AO	cured	5.37
LFC-24	J	EB	cured	5.37
LFC-24	J	SDS	cured	5.37

Contd.

Table 19 (Contd.)

1	2	3	4				
MSP-7 control	--	--	63.10	17.78	6.76		
MSP-7	A	SDS	cured	17.78	6.76		
MSP-7	AO	EB	cured	cured	6.76		
MSP-7	O	AO	cured	17.78	6.76		
MSP-7	AT	Temp	cured	17.78	6.76		
980	C	SDS'	cured	14.13	cured	3.89	cured
980	A	SDS	cured	14.13	cured	3.89	cured
980 control	--	--	107.2	14.13	5.37	3.89	3.54
980	OC	AO	cured	14.13	cured	cured	cured
980	CSu	EB	cured	cured	cured	cured	cured
980	AOSu	Temp	cured	cured	cured	cured	cured
RLFB control	--	--	79.43	5.01	1.95	1.47	
RLFB	QSOSuT	Temp	cured	5.01	cured	cured	
MS-Aloor control	--	--	67.61	5.49	4.07		
MS-Aloor	S	EB	cured	cured	cured		

Table 20. Various characteristics and plasmid profiles of cured *E. coli* isolates

E. coli isolates	Antibiogram	Resistogram	Enterotoxin	Hemolysin	Plasmid profile	Resistance tried in conjugation	Conjugal transfer of			Plasmid profile of transconjugants	Curing of			Profiles of cured isolates			
							R-factor	*Hly*	*Ent*		R-factor	*Hly*	*Ent*	EB	AO	SDS	Temp.
MSP.7	AORT	E	–	+	63.10, 17.78 6.76	AO	None	+	NA	63.10	+	+	NA	6.76	17.78, 6.76	17.78, 6.76	17.78, 6.76
MS-Aloor	RSSu	E	+	+	67.61, 5.49, 4.07	SSu	Su	+	–	67.61	+	+	+	Nil	ND	ND	ND
980	ACORSu	BE	+	+	107.2, 14.13, 5.37, 3.89, 3.54	COSu	O	+	–	107.2	+	+	+	Nil	14.13	14.13, 3.89	Nil
RLFB	CQRSSuT	AE	+	–	77.43, 5.01, 1.95, 1.47	CQRSSu	SSu	NA	–	79.43	+	NA	+	ND	ND	ND	5.01
LFB-5	RSuT	EF	–	–	65.0, 5.01	SuT	None	NA	NA	ND	+	NA	NA	Nil	ND	ND	ND
V6-III	CFNaORT	EF	–	–	14.13	ND	NA	NA	NA	ND	+	NA	NA	Nil	ND	ND	ND
LFB 524	AFJORSSu	AE	–	–	10.00	FSSu	None	NA	NA	ND	+	NA	NA	ND	Nil	Nil	ND
LFC-24	ACJQRSSuT	BEF	–	–	45.0, 5.37	ACJQSuT	AQSu	NA	NA	ND	+	NA	NA	5.37	5.37	5.37	ND
979	NaO	BE	–	–	6.76	ND	NA	NA	NA	ND	+	NA	NA	Nil	ND	ND	ND

NA – Not applicable

ND – Not done

elimination. In isolate *E. coli* 980, EB eliminated F'*lac* plasmid at 40.4 per cent and elevated temperature at 43.85 per cent frequency, whereas in *E. coli* MS-Aloor it was 62.68 per cent by AO and in case of *E. coli* 979 it was 75.52 per cent by SDS treatment.

4.8.2.3 Curing of enterotoxin producing (Ent) plasmids

The cured isolates of four enterotoxigenic *E. coli* did not produce enterotoxin when tested by RLIL.

4.8.3 Transferability of cured isolates

None of the cured isolates when checked for the conjugal transferability of antibiotic resistance, and hemolysin production could transfer any of these characters, to the recipient *E. coli*.

4.9 Plasmid DNA photography

The clear colour photographs of the plasmid DNA profiles were obtained with single lens reflex camera. The ninety seconds exposure of the film resulted in clear photographs. Better resolution of different plasmid DNA bands was appreciated on the prints after processing the film by film and print processors.

Discussion

DISCUSSION

Among the organisms causing mastitis, the role of *E. coli* as a causative agent is quite significant. In the present study the incidence of *E. coli* recorded was 15.33 per cent and the percentage of *E. coli* among Gram negative bacilli causing mastitis was 35.38. Similar findings were reported by Pal *et al.* (1988) and Todhunter *et al.* (1991). Prabhakar *et al.* (1988) and Bansal *et al.* (1990) reported *E. coli* as the second most common agent causing bovine mastitis.

5.1 Antibiogram testing

In the present study among the 46 *E. coli* isolates tested against various antibiotics and chemotherapeutic agents 78.26 per cent exhibited resistance to rifampicin, 50 per cent to oxytetracycline, 36.95 per cent to Su, 34.78 per cent to T, 23.91 per cent to S, 21.73 per cent to A and C, 19.56 per cent to Na and 13.04 per cent to F. However, the resistance was found to be very low in case of Q (8.69%) and J (6.52%). Kanamycin and norfloxacin showed cent per cent susceptibility (Table 2).

Premkumar David *et al.* (1991) observed 100 per cent resistance in avian *E. coli* to rifampicin and the results of the present study generally corroborate with their findings.

Jackson (1981), Prabhakar et al. (1988), Barrow and Hill (1989) observed considerable resistance to tetracycline in their studies on E. coli isolated from mastitic milk. Prabhakar et al. (1988) noticed resistance to oxytetracycline also Adetosoye (1980b), Saida et al.(1981). Panhotra et al. (1982), Punnoose (1982), Tewari and Agarwal (1983), Prabhakar and Sud (1984), Harnett and Gyles (1984), Hosoda et al. (1990) and Kaura et al. (1991) observed resistance to tetracylines ranging from 22 to 58 per cent in E. coli isolated from livestock and poultry. These findings compare well with the results of the present study.

Barrow and Hill (1989) found that 15 per cent of E. coli from mastitis milk samples were resistant to Su which is lower than the results obtained in the present study. But Gianelli et al. (1979b), Adetosoye (1980b), Saida et al. (1981), Punnoose (1982), Panhotra et al. (1982), Tewari and Agarwal (1983) and Hosoda et al. (1990) reported resistance to Su varying from 15-58 per cent in E. coli isolated from animals and birds.

Jackson (1991) observed 17.3 per cent resistance to streptomycin. While Prabhakar et al. (1988) observed high resistance to streptomycin among E. coli isolated from milk samples. While Gianelli et al. (1979b), Adetosoye (1980b), Saida et al. (1981), Panhotra et al. (1982), Tewari and

Agarwal (1983), Prabhakar and Sud (1984), Harnett and Gyles (1984) and Hosoda et. al. (1990) recorded resistance to streptomycin, varying from 15.6 to 52.9 per cent among E. coli isolated from livestock and poultry.

Jackson (1981) observed 11.4 per cent resistance to ampicillin while Prabhakar et al. (1988) observed some degree of resistance to ampicillin. Barrow and Hill (1989) observed 14 per cent resistance to A among E. coli isolated from milk, while Saida et al. (1981), Tewari and Agarwal (1983), Prabhakar and Sud (1984), Harnett and Gyles (1984), Grewal and Tiwari (1990), and Mahipal Singh (1992) noticed resistance to A varying from 24 to 49.7 per cent in E. coli isolated from animals and poultry.

Jackson (1981) and Barrow and Hill (1989) noticed 9.5 per cent and 5 per cent resistance respectively, to chloramphenicol in E. coli isolated from bovine mastitis milk. Whereas the resistance to C ranged from 5.8 to 58 per cent in E. coli isolated from animals and birds (Gianelli et al., 1979b; Saida et al., 1981; Panhotra et al. 1982; Tewari and Agarwal, 1983; Prabhakar and Sud, 1984; Kovalovo et al. 1980; Hosoda et al., 1990 Kaura et al., 1991). The results of the present study on antibiotic resistance are consistent with the results of the above workers.

In the present study 19.56 per cent of *E. coli* were resistant to nalidixic acid but a search of literature did not reveal the presence of Na resistant *E. coli* in mastitis milk. However, Hosoda *et al*. (1990) reported 28 per cent resistance among *E. coli* isolated from calves.

In the present study three *E. coli* isolates (6.52%) were sensitive to all the 13 antibiotics tested while nine (19.56%) were resistant to single antibiotic and 34 (73.91%) isolates were resistant to more than one antibiotic. Among the 43 resistant organisms, 20.93 per cent were single antibiotic resistant while 79.06 per cent were multiple drug resistant ranging from 2 to 9 antibiotics in different combinations. Among the *E. coli* tested, OR resistance was the most common (6 isolates) among the double antibiotic resistant organisms. Seven isolates were triple resistant (Table 3). OR resistance either alone or alongwith other combinations occurred in 23 of 34 (67.6%) MDR isolates whereas RSu and RT resistances occurred in 38.2 per cent and 38.3 per cent respectively. ORT resistance either alone or with other combinations occurred in 23.52 per cent cases whereas RSSu and ORSu resistances were noticed in 20.9 per cent cases each. ORTSu/ORSSu/ARSSu/AORSu/ORSuT resistances were noticed either alone or in combination in 11.76 per cent cases. Whereas CFNaORT resistance in 8.82 per cent cases and ACRSSu, AORSSu,

ORSSuT resistances occurred either alone or in combinations in 5.88 per cent cases each. AFJORSSu resistance also occurred in 2 (5.88%) cases and ACFNaOQRSuT resistance was noticed in one (2.94%) isolate. On the whole 26 different patterns of resistances were observed in the present study, among the 43 isolates.

Adetosoye (1980b), Saida et al. (1981), Punnoose (1982), Prabhakar and Sood (1984), and Harnett and Gyles (1984) observed various percentages of quadruple, resistance in different combinations as the most common pattern. While Tewari and Agarwal (1983) observed quintuple resistance as the most common pattern in animal E. coli. In the present study double resistance in different combinations was most common (23.91%) followed by triple resistance in different combinations (15.21%) and quintuple resistance (13.04%). Punnoose (1982) observed resistance ranging from 1-7 antibiotics and chemotherapeutic agents, while in the present study resistance ranging from 1-9 antibiotics and chemotherapeutic agents was noticed.

The results of the present study clearly coincide with the findings of the above workers on the occurrence of multiple drug resistant E. coli, but the patterns of resistance (double to nonuple) observed in the present study did not have cent per cent correlation with the previous

reports, since the organisms used in the present study have come from diverse geographical and environmental conditions. The indiscriminate use of antibiotics for combating various ailments in animals especially in the treatment of mastitis, often leads to the emergence of drug resistant bacteria (Premkumar David *et al*., 1991). Since the use of drug varies from place to place, the emergence of drug resistant bacteria will also change accordingly. This may also be a factor for the diverse multiple resistance noticed.

Adetosoye (1980b) observed nineteen patterns of antibiotic resistance to 414 isolates of *E. coli* using eight antibiotics while Premkumar David *et al*. (1991) observed 19 patterns of resistance in 20 *E. coli* isolates using 32 antibiotics. But in the present study 26 patterns of resistance in 43 *E. coli* using 13 antibiotics were obtained.

A comparison of the patterns of drug resistance observed in the study and the previous work clearly established the existence of different patterns. But the pattern of drug resistance depends on the antibiotics used and also the source of the organism and the differences in the patterns encountered could be attributed to these factors.

Antibiotic sensitivity is used as an epidemiological marker in the study of bacteria. In the present study 26

different resistance patterns using 13 antibiotics and chemotherapeutic agents were noticed among 43 E. coli isolates with the help of antibiotic sensitivity test. This gave a reliability of 60.46 per cent in differentiating 43 isolates. Old et al. (1980) and Premkumar David et al. (1991) used antibiotic resistance patterns for differentiating E. coli strains, and the results of the present study concur with those of above workers. This method is considered to be easy, sensitive, reproducible and cost effective than biotyping, resistotyping, phage typing or serotyping and can be used as an adjunct to other epidemiologal typing methods.

5.2 Resistogram testing

The resistograms of 46 E. coli isolates from mastitis were studied by using seven different heavy metal and quaternary ammonium compounds. The organisms revealed high degree of resistance (100%) to lead oxide. Novick and Roth (1968) and Manohar Paul (1992) observed high degree of resistance in Staphylococci and Harnett and Gyles (1984) observed high degree of resistance against lead ions in E. coli of animal origin.

The resistogram study of 46 E. coli isolates to antimony chloride indicated that 32.6 per cent of the organisms were resistant to antimony compounds. Novick and

Roth (1968) reported resistance to antimony compounds in *S. aureus*. In the present study 30.43 per cent of isolates were resistant to copper sulphate. Smith *et al*. (1978), Harnett and Gyles (1984), Premkumar David *et al*. (1991) observed resistance to copper sulphate in *E. coli* of livestock and poultry, whereas Barrow and Hill (1989) observed inhibitory effect of copper sulphate discs, on *E. coli* isolated from mastitis.

In the present study 19.56 per cent *E. coli* were resistant to silver nitrate. Grewal and Tiwari (1990) recorded resistance to silver nitrate in *E. coli*, whereas Harnett and Gyles (1984) recorded complete susceptibility in bovine enterotoxigenic *E. coli* and porcine *E. coli*.

Resistance to cetrimide was found to be very low (2.17%) in the present study. This might be due to the low frequency of exposure of this chemical to the organisms in the present study. Emslie (1985) recorded very low resistance of Staphylococci to cetrimide while Manohar Paul (1992) observed a slightly higher resistance (30%) in Staphylococci.

None of the *E. coli* isolates studied was resistant to cadmium chloride and mercuric chloride. This may be due to the fact that these isolates might not have been exposed to these compounds. Similarly Barrow and Hill (1989) did not

observe any resistance to cadmium chloride in *E. coli* isolated from mastitis, though marginal resistance to mercuric chloride was demonstrated. However, there are reports of resistance to cadmium in *E. coli* of animal origin as observed by Harnett and Gyles (1984). Grewal and Tiwari (1990) also reported more than 50 per cent resistance to mercuric chloride.

The resistotyping of 46 *E. coli* isolates resulted in nine different patterns of heavy metal resistance (Table 6). Thus, in the present study, this test has the reliability of identifying the strains to the tune of 19.56 per cent, which is less than the one reported by Premkumar David *et al.* (1991). The probable reason of less number of resistance patterns may be due to the cent per cent susceptibility of organisms to cadmium and mercury (two of seven) compounds used.

5.3 Correlation between antibiotic and heavy metal resistance

Of the 43 antibiotic resistant isolates, 36, 23, 17, 16, 11, 10, 10, 9, 6, 4, and 3 isolates were resistant to R, O, Su, T, S, A, C, Na, F, Q and J respectively making 145 individual resistances (Table 7). Among these, lead revealed 100 per cent (145/145) resistance, antimony 41.3, copper 37.2, silver 20.6, cetrimide 6.2 per cent.

Since all the isolates were resistant to lead, there appears to be 100 per cent correlation between various antibiotic resistances and lead resistance. The rifampicin resistant organisms showed 33.33 per cent correlation between copper (Cu) and antimony (Sb), whereas oxytetracycline resistant organisms showed 73.9 and 43.47 per cent correlation with antimony and copper respectively. Sulphadiazine resistant *E. coli* showed 41.17 and 23.52 per cent correlation with Cu and Sb respectively. Tetracycline resistant organisms showed 37.5 per cent correlation with Cu and Sb, ampicillin showed 30 per cent correlation with Cu and 20 per cent with Sb and Ag, chloramphenicol showed 50 per cent with Sb and 30 per cent each with Cu and Ag. Nalidixic acid resistant organisms showed 44.44 per cent correlation with Sb and 33.33 per cent with Ag. Furazolidone resistance showed 50 per cent correlation with Sb and Cu, co-trimoxazole resistant *E. coli* showed 75 per cent correlation with Sb and 25 per cent with Cu, Ag and cetrimide. Gentamicin resistant organisms revealed 66.66 per cent correlation with Cu and 33.33 per cent with Sb and Ag.

It could be concluded from the present study that there is a correlation between antibiotic resistance and lead, antimony and copper in descending order. Cenci *et al*. (1982), Harnett and Gyles (1984) and Grewal and Tiwari (1990) observed

correlation between antibiotic and metal ions. The present findings concur with their results.

5.4 Hemolysin production

Three of the 46 (6.52%) *E. coli* isolates were positive for α-hemolysin production (Table 8). Barrow and Hill (1989) observed that 5 per cent of *E. coli* isolated from bovine mastitis were hemolytic, whereas Smith and Halls (1967a), and Gyles *et al*. (1971) had observed higher percentage of hemolytic *E. coli* from bovine and porcine sources. Gyles *et al*. (1971) reported higher incidence of hemolytic *E. coli* among enteropathogenic than non-enteropathogenic *E. coli*. But in the present study 2 of 13 (15.38%) enterotoxigenic *E. coli* were hemolytic too. Of three hemolytic isolates, two (66.66%) were enterotoxigenic.

Smith and Linggood (1971) noticed the association of hemolysin production and enterotoxin (LT) in *E. coli* of bovine origin. Barrow and Hill (1989) also reported the association of hemolysin and enterotoxin in *E. coli* isolated from mastitis. Hence, the present finding concurs with the above workers.

5.5 Enterotoxin production

Thirteen of the forty six (28.26%) *E. coli* isolated

from bovine mastitis were enterotoxigenic (Table 8). Smith and Hall (1967b; 1968) recognized the enterotoxin of *E. coli* and opined that plasmid *Ent* was responsible for the production of enterotoxin. Detection of enterotoxin (LT) was carried out using rabbit ligated ileal loop assay based on dilatation index of De *et al.* (1956), McNaught and Roberts (1958), Ronneberg (1986) and Bergdoll (1988). Barrow and Hill (1989), Alkaff *et al.* (1991) and Neneth *et al.* (1991) observed 1, 6 and 25.35 per cent enterotoxigenic *E. coli* respectively, among *E. coli* from bovine mastitis. The results of the present study generally agree with the above findings.

In the present observation two of the thirteen (15.38%) enterotoxigenic *E. coli* were hemolytic. Similar observations were also recorded by Smith and Linggood (1971), Prada *et al.* (1991) and Wasteson *et al.* (1992).

5.6 *In vitro* transfer of antibiotic resistance

Among the 24 isolates of *E. coli* having resistance ranging from 1-6 antibiotics, 14 (58.33%) transferred drug resistance either alone or in combination (Table 11). Among the 13 sulphadiazine resistant isolates 9 (69.23%) transferred Su resistance to the recipient; of ten O resistant isolates 5 (50%) transferred O resistance, of 5 ampicillin resistant, 2 (40%) transferred A resistance, of 5 S resistant 2 (40%)

transferred S resistance, of 3 Q resistant 1 (33.33%) transferred Q resistance and of four C resistant 1 (25%) transferred C resistance.

Davies and Stewart (1978), Crosa and Falkow (1981), Punnoose (1982) and Barrow and Hill (1989) opined that ability to transfer drug resistance was an indication of the presence of R plasmids in bacteria. The ability of drug resistant organisms to transfer their resistance to recipient bacteria indicates that the drug resistance is plasmid borne. Kinjo (1974) observed 42.21 and 36.36 per cent transfer of drug resistance in *E. coli* isolated from swines and fowls respectively. While Nakamura *et al*. (1978) observed the transfer of drug resistance of *E. coli* from bovines and swines at 57.7 and 52.2 per cent respectively. Adetosoye (1980b) observed a transfer rate of 31.5 per cent only in *E. coli* isolated from clinically healthy livestock. Barrow and Hill (1989) studied the antibiotic transfer of *E. coli* isolated from milk and found that 41 per cent transferred their drug resistance to recipient. Therefore the result of the present study indicating 58.33 per cent transfer of drug resistance goes very well with the previous observations.

Gianelli *et al*. (1979 a) have observed 75 per cent transfer of S and O resistance, 57 per cent transfer of Su resistance, and 23 per cent transfer of C resistance, while

Gianelli et al. (1979b) observed slightly lower percentage of transfer of antibiotic resistances such as S, 42 per cent; O, 29.5 per cent; C and Su, 23 per cent. Khanna et al. (1981) observed only 12.3 per cent of transfer of O and S resistances. Though there is no complete unanimity in the percentage of transfer observed by various authors, the results of the present study fall within their range. There is similarity of the percentage of transfer of C and S and to certain extent in case of O and Su.

5.7 *In vitro* transfer of hemolysin producing character

All the three hemolytic isolates of *E. coli* transferred the hemolysin production by conjugation but at different frequencies viz. 52.71, 66.42 and 82.75 per cent (Table 12). Noegel et al. (1981) observed the transfer of hemolysin production from *E. coli* to recipient cell and observed it to be plasmid borne. Similar observations were made by Klimuszku et al. (1987), who observed transfer in 30 per cent cases. This was further confirmed by Klimuszku et al. (1989) by *in vivo* transfer of *Hly* plasmid in pig intestine. The results of the present work indicate the possibility of existence of hemolytic character on plasmid as evidenced by the previous workers. Further the transferable nature of the character observed in the present study fully concurs with the observation of above workers.

Two of three hemolytic isolates were enterotoxigenic but, they could not transfer the enterotoxin producing character to recipient organisms though the hemolytic character was transferred. Franklin *et al*. (1981) observed that genes for enterotoxin production, K 88 antigen, colicin and antibiotic resistance were located on different plasmids, and could be transferred independently of one another. While Bertin (1992) could transfer only hemolysin production from hemolytic enterotoxigenic *E. coli* which also clearly supports the present findings.

5.8 Conjugal transfer of enterotoxin producing character

None of the eleven enterotoxin producing *E. coli* tested, could transfer the character to recipient organisms as evidenced by lack of dilatation and accumulation of fluid by RLIL assay. Gyles *et al*. (1974) observed production of enterotoxin in *E. coli* isolated from disease conditions only. Baldev Raj (1991) could transfer enterotoxin producing character of *E. coli* isolated from diarrhoeic cases of bovines in 5.5 per cent cases whereas Bertin (1992) could not transfer enterotoxin producing character of antibiotic resistant, hemolysin producing enterotoxigenic *E. coli*. Singh *et al*. (1992) reported 7.69 per cent transfer of enterotoxin producing character in *E. coli* isolated from various disease conditions of animals.

The non-transfer of enterotoxin producing character observed in the present study could probably be due to the existence of genes governing enterotoxin production on bacterial chromosome as suggested by Gyles et al. (1974), or could be due to their presence on low molecular weight plasmids or on non-conjugative plasmids, low frequency of transfer, failure of replication, or gene expression, absence of effective pair formation or surface exclusion as suggested by Broda (1979).

5.9 Plasmid profiles of bovine mastitis *E. coli*

The plasmids are known to be responsible for the various characters such as drug resistance, toxin production, hemolysin production and colonization antigens (Gonzalez and Blanco, 1986). To trace the role of plasmid in all these characters the plasmid profile is essential. In the present study 40 *E. coli* isolates were processed for their plasmid analyses. The presence of plasmids was confirmed with the agarose gel electrophoresis. Thirty-eight of the forty *E. coli* isolates harboured the plasmids, ranging from one to five in number and of different molecular sizes ranging from 1.47 to 128.8 Kb (Table 14). Avila and de la Cruz (1988) opined that the plasmids of 30 Kb and above are large plasmids. In the present study twenty isolates had only small, seven harboured only large, and 11 had large and small

plasmids. The two isolates without any plasmids were also negative for enterotoxin and hemolysin.

Baumgartner et al. (1983) isolated large plasmids from antibiotic resistant E. coli isolated from bovine mastitis. Similar type of observation was also recorded by Gonzalez and Blanco (1986) from E. coli, and Manohar Paul (1992) from Staphylococci isolated from mastitis. The occurrence of large plasmids observed in the present study is in agreement with the observations of above workers.

Manohar Paul (1992) could not come across any plasmids in few staphylococcal samples harbouring antibiotic resistance. Absence of plasmids in two samples harbouring antibiotic resistance observed in the present study is in confirmity with the findings of above workers. It leads to the possibility of the antibiotic resistant gene harbouring on chromosome.

Of the thirty eight plasmid harbouring E. coli isolates, altogether 35 different patterns of plasmid profiles were obtained. No plasmid was found common to all the isolates. Three different plasmid profiles represented two isolates per profile, such as plasmid profile 6.06 Kb was seen in isolate GNI 25491 and GNR 20, while H1 and MD isolates had 5.6 and 5.1 Kb plasmids whereas LFB 524 and LF 82 had 10 Kb

plasmid in common. The antibiogram and resistogram of GNI 25491 was similar to GNR 20, H1 was similar to MD and LFB 524 similar to LF 82 (Table 14.) The isolates GNI 25491 and GNR 20 were enterotoxigenic also. Thus the two isolates in each plasmid profile pattern are similar to each other, and are apparently representatives of the same strain.

Pai and Joshi (1984) opined that the comparison of molecular properties of resistant plasmids may help in locating the source. Whereas Wasteson et al. (1992) were able to group E. coli, based on plasmid profiling and restriction endonuclease digestion of total DNA for identifying bacterial strains when phenotypic character is unsuitable. They have further observed that in the genetic characterization of selected strains, by total DNA restriction digestion and plasmid profiling, the plasmid profiling is a more suitable method for epidemiological investigation of E. coli strains. In the present investigation 40 E. coli isolates were categorized into 35 different groups based on plasmid profiling and hence the results of the present investigation coincide with the observations of the above authors. The similarity of characters (same antibiogram, resistogram and enterotoxigenic character) exhibited by two organisms each, showing identical plasmid profile, clearly concurs with the observation of Wasteson et al. (1992).

Similarity of plasmid profiles in organisms having same resistogram and antibiogram further lends support to the view that plasmid profiling could be used as an effective method for discriminating the strains where phenotypic characterization is indecisive. So plasmid profiling could be employed as a means of identifying related or unrelated isolates in a particular geographical area. The present findings are in agreement with Wasteson et al. (1992).

5.9.1 Plasmid profiles of hemolytic strains

The plasmid profiles of three hemolytic strains revealed that isolate E. coli 980 (Hly^{+} Ent^{+} ACORSu) harboured five different plasmids of 107.2, 14.13, 5.37, 3.89 and 3.54 Kb; E. coli MS-Aloor (Hly^{+}. Ent^{+}, RSSu) contained 67.61, 5.49, 4.07 Kb plasmids and E. coli MSP-7 (Hly^{+}, Ent^{-}, AORT) possessed 63.10, 17.78 and 6.78 Kb plasmids (Table 15) Prada et al. (1991) and Wasteson et al. (1992) observed high molecular plasmids in hemolytic E. coli and the occurrence of larger plasmids seen in the present study is in confirmity with the findings of above authors.

5.9.2 Plasmid profiles of enterotoxigenic E. coli

Among the 13 enterotoxigenic strains used for plasmid profile analysis six showed small plasmids of less than 30 Kb, two showed large plasmids only whereas remaining revealed

large as well as small plasmids. One isolate (E. coli 2) among the 13, was sensitive to all the drugs and non-hemolytic but enterotoxigenic and harboured only a small plasmid of 5.7 Kb. Gyles et al. (1974) reported that the size of enterotoxigenic plasmid ranged from 21 to 80 MDa whereas Baldev Raj (1991) observed the size of the enterotoxigenic plasmid (Ent) to be 38 MDa or above but has also reported smaller Ent plasmids of less than 7 MDa. The results of the present work partially agree with Gyles et al. (1974) and fully agree with Baldev Raj (1991).

5.9.3 Plasmid profiles of transconjugants

Among the 24 antibiotic resistant E. coli isolates from bovine mastitis, 14 transferred antibiotic resistance to the recipient. The transfer of hemolysin producing character (Hly) was noticed in all the three isolates. None of the eleven enterotoxigenic E. coli tested, could transfer the property to the recipient. The plasmid profiles of nine transconjugants and wild type of E. coli (donor) are furnished in Table 16 . It was observed that only plasmids having high molecular size of 45 Kb and above appeared in the transconjugants showing antibiotic resistance and Hly character. It was also observed that in case of three Hly^+ transconjugants, all the three possessed high molecular size

plasmids (107.2, 67.61 and 63.10 kg). Among three Hly^{+} transconjugants only two revealed antibiotic resistance character.

Gyles et al. (1974), Pai and Joshi (1984), and Avila and de la Cruz (1988) while studying the transfer of antibiotic resistance, observed that only plasmids of 30 Kb and above were transferable to recipient strains. In the present study only larger plasmids could be transferred. Pai and Joshi (1984), and Avila and de la Cruz (1988) opined that those plasmids with molecular size of 30 Kb and above could be considered as large, plasmids. In the present observations, no plasmid between the molecular size of 30 to 45 Kb existed in the isolates and the minimum size of "large plasmids" seen was 45 Kb. The results of the present work are strengthened by the observations of the above workers.

It was observed in the present study that the size of the plasmid transferred remained unchanged. Similar were the observations of Pai and Joshi (1984).

The plasmid profiles of three hemolytic transconjugants indicated that they contain only high molecular size 107.2, 67.61 and 63.1 Kb in three different isolates. Prada et al. (1991) observed that plasmids responsible for hemolytic character were having the molecular

size 48 MDa (72 Kb) and above. The present finding is in confirmity with Pai and Joshi (1984), Avila and de la Cruz (1988) and Prada *et al*. (1991).

In the present study in case of two hemolytic isolates (980 and MS-Aloor) the hemolytic character was co-transferred with oxytetracycline and sulphadiazine resistance respectively, and this is in agreement with the findings of Harnett and Gyles (1984). It could be presumed from the present findings that the genes responsible for Hly character and antibiotic resistance are harboured on a single plasmid.

5.10 Curing of *E. coli* plasmids

Curing of certain characters in bacteria by chemical or physical methods is being used as a confirmatory test for the presence of these characters on the plasmids. The chemical agents used in the present study are EB, AO and SDS, and elevated temperature was used as a physical method.

5.10.1 Curing with ethidium bromide

Out of the eleven *E. coli* isolates tested, nine lost the resistance character when incubated with EB. The percentage of loss increased with the period of incubation, in certain cases (Table 17). Poppe and Gyles (1988) used EB as a curing agent on Salmonella for 1-7 days and observed that EB

was not an efficient curing agent. Reddy (1990) also reported similar findings, whereas Bouanchaud et al. (1969) Subramanyam and Agarwal (1982) and Kaur et al. (1985) obtained substantial degree of elimination of plasmids when treated with EB, and the results of the present work concur with the above three authors. The resistance for F was not eliminated in the two cases tested. It was observed in conjugation studies that resistance for F was not transferred. Failure of curing by EB together with the nontransfer of F resistance in conjugation studies clearly establish that the F resistance is not plasmid borne. This finding is supported by Smith and Halls (1966); Smith (1967) and Davies (1979).

In the present study EB eliminated the Na resistance of two E. coli isolates tested (979 and V6 III). These isolates were not used in the conjugation studies as the recipient used was a nalidixic acid resistant marker. Plasmid borne nature of nalidixic acid resistance is not generally reported, and there are the reports of only chromosomal resistance of nalidixic acid; (Davies, 1979). Though the present study indicates the plasmid borne nature of Na resistance as seen by curing with EB, further study is required to make a conclusion (Table 17). The results of the present study indicate that EB is a better curing agent.

It was observed that EB also cured F'*lac* factor (40.4% frequency) responsible for the lactose fermentation. Bouanchaud *et al*. (1969) also reported F'*lac* curing in *E. coli*. The elimination or the curing of drug resistance and lactose fermentation character (F'*lac*) was due to the interference with the replication of the plasmid DNA responsible for these characters (Carlton and Brown, 1981).

5.10.2 Curing with acridine orange

Of the ten *E. coli* isolates tested for the elimination of plasmids by AO treatment, only four were cured either partially or completely, of their antibiotic resistance (Table 17). Gentamicin resistance was cured in all the isolates but ACOSu resistance was cured in some cases only. In the present study the F resistance was not eliminated in any of the isolates. The F resistance was not transferred in conjugation studies also. This clearly indicates that the resistance is not plasmid borne as discussed under EB curing.

It was noticed that the F'*lac* was cured by AO in one isolate at 62.68 per cent frequency. Similar observations were recorded by Watanabe and Fukasawa (1961b) and Salisbury *et al*. (1972).

The present observation revealed that AO is less

efficient as a curing agent. Similar findings were reported by Hahn and Ciak (1976) and Poppe and Gyles (1988).

5.10.3 Curing with sodium dodecyl sulphate

Out of the ten isolates, four were cured of their resistances either partially or completely by SDS (Table 17). Gentamicin and ampicillin resistances were cured in all the cases whereas O and C were cured in some cases, while Su resistance was not cured in any of the cases. Tomoeda et al. (1968) postulated that SDS might gain access to the cytoplasmic membrane through the pili and the plasmids which are attached to the membrane close to the pili might be damaged by SDS. The present study indicates that SDS is an effective curing agent but less efficient than EB. The observations made by Tomoeda et al. (1968) and Kaur et al. (1985) indicated that SDS is an effective curing agent and their observations support the results of the present work. F'lac was eliminated in one case at 75.52 per cent frequency. Elimination of F'lac by SDS was also observed by Tomoeda et al. (1968).

5.10.4 Curing at elevated temperature

Seven E. coli isolates were exposed to elevated temperature (45°C) for one to ten days to study the effect of elimination of antibiotic resistance (Table 18). No

elimination of antibiotic resistance was observed at 24 h of incubation, but the frequency of elimination increased from 5th to 10th day. The results of the present study clearly indicate that this method is more efficient for plasmid curing when compared to chemical methods May, *et al.* (1964), Terawaki *et al.* (1967), Robinson *et al.* (1980) and Poppe and Gyles (1988) had observed elimination of antibiotic resistance on prolonged incubation. The result of the present work fully concurs with the observations of the above workers.

Terawaki (1967) and Robinson (1980) postulated the existence of temperature sensitive resistance factor in *E. coli*. They were of the opinion that the loss of drug resistance was due to the failure of plasmid DNA replication. The possible reason for the elimination of plasmid observed in the present study could be due to the failure of plasmid DNA replication as opined by the previous workers. In the present experiment the plasmids were more readily eliminated by incubation at 45°C, than by chemical agents and all the seven *E. coli* isolates tested were cured of various drug resistances at different frequencies. It was noticed that both the small and large plasmids were eliminated when exposed to elevated temperature (Table 19). The present findings concur with those of Poppe and Gyles (1988).

5.10.5 Curing of hemolysin producing plasmids

Three hemolytic *E. coli* were used to study the elimination of *Hly* plasmid using EB, AO, SDS and elevated temperature methods. The *Hly* plasmid was seen eliminated from all the three isolates by these methods, as confirmed by the growth of non-hemolytic *E. coli* colonies on sheep blood agar. Prada *et al*. (1991) have reported 60.5 per cent curing of *Hly* plasmid in *E. coli* by EB treatment. Screening of literature did not reveal any previous work using AO, SDS and elevated temperature methods for the elimination of *Hly* plasmid. The results of the present work concur with that of Prada *et al*. (1991).

5.10.6 Curing of enterotoxin producing plasmids

Only four *E. coli* isolates (MS-Aloor, RLFB, 980 and FD-22) were subjected to curing by various agents. Of *E. coli*, MS-Aloor, EB-cured; *E. coli* RLFB, elevated temperature-cured and *E. coli* 980, EB, AO, SDS and elevated temperature cured colonies, were studied for plasmid profile and enterotoxin production by RLIL assay. The enterotoxigenic *E. coli* subjected to curing did not reveal the production of enterotoxin by RLIL assay. It shows that *Ent* character is cured from bacteria. It supports the assumption that the *Ent*

character is plasmid borne. Perusal of literature did not reveal any previous work on similar lines.

5.11 Plasmid profiles of Hly cured E. coli

A comparative study of the plasmid profiles of nine wild and their cured strains was made in the present study. Only three of the nine, revealed hemolytic character and all the three transferred the hemolytic character to recipient E. coli. Only large plasmids, were transferred to the recipient. The original three wild strains contained large as well as small plasmids whereas the cured strains contained only small plasmids. In certain cases especially with EB curing and to certain extent with high temperature curing even the small plasmids were eliminated (Table 20). Three cured strains were found to have lost the hemolytic activity, when cultivated on sheep blood agar. Prada et al. (1991) observed the curing of Hly plasmid by EB in five of eight cases and further reported the loss of high molecular size plasmids in such cured strains. In the present study loss of large plasmid was observed on curing which concurs with the finding of Prada et al. (1991). A search of literature did not show any report indicating the use of AO, SDS and elevated temperature for curing Hly plasmid.

The results of the present work viz. the transferability of Hly by conjugation, curing of Hly plasmid with subsequent loss of high molecular size plasmids in cured strains and the lack of hemolysin production by the cured strains on sheep blood agar clearly establish that the hemolytic characters studied in the present work are plasmid borne. The observations are in confirmity with those of Harnett and Gyles (1984), Prada *et al*. (1991).

5.12 Plasmid profiles of Ent cured *E. coli*

Of the nine strains tested for curing, three were enterotoxin producers. Ent character was not transferred in mating experiment. The plasmid profiles of these wild enterotoxin producing strains revealed that they harboured large as well as small plasmids (Tables 19 and 20). In *E. coli* MS-Aloor EB curing has resulted in elimination of all the three plasmids present in wild strain. In the case of *E. coli* RLFB elevated temperature cured three of four plasmids. While in *E. coli* 980 EB and elevated temperature cured all the five plasmids whereas SDS and AO cured three and four out of five plasmids respectively. The cured isolates did not produce enterotoxin when tested by RLIL assay. Perusal of literature did not reveal any previous work regarding plasmid profile studies of cured enterotoxigenic *E. coli* isolates. The results of the present study throw

light on the possibility of plasmid borne nature of enterotoxin producing character, but further work is needed.

5.13 Plasmid profiles of R-factor cured *E.coli*

Of the nine *E. coli* isolates cured of various plasmids, three harboured only low molecular size plasmids and the rest carried large and small plasmids before curing (Table 19). *E. coli* LFB-5 lost Su resistance by EB treatment eliminating 65 and 5.01 Kb plasmids. *E. coli* V6 III lost nalidixic acid resistance and the only plasmid (14.13 Kb) harboured when treated with EB. In *E. coli* 979, EB treatment cured nalidixic acid resistance and eliminated the only plasmid (6.76 Kb) harboured by it. In the case of LFB-24, curing by EB, AO and SDS eliminated gentamicin resistance and also resulted in elimination of 45 Kb plasmid, indicating the possibility of location of gentamicin resistance on 45 Kb plasmid. In LFB 524, SDS and AO eliminated the only plasmid (10 Kb) present alongwith gentamicin resistance. The curing of one or the other antibiotic resistance was observed in all the nine isolates, irrespective of the size of the plasmids and whether the resistance was transferred by conjugation or not. Study of the plasmid profiles of the isolates cured of drug resistance, indicates that, the large plasmids are invariably eliminated in all the cases while the smaller plasmids were eliminated in certain cases. In three wild

antibiotic resistant *E. coli*, harbouring only small plasmids, the curing eliminated all these plasmids (Tables 19 and 20). The findings are in concurrence with Poppe and Gyles (1988) who observed the elimination of small and large plasmids by chemical and physical curing agents.

5.14 Correlation between *Hly*, R-factor and *Ent* characters *E. coli*

Among the three *Hly* positive *E. coli* isolates (MSP-7, MS-Aloor and 980) the *Hly* character was transferred to recipient in all the cases. All the three *E. coli* were resistant to different antibiotics as well. But only two (MS-Aloor and 980) transferred one antibiotic resistance each to the recipient. All the three harboured large as well as small plasmids (Table 20). The recipient, *E. coli* K 12 ML I410 did not harbour any plasmid, but the transconjugants contained one large plasmid each, which was transferred from donor *E. coli*. It could be concluded that in case of two *E. coli* isolates (MS-Aloor and 980), the genes responsible for *Hly* character and antibiotic resistance were co-transferred as they are borne on one and the same large plasmid. In *E. coli* MS-Aloor, EB treatment cured the resistance against streptomycin alongwith hemolytic activity with the loss of three plasmids of 67.61, 5.49, 4.07 Kb. Moreover the enterotoxin production was also noticed to have lost. In

E. coli MSP-7, SDS cured, ampicillin resistance and hemolytic activity with the loss of 63.1 Kb plasmid. Similarly AO cured oxytetracycline and hemolytic activity and elevated temperature cured ampicillin and tetracycline resistance alongwith hemolytic activity with the loss of 63.1 Kb plasmid. Ethidium bromide cured resistance against ampicillin and oxytetracycline with the loss of 63.1 and 17.78 Kb plasmids alongwith the loss of hemolytic activity.

In *E. coli* 980, SDS cured chloramphenicol and ampicillin resistances with the loss of 107.2, 5.39 and 3.54 Kb plasmids. Acridine orange cured oxytetracycline and chloramphenicol resistances by eliminating 107.2, 5.37, 3.89 and 3.54 Kb plasmids. Elevated temperature cured AOSu resistance and EB cured CSu resistance and hemolytic character by curing all the five plasmids. By all the curing agents the hemolysin producing activity and enterotoxin production were noticed to have lost. Harnett and Gyles (1984) and Baldev Raj (1991) demonstrated the co-transfer of antibiotic resistance with other characters. The results of the present work are similar to the observations of above authors.

None of the eleven enterotoxigenic *E. coli* transferred the *Ent* character to the recipient by conjugation. Although six of these transferred one or more antibiotic resistance(s) and two of the six also transferred *Hly* character to the

recipient. The plasmid profiles of nine isolates were studied after conjugal transfer. Of these nine, only four were enterotoxigenic. All the four isolates viz., MS-Aloor, 980, RLFB and E 3-25 harboured large as well as small plasmids. The plasmid profiles of the transconjugants of these isolates revealed the presence of large plasmids only. Since the Ent character was not transferred to the recipient alongwith the antibiotic resistance character it could be presumed that Ent character might have not borne by these large plasmids. The plasmid profiles of *E. coli*, MS-Aloor and 980 revealed that all the plasmids were cured by EB while in *E. coli* RLFB three of four plasmids were cured by EB, (Table 19). The RLIL assay of the cured isolates did not show any indication of enterotoxin production. The elimination of plasmids and absence of enterotoxin production in cured isolates by RLIL assay further supplements the statement that enterotoxin production is plasmid borne. Gyles *et al*. (1971), Harnett and Gyles (1984), Baldev Raj (1991) and Singh *et al*. (1992) have observed the plasmid borne nature of enterotoxin and the present study concurs with their findings.

In the present work, the Ent production was not transferred alongwith the antibiotic resistance character. This points to the possibility of the existence of this character on two different plasmids and hence absence of

co-transfer. Baldev Raj (1991) and Singh *et al*. (1992) observed co-transfer of enterotoxin production and antibiotic resistance only in one case each, but Bertin (1992) could not report any co-transfer of antibiotic resistance and enterotoxin production in conjugation experiment. This supports the present finding about the absence of co-transfer of antibiotic resistance and enterotoxin production.

In the present study there is co-transfer of antibiotic resistance and hemolysin production by conjugation. Though the transfer of *Ent* character was not observed, the elimination of *Ent* character alongwith antibiotic resistance and hemolysin production was observed in *E. coli* subjected to various curing agents indicating co-elimination of these characters. This is further supported by the plasmid profile analysis of wild, transconjugants and cured isolates. The plasmid profile analysis of wild and cured isolates clearly indicated the elimination of large as well as small plasmids. The phenotypic characterization of wild and cured isolates further gives credence to the plasmid borne nature of three characters.

5.15 Plasmid DNA photography

The photographs of the plasmid profiles were taken with a 35 mm single lense reflex camera, using 100 ASA Konica

135/36 colour film. The photographs were taken at f/16 aperture exposing the film for 90 sec. The film was developed and printed on film and print processors. Premkumar David *et al*. (1991) reported photographic technique with exposure at f/22 aperture for about 60-90 sec., but the film was developed and printed manually. Thus the present photographic technique helps replacing costly polaroid camera - built in - transilluminator which is a constraint in developing countries. Moreover the resolution, colour contrast and life of photographs taken by this technique are better than polaroid camera prints. The added advantage of the technique applied in the present study is the availability of a negative as a permanent record for further use, which is not feasible with polaroid camera photography.

Summary

SUMMARY

The incidence of *E. coli* mastitis was found to be 15.33 per cent. Of the 130 isolates of Gram negative bacilli 46 (35.38%) were *E. coli*. Among forty-six, forty-three were resistant and three were sensitive to thirteen antibiotics and chemotherapeutic agents tested by agar diffusion method. Among the 43 resistant isolates, multiple drug resistance (MDR) ranging from two to nine drugs was seen in 34 and single drug resistance was noticed in nine isolates. The maximum resistance of 78.26 per cent was recorded against rifampicin, followed by oxytetracycline (50%), tetracycline (37.78%), sulphadiazine (36.95%), streptomycin (23.91%), ampicillin and chloramphenicol (21.7%), nalidixic acid (19.56%), furazolidone (13.04%), co-trimoxazole (8.79%) and gentamicin (6.52%). There was cent per cent sensitivity to kanamycin and norfloxacin.

High degree of rifampicin resistance (78.26%) and moderate nalidixic acid resistance (19.56%) are some of the interesting findings. Among the MDR organisms, oxytetracycline and rifampicin resistance occurred in 76.2 per cent cases and oxytetracycline -rifampicin - tetracycline resistance recorded in 23.5 per cent cases were some of the significant findings.

Twenty-six different patterns of antibiotic resistance were noticed among 43 E. coli isolates giving a reliability of 60.46 per cent in differentiating the isolates. Though antibiogram was used as an epidemiological marker in the past, it was found to vary depending upon the number of antibiotics used. So antibiogram could only be used as an adjunct to plasmid profiling in epidemiological studies.

The results of the present work clearly indicated that norfloxacin, kanamycin, gentamicin, co-trimoxazole, furazolidone are the drugs of choice for the treatment of mastitis due to E. coli. The drugs like oxytetracycline, tetracycline, sulphadiazine, streptomycin, ampicillin, chloramphenicol and nalidixic acid could be employed for the treatment of mastitis due to E. coli, only after proper antibiotic sensitivity studies, in order to avoid dissemination of drug resistant E. coli. The use of rifampicin was not recommended for the treatment of E. coli mastitis.

Resistograms of 46 E. coli isolates were studied using seven heavy metal and quaternary ammonium compounds. The organisms evinced cent per cent resistance to lead, followed by antimony 32.6, copper 30.43, silver 19.56 per cent. Very low resistance (2.17%) was noticed against cetrimide. The organisms were fully susceptible to cadmium and mercury. Forty six E. coli isolates were differentiated in nine

different patterns with a reliability of 19.56 per cent in differentiating the strains. Hence the method was found to be least sensitive among the various methods employed for differentiation of the E. coli isolates.

There was a correlation between the antibiotic resistance and heavy metal ion resistance such as lead, antimony and copper, in descending order.

Of the 46 E. coli three (6.52%) were found to be hemolytic as they produced α-hemolysin on sheep blood agar. Two of three hemolytic strains were enterotoxigenic.

Thirteen of the 46 (28.26%) E.coli isolates tested by rabbit ligated ileal loop assay were found to be enterotoxigenic. Two of the thirteen (15.38%), enterotoxigenic isolates were also hemolytic. All these thirteen isolates were resistant to antibiotics and heavy metal ions.

Fourteen of the 24 (58.33%) drug resistant E. coli transferred drug resistance against one or more antibiotics to the recipient organism. In none of the cases, the furazolidone resistance was transferred.

All the three hemolytic E. coli isolates transferred the Hly character by conjugation to the recipient, at different frequencies, indicating the plasmid borne nature of

hemolysin production. Two of the three hemolytic E. coli were also enterotoxigenic but they could not transfer the enterotoxin producing character along with hemolytic character indicating that the genes for the production of hemolysin and enterotoxin could be located on different plasmids. None of the enterotoxin producing E. coli could transfer the character to recipient by conjugation.

Analysis of the 40 wild E. coli isolates from mastitis revealed diverse characters with respect to the number and molecular size of the plasmids. The number of plasmids varied from one to five with molecular size ranging from 1.47 to 128.8 Kb. Two strains were devoid of any plasmid. Among thirty-eight plasmid harbouring E. coli altogether 35 different plasmid profile patterns were obtained. Six E. coli isolates had only three different plasmid profiles, each profile representing two isolates. Both the isolates with similar plasmid profile revealed similar antibiogram, resistogram and Ent character, indicating the similarity between them and thus they could be the representatives of the same strain. Thus plasmid profiling would be a valuable epidemiological tool to differentiate and identify the strains of E. coli.

Study of plasmid profiles of hemolytic strains revealed that they contained large as well as small plasmids. On conjugation, only large plasmids along with hemolytic

character and drug resistance were transferred to the recipient. It was observed that the plasmids carrying genes for hemolysin production were also harbouring antibiotic resistance genes.

Of the eleven enterotoxigenic _E. coli_ (ETEC) studied for conjugal transfer of Ent character, six harboured small plasmids only, whereas the rest harboured both large and small plasmids. _Ent_ character was not transferred by conjugation, which could be due to its location on small plasmids which are non-conjugative and hence not transferred. Two of the ETEC strains which were hemolytic, transferred the _Hly_ character, but not the _Ent_ character. This indicated that _Ent_ and _Hly_ characters were borne on different plasmids.

Ethidium bromide at 100 µg/ml was found to be the best curing agent among the various chemicals used. The degree of curing increased with the time of exposure to the chemical. It cured antibiotic resistance, _Hly_ and _Ent_ characters. It also cured F'_lac_ plasmid in one case. The furazolidone resistance was not cured by any of the chemical curing agents tested. Moreover, it was not transferred by conjugation, which indicated that the F resistance was not plasmid borne. The nalidixic acid resistance was eliminated at high frequencies by EB indicating the plasmid borne nature of nalidixic acid resistance.

As compared to EB, AO and SDS were less effective curing agents. But Hly and Ent characters were cured by both the agents in some cases. In about half the number of cases antibiotic resistance was also cured by AO and SDS. Exposure to elevated temperature (45°C) was found to cure antibiotic resistances in all the cases tried. The rate of curing increased from fifth day onwards. It also cured Hly and Ent plasmids in all the cases tested and F'lac in one case.

Study of the plasmid profiles of cured strains revealed that the large plasmids were invariably eliminated in all the cases but few small plasmids were seen in cured isolates irrespective of the method of curing. The study of the phenotypic characters of the cured samples indicated that hemolysin production, enterotoxin production and antibiotic resistance characters were eliminated by curing, thus supporting the plasmid borne nature of these characters.

The conjugation and curing along with the study of the plasmid profiles of wild, transconjugants and cured strains established the correlation between virulence factors and plasmids. The transfer of antibiotic resistance and Hly character by conjugation, detection of single large plasmid in transconjugant by plasmid profiling, elimination of antibiotic resistance and Hly character by curing, absence of large plasmids in cured isolates as evidenced by plasmid profile studies clearly indicated that the Hly character and the

antibiotic resistance were located on one and the same plasmid and were co-transferred by conjugation.

A photographic technique for plasmid DNA was developed using ordinary single lens reflex (SLR) camera. The colour photographs with better resolution were obtained by exposing 100 ASA film at f/16 aperture for 90 sec. The developing of film and printing of the photographs were done on film and print processors.

The results obtained from the present study led to the following conclusions.

The indiscriminate usage of different antibiotics for the treatment of mastitis should be dispensed with and antibiotic sensitivity test should be conducted prior to the treatment for mastitis. The drugs identified were kanamycin, norfloxacin, gentamicin, co-trimoxazole, furazolidone and nalidixic acid.

The antibiogram and resistogram could serve as an adjunct to plasmid profile analysis in epidemiological studies.

The production of hemolysin, enterotoxin and, antibiotic resistance were found to be plasmid borne.

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Ethidium bromide was found to be the best chemical curing agent. Elevated temperature showed the best curing effect among physical and chemical methods.

Only large plasmids could be transferred by conjugation.

Plasmid fingerprinting could be used as a rapid, sensitive, economical and reproducible epidemiological tool in differentiating and identifying *E. coli* strains.

The hemolysin producing genes were located on self transmissible larger plasmids whereas antibiotic resistant genes were harboured on large as well as small plasmids.

There was a correlation between hemolysin production and antibiotic resistance since both the characters were co-transferred and co-eliminated.

The plasmid DNA photographic technique with ordinary single lens reflex camera was found to serve as a substitute to polaroid camera system.

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CHARACTERIZATION OF PLASMIDS OF *Escherichia coli* ISOLATED FROM MASTITIS

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ABSTRACT OF A THESIS

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ABSTRACT

Escherichia coli were isolated in 15.33 per cent cases of mastitis. Of the 46 *E. coli* isolated 43 were resistant to one to nine antibiotics and three were sensitive to all the 13 antibiotics tested. The organisms were resistant to rifampicin (78.26%) followed by oxytetracycline (50%), tetracycline (37.78%), nalidixic acid (19.56%), co-trimoxasole (8.69%) and gentamicin (6.52%). All the organisms were susceptible to kamamycin and norfloxacin. Among the multiple drug resistance oxytetracycline - rifampicin (OR) resistance was noticed in 76.2% cases. Twenty-six different patterns of antibiotic resistance were noticed among 43 *E. coli* isolates giving a reliability of 60.46 per cent in differentiating the isolates. Hence, antibiogram could only be used as an adjunct to plasmid profiling in epidemiological studies.

The resistograms revealed cent per cent resistance to lead, followed by antimony (32.6%), copper (30.43%), silver (19.56%) and cetrimide (2.17%). All the isolates were sensitive to cadmium and mercury. Among the 46 *E. coli* isolates, 9 different resistogram patterns were obtained giving reliability of 19.56 per cent in differentiating the strains.

A correlation between the antibiotics and heavy metal resistance such as lead, antimony and copper, was observed in descending order.

Of the forty-six E. coli isolates three (6.52%) were hemolytic on sheep blood agar. Two of the three hemolytic strains were also enterotoxigenic.

Thirteen of the 46 (28.26%) E. coli isolates were enterotoxigenic, when tested by rabbit ligated ileal loop assay. Two of the thirteen (15.38%) enterotoxigenic isolates were also hemolytic.

Fourteen of the 24 (58.33%) drug resistant E. coli transferred drug resistance against one or more antibiotics to the recipient organism. In none of the cases the furazolidone resistance was transferred.

All the three hemolytic E. coli isolates transferred the hemolytic character by conjugation indicating the plasmid borne nature of hemolysin production. None of the enterotoxin producing E. coli could transfer the character to recipient by conjugation.

Of the 40 wild E. coli screened for plasmid profiles two were devoid of any plasmid. The number of plasmids varied from one to five with molecular size ranging from 1.47 to

128.8 Kb. Among the thirty-eight plasmid bearing *E. coli* isolates, altogether 35 different plasmid profile patterns were obtained as six *E. coli* isolates showed three different plasmid profiles. The isolates with similar plasmid profiles revealed similar antibiogram, resistogram and *Ent* character.

The hemolytic strains contained large as well as small plasmids. Only large plasmids, along with hemolytic character and drug resistance were transferred to the recipient. It indicated that the plasmids carrying genes for hemolysin production also harboured antibiotic resistance genes. In case of enterotoxigenic *E. coli* (ETEC) the *Ent* character was not transferred by conjugation, which could be due to the location of the character on small plasmids which are nonconjugative. Two of the three ETEC strains which were hemolytic, transferred the *Hly* character, but not the *Ent* character to the recipient *E. coli*.

Ethidium bromide was found to be the best curing agent among the chemicals used. The degree of curing increased with the time of exposure. Ethidium bromide cured R-factors, *Hly*, *Ent* and F'*lac* characters. Furazolidone resistance was neither transferred by conjugation nor cured. But nalidixic acid resistance was eliminated by EB. Elevated temperature (45°C) cured antibiotic resistance *Hly*, *Ent*, F'*lac* characters. The

rate of curing increased from fifth day onwards. Large plasmids were eliminated in all the cases.

The study of, transfer of plasmids by conjugation, their elimination by curing, and plasmid profiles of wild, transconjugant and cured strains of E. coli revealed correlation between plasmid and virulence characters. There was a definite correlation between Hly character and antibiotic resistance as they were co-transferred on conjugation and the transconjugants harboured only one large plasmid exhibiting these two characters.

The photographic technique developed with ordinary single lens reflex camera, resulted in colour photographs with better resolution. This technique could serve as a substitute to polaroid camera system.

The results obtained from the present study led to the following conclusions.

* Indiscriminate use of antibiotics should be dispensed with and antibiogram should be carried out prior to treatment. The drugs of choice were kanamycin, norfloxacin, gentamicin, co-trimoxazole, furazolidone and nalidixic acid.

* The antibiogram and resistogram could serve as an adjunct to plasmid profile analysis in epidemiological studies.

* The role of plasmids in the production of hemolysin, enterotoxin and the antibiotic resistance was established.

* Ethidium bromide was found to be the best chemical curing agent, whereas elevated temperature (45°C) showed the best curing effect among physical and chemical methods of curing.

* Only larger plasmids could be transferred by conjugation. Plasmid profile analysis could be used in differentiating and identifying E. coli strains and thus serves as a better epidemiological tool.

* There was a correlation between hemolysin production and antibiotic resistance since both the characters were co-transferred and co-eliminated.

* Photographic technique of plasmid DNA with ordinary single lens reflex camera was found to serve as a substitute to polaroid camera system.