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Mechanism of ciprofloxacin resistance in *Shigella dysenteriae*

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Bacterial resistance to quinolones has emerged rapidly and such resistance has traditionally been attributed to the chromosomally mediated mechanisms that alter the quinolone targets and/or over-produce multidrug resistance efflux pumps. In the present investigation possible mechanism of ciprofloxacin (Cp) resistance in *Shigella dysenteriae* was studied. While the growth of sensitive *S. dysenteriae* was completely inhibited at 1 µg /ml concentration of ciprofloxacin, the resistant strain tolerated even 10 µg /ml concentration of ciprofloxacin. Mechanism of resistance was found to be the presence of plasmid.

Key words: Ciprofloxacin, *Shigella dysenteriae,* quinolone, plasmid, Resistance, SDS.

INTRODUCTION

Microbial resistance to antimicrobials is emerging as an important public health problem in both hospitals and the community. The 20th century has been considered the antimicrobial era whereas the 21st century may well present the post-antimicrobial era. The reason for this dramatic change, should it come to pass, is the development of bacterial resistance to antimicrobial agents. This emerging resistance is now challenging the clinical utility of many antimicrobial agents such that the chemotherapy of hospitalized patients with serious infections has been compromised. If the problem with resistance is to be successfully dealt with by clinicians, the mechanisms of such resistance must be known and understood. An understanding of these important microbial resistance mechanisms will help the clinician identify circumstances in which resistance may be a problem, as well as, evaluating the potential usefulness of an alternate antimicrobial agent against resistant microbes (Stratton, 2000).

Plasmid-mediated resistance to quinolones was first reported in 1998 in a *Klebsiella pneumonia* clinical strain solated in 1994 in Birmingham, Ala (Ghosh et al. 1997). Ciprofloxacin, a new fluoroquinolone, is a potent, broad spectrum antibacterial agent. It rapidly blocks spectrum of antibacterial agents. It rapidly blocks bacterial DNA replication by inhibiting DNA gyrase an essential prokaryotic enzyme that catalyzes chromosomal DNA supercoiling (Bhutta et al., 1999). Spence in Spence and Towner (2003) compared the *in vitro* activity of Moxifloxacin and Ciprofloxacin against 226 nosocomial isolates of *Acinetobacter baumanii* out of them 49% were resistant to Ciprofloxacin and 39.4% were Moxifloxacin resistant. A *Gyr A* mutation at *Ser*-83 was found in all ciprofloxacin resistant isolates.

Routine surveillance of antimicrobial susceptibility to all classes of clinically used agents is necessary to detect resistance trends in different parts of the world, detecting the emergence of new resistance mechanisms that guide infection control measures and public health guidelines such trends may help in identifying outbreaks of resistant organisms. Such a check seems to be the best way to find appropriate antibiotic regimens (Ashtiani et al.,

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2009).

Resistance has emerged even to newer, more potent antimicrobial agents. Therefore, to report resistance rates to antimicrobial agents, Ashtiani et al. (2009) isolated 2487 stool cultures from tertiary care hospital between 1996 and 2000, 2001 and 2005, out of the fecal cultures 53% of the cultures were *Shigella* spp. and 28% of the isolates were *Salmonella* spp. Resistance to antimicrobial agents increased among most of the pathogens between 2001 and 2005. An increase in the rate of resistance was observed in *Shigella* spp. for kanamycin and Ceftazidime and among *Salmonella* spp. for Nalidixic acid and Ceftazime (Ashtiani et al., 2009).

S. dysenteriae is a causative agent of bacillary dysenteries and the emergence of the drug resistance pattern was found to be these species. Infections caused by drug resistant *S. dysenteriae* are often serious sometimes even life threatening. The aim of the present investigations was to detect the exact mechanism of resistance to ciprofloxacin in *S. dysenteriae*.

Antibiotic resistance and associated genes are ubiquitous and ancient, with most genes that encode resistance in human pathogens having originated in bacteria from the natural environment (eg, ß-lactamases and fluoroquinolones resistance genes, such as qnr) (Finley et al., 2013). Consumption of raw vegetables represents a route of human exposure to antibiotic-resistant bacteria and resistance determinants naturally present in soil (Marti et al., 2013).

MATERIALS AND METHODS

Bacterial strain and its cultivation

The organism used in this study was one resistant *S. dysenteriae strain*, obtained from Agarkar research institute, Pune, India. One sensitive strain *S. dysenteriae* was obtained from Grant Medical College, Mumbai. *S. dysenteriae* cells were cultured on Nutrient agar (NA) (HiMedia, India) slants containing Beef extract 0.5; Peptone 2.5; Sodium chloride 2.5; Agar15 in a liter of distilled water pH was maintained at 7.4, slant were incubated at 37°C for 24 h.

Detection of antibiotic resistance and sensitivity in bacteria

A Standard agar diffusion method in the study of Reddish et al. (1929) was used here. Solution of ciprofloxacin in different concentrations was added to a well cut in the Muller Hinton Agar (MH) (HiMedia, India). MH agar plates were inoculated with McFarland 0.5 standard of each resistant and sensitive organism. Plates were incubated for 16 to 18 h at 37°C. Zone of inhibition was measured in millimeter scale and compared by following the NCCL standard. Tube dilution method in the study of Piddock (1990) was applied here. One hundred microgram (100 µg) of ciprofloxacin stock solution was prepared in distilled water and sterilized by filtration (Millipore filter size 0.45 nm was used). Nutrient broth was used as media. Different concentrations of ciprofloxacin were prepared in tubes. The innoculum was 10^8 cfu/ml (equivalent to Mc Farland 0.5 standard). Incubation was carried out at 37°C for 72 h on rotatory shaker. Turbidity was measured on LUMICHEM 20 at 540 nm. Results were compared with sensitive strain.

Isolation of plasmid

Plasmid DNA was isolated from *Shigella dysenteriae* resistant strain by alkaline lysis method (Lavery et al., 1997). Overnight cultures in Luria Bertani (LB) broth (1.5 ml) were harvested by centrifugation at 14,000 g for 3 min at room temperature. The cells were re-suspended in 100 µl of solution A (25 mM Tris HCL, 10 Mmedta, 50 mMGlucose + Lysozyme 20 mg/ml) and incubated at 37°C for 1 h. Freshly prepared 200 µl of solution B (0.2 M NaOH +1%SDS) was added for lysis of cells and mixed thoroughly by vortexing. It was then kept on ice for 5 min after which, 150 µl of solution C (5 M Potassium acetate) was added. It was then kept on crushed ice for further 15 min. Centrifugation of tubes was done at 14 000 g for 5 min at 4°C. The supernatant was transferred to new microfuge tubes. DNA was extracted with phenol: chloroform (25:24 ml) mixture and precipitation with the ice-cold absolute ethanol. After centrifugation, the supernatant was discarded and extracted DNA was air dried and dissolved in 50 µl of 50 mM Tris EDTA Buffer pH 8.0.

Spectrophotometric method

Qualitative determination of DNA was carried out by spectrophotometric method given by Maniatis et al. (1982).

Agarose gel electrophoresis

Agarose gel electrophoresis of plasmid was conducted for separation and analysis of nucleic acid using the method in the study of Meyers et al. (1976). Plasmid DNA (1 to 2 µg/ml) from *Shigella dysenteriae* resistant strain was electrophoresed in 2% agarose slab gels at 50V in Tris Acetate buffer(TAE) running buffer (0.15 m Tris base, 0.5M EDTA, 1 M Glacial acetic acid). Stained with ethidium bromide and photographed under U. V. illumination. The size of plasmid was estimated by comparing mobility with standard plasmid marker (pBR322/Hae III Digest, Banglore Genei, Banglore, INDIA).

Elimination of resistance due to plasmid

Elimination of resistance due to plasmid was carried out using the treatment of sodium dodecyl sulfate (SDS) and that of Tomoeda et al. (1968). An overnight culture of resistant (R) cells in penassay broth containing 10 µg/ml of ciprofloxacin was diluted to10^3 cells/ml in broth and add to the tubes containing 10% (w/v) SDS and shaken at 37°C. After appropriate dilution in saline cells were plated on nutrient agar. All colonies on the plate were tested for their ciprofloxacin resistance character on nutrient agar containing different concentrations of ciprofloxacin.

RESULTS

In agar diffusion test, when 0.25 and 0.5 µg/ml concentration of ciprofloxacin was tested, no zone of inhibition was observed in sensitive strain. When 1 to 10 µg/ml concentration of ciprofloxacin in sensitive *S. dysenteriae* strain was tested, zones of inhibition in each case were 21 to 29 mm (Figure 1; Table I). The resistant strain showed no zone of inhibition at 0.25 to 10 µg/ml concentration of ciprofloxacin (Figure 2). Effect of ciprofloxacin was studied on growth of *S. dysenteriae* sensitive strain and resistant strains. The growth in terms of turbidity was measured at 12 h interval at 540 nm

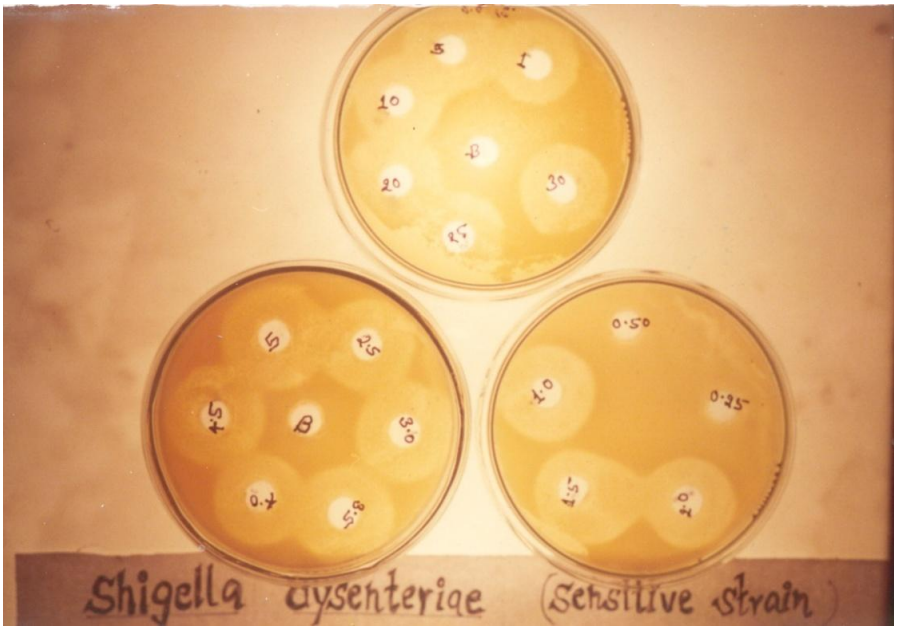

Figure 1. *S. dysenteriae* sensitive strain showing sensitivity to 1 to 10 µg/ml concentration of Ciprofloxacin.

Table 1. Diameter of zone of inhibition in *Shigella dysenteriae* to concentrations of Ciprofloxacin.

Concentrations of Ciprofloxacin (µg/ml)	Resistant strain (mm)	Sensitive strain (mm)
0.25	No zone of inhibition	No zone of inhibition
0.5	“	“
1	“	21
1.5	“	21.5
2.0	“	22
2.5	“	22.5
3.0	“	23
3.5	“	23.5
4.0	“	24
4.5	“	24.5
5	“	25
6	“	26
7	“	27
8	“	28
9	“	28.5
10	“	29

using colorimeter. Increased turbidity was observed in resistant strain and sensitive strain showed no turbidity. The inhibition in the growth of sensitive *S. dysenteriae* was 60 to 80% (that is, growth up to 20 to 40%) at 0.1 to 0.7 µg/ml concentration of ciprofloxacin, as compared to the control set. At 1 µg/ml concentration of ciprofloxacin, the growth of sensitive strain was completely inhibited (Figure 3) while the inhibition in the growth of resistant strain was 10 to 30% (that is, growth up to 70 to 90%) at 1 to 10 µg/ml concentration of ciprofloxacin as compared to the control set.

The resistant strain tolerated up to 10 µg/ml concentration of ciprofloxacin (Figure 4). Plasmid was isolated from resistant *S. dysenteriae* strain. Plasmid was not detected in sensitive strain. Agarose gel electrophoresis of plasmid DNA was carried out. The plasmid band was compared with standard plasmid marker. The single band was detected in Lane 2 (that is isolated plasmid DNA from resistant *S. dysenteriae*). The standard plasmid marker pBR322 /Hae III Digest showed

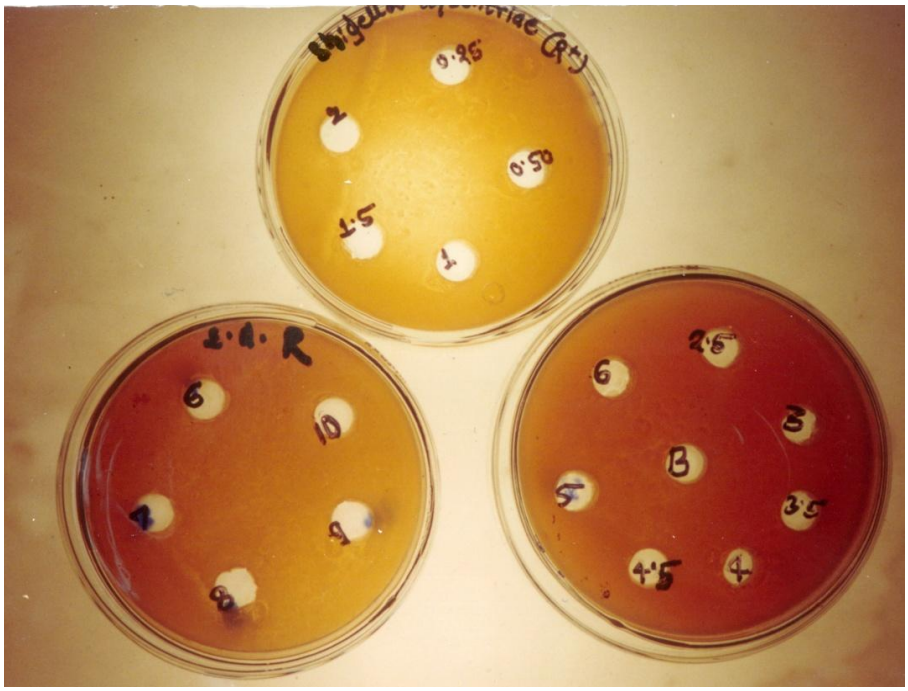

Figure 2. *S. dysenteriae* resistant strain showing resistance to 1 to 10 µg/ml concentration of Ciprofloxacin.

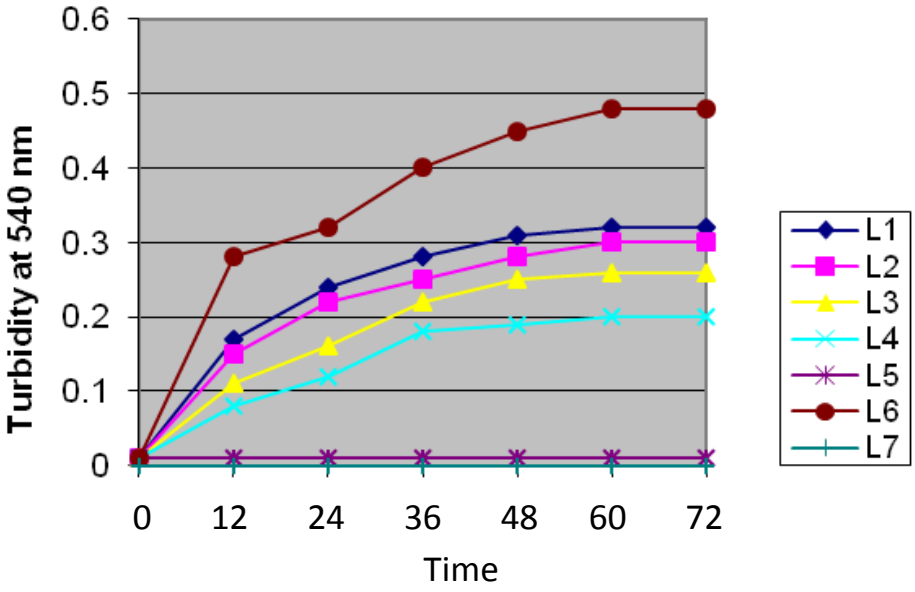

Figure 3. *S. dysenteriae* sensitive strain showing sensitivity to 1 µg/ml concentration of ciprofloxacilin. Different concentrations of ciprofloxacin were, L1- 0.1 µg/ml; L2-0.3 µg/ml; L3-0.5 µg/ml; L4-0.7 µg/ml; L5-1 µg/ml; L6-control; L7- blank.

bands (Figure 5). The size of isolated plasmid was 587(bp). It seems that mechanism of resistance was found to be the presence of plasmid.

Also the concentration of DNA in the S. *dysenteriae* was calculated. Concentration of DNA in sample solution was 1064 µg/ml. The ratio of protein to extracted DNA was 1.87 that means extraction of DNA was pure. *S. dysenteriae* resistant cell carrying plasmid was treated with sodium dodecyl sulphate at 2 to 10% concentrations. Sodium dodecyl sulphate was effective in producing

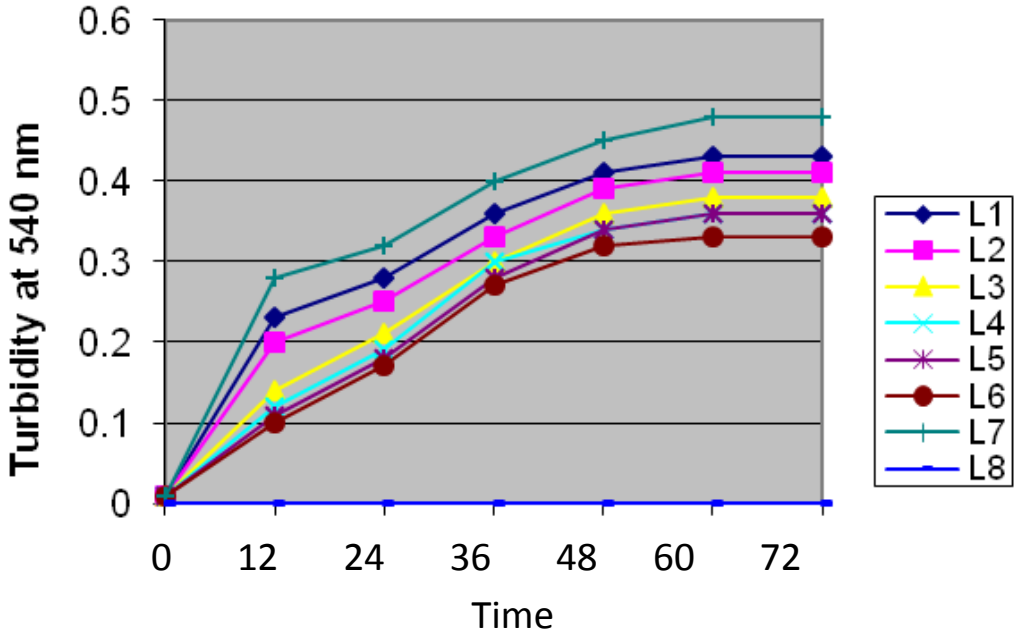

Figure 4. *S. dysenteriae* resistant strain showing resistance to 10µg/ml concentration of ciprofloxacin. Different concentrations of ciprofloxacin were, L1- 1 µg/ml; L2-2.5 µg/ml; L3-5 µg/ml; L4-7 µg/ml; L5-9 µg/ml; L6-10 µg/ml; L7-control; L8- blank.

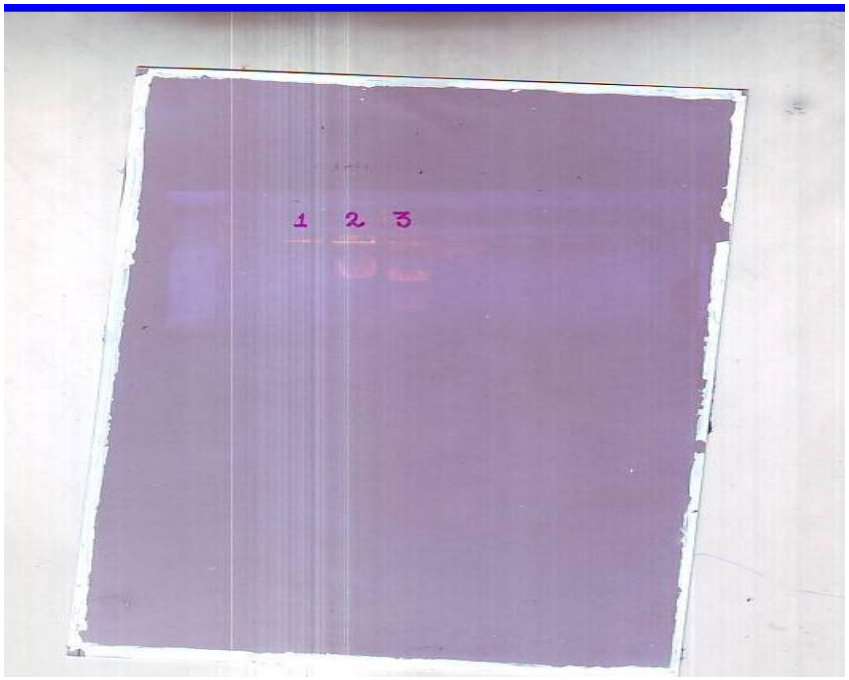

Figure 5. Agarose gel electrophoresis of plasmid DNA of *Shigella dysenteriae* resistant to ciprofloxacilin, Ampicillin and Zinc, showed single band (Lane 2) and Standard plasmid marker pBR322 DNA / Hae III Digest showed bands of different molecular sizes (Lane 3).

susceptible cells. The growth of resistant strain was 19 to 33% at 2 to10% SDS concentration as compared to control set. After SDS treatments, resistant strains were analyzed at interval for the presence or absence of drug resistance against different concentrations of ciproflo-xacin. The resistance was lost and the resistant strain

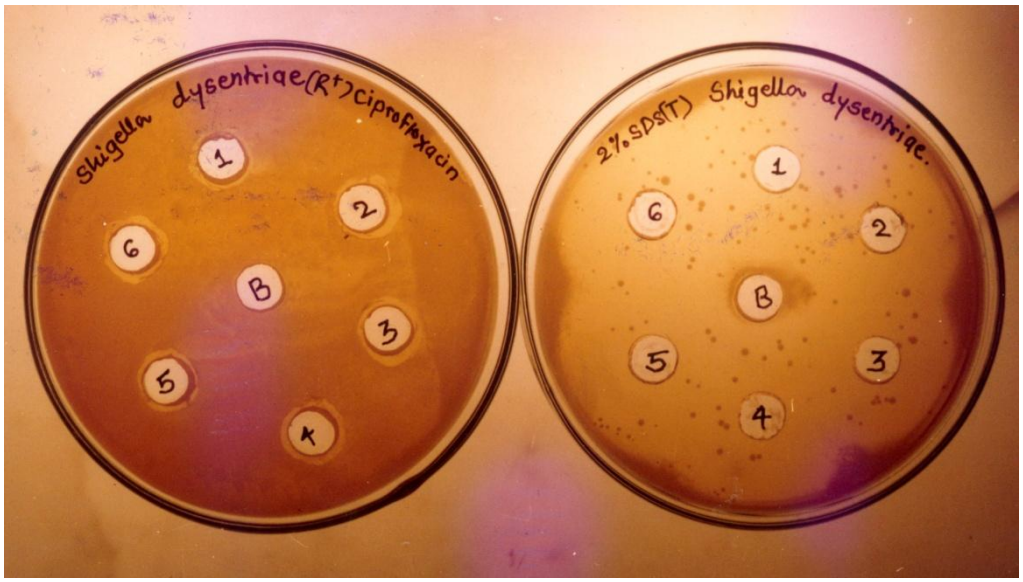

Figure 6. Elimination of resistance factor by treatment of Sodium dodecyl sulfate in *Shigella dysenteriae.*

strain was changed to sensitive strain (Figure 6).

DISCUSSION

The present investigation deals with the mechanism of ciprofloxacin resistance in *S. dysenteriae*. The *S. dysenteriae* resistant strain showed resistant to 10 µg/ml concentration of ciprofloxacin with no zone of inhibition, which was much superior result than the observation made by Cormican and Jones (1995), where they reported the MIC of ciprofloxacin as ≥4 µg/ml. Therefore it is indicated that the strain used in the present study seems to be more resistant. However, sensitive strain showed 21 mm zone of inhibition to1 µg/ml of ciprofloxacin. Hence, it indicates that the efficiency of ciprofloxacin varies according to resistant and non-resistant strains.

In the present investigation, plasmid was isolated from *S. dysenteriae* resistant strain while in sensitive strain, no plasmid was found. On elimination of plasmid from resistant strain sensitivity was observed in *S. dysenteriae*. This indicates that plasmid was responsible for resistance development in the microorganisms. The plasmid profile of the strain was studied. The single DNA band was detected on agarose gel electrophoresis. The plasmid molecular size of *S. dysenteriae* resistant strain was 587bp. This was in agreement with the studies of other workers, Ghosh et al. (1997) isolated plasmid from *A. aminolytica* and GS19h each possessed more than one plasmid. Kessie et al. (1998) reported that, *Staphylococcus lenticus* carried small plasmids of molecular size 2.0, 2.3, 2.7, 4.3 and 4.4 kb and *S. hylicus* isolated, harbored up to seven small plasmids ranging from 2.0 to 4.7 kb.

In the present study, elimination of resistance due to plasmid was carried out using sodium dodecyl sulphate (SDS) treatment. An anionic surface activating agent sodium dodecyl sulphate eliminated plasmid carrying resistance factor in *S. dysenteriae*. After SDS treatment, resistant cells lost their resistant character and changed to sensitive cells. SDS treated cells showed same zone of inhibition as that of sensitive strain. This is in agreement with the observations made by Tomoeda et al. (1968) that the action of SDS proved to be effective enough to eliminate R factor present in bacteria at frequencies up to 100%. Other workers in curing plasmid used different reagents. Rotimi and Duerden (1982) reported action of acridine dye on *E.coli* R^+ and *Bacteroides fragillis* cells leading to complete loss of R-factor. Pan et al. (1981), Attfield and Pinney (1985) used mytomicin C and bleomycin treatment for curing multi-copy plasmid. The relation of plasmid in development resistance was studied by Pan et al. (1981) in *Enterobacter aerogenes* against mercury.

Furthermore, he related mercury resistance due to the synthesis of outer member proteins coated by genes on plasmid. Denis and Moreau (1993) reported decrease in permeability in resistant strains compared with sensitive, due to modification of outer member proteins that was often evolved with resistance. Observation of Tran and Jacoby (2002) also supports the present finding, who reported a multi resistance plasmid that encodes transferable resistance to quinolones. Therefore it indicates that plasmid elimination which synthesizing membrane protein was responsible for drug resistance. It seems

that similar reason could be responsible for the ciprofloxacin resistance in the *Shigella dysenteriae* in the present study. The formation of the plasmid in the bacterial cell is mainly responsible for ciprofloxacin resistance in microorganisms.

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