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Full Length Research Paper

Mechanism of action of pefloxacin on surface morphology, DNA gyrase activity and dehydrogenase enzymes of *Klebsiella aerogenes*

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The aim of the present study was to investigate susceptibility of *Klebsiella aerogenes* towards pefloxacin. The MIC determined by broth dilution method and Hi-Comb method was 0.1 µg/ml. Morphological alterations on the cell surface of the *K. aerogenes* was shown by scanning electron microscopy (SEM) after the treatment with pefloxacin. It was observed that the site of pefloxacin action was intracellular and it caused surface alterations. The present investigation also showed the effect of Quinolone pefloxacin on DNA gyrase activity of *K. aerogenes*. DNA gyrase was purified by affinity chromatography and inhibition of pefloxacin on supercoiling activity of DNA gyrase was studied. Emphasis was also given on the inhibition effect of pefloxacin on dehydrogenase activity of *K. aerogenes*.

Key words: Pefloxacin, *Klebsiella aerogenes*, scanning electron microscopy (SEM), deoxyribonucleic acid (DNA) gyrase, dehydrogenases, Hi-Comb method, minimum inhibitory concentration (MIC).

INTRODUCTION

Klebsiella spp. is opportunistic pathogen, which primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus or chronic pulmonary obstruction. *Klebsiella* accounts for 6 to 17% of all nosocomial urinary tract infections (UTI) and shows an even higher incidence in specific groups of patients at risk, for example, patients with neuropathic bladders or with diabetes mellitus (Bennett et al., 1995; Lye et al., 1992).

Since nalidixic acid and its first analogs, pipemidic acid and oxolinic acid, were found to have good activity against Gram-negative bacteria involved in UTI, the quinolone class has been intensively studied and many new active products have been synthesized (Albrecht, 1977; Domagala et al., 1986). They are characterized by broad spectrum activity with oral efficacy. These agents have been shown to be specific inhibitors of the A subunit of the bacterial topoisomerase deoxyribonucleic acid (DNA) gyrase, the Gyr B protein being inhibited by coumermycin A_1 and novobiocin (Gellert et al., 1976; Hooper et al., 1982).

DNA gyrase are topoisomerases catalyze the supercoiling of relaxed closed circular DNA coupled to the hydrolysis of Adenosine triphosphate, ATP (Wang and Liu, 1979). Quinolone antimicrobial agents form a complex with gyrase and DNA that blocks replication fork movement (Drlica, 1984; Drlica et al., 1980). To understand *in vivo* mechanism of pefloxacin, we examined the contribution of enzyme inhibition to drug action against *K. aerogenes*.

In this report, sensitivity of *Klebsiella aerogenes* against quinolone pefloxacin was studied by broth dilution method and Hi-Comb method (Hi-media) and MIC was determined. Morphological alterations on the cell surface of *K. aerogenes* were studied after treatment with pefloxacin by scanning electron microscope (SEM).

Emphasis was also given on inhibition of dehydrogenases enzymes of organism by the antibiotic.

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Abbreviations: **SEM**, Scanning electron microscope; **UTI**, urinary tract infections; **MIC**, minimum inhibitory concentration.

MATERIALS AND METHODS

Bacterial strain, culture media and drug

K. aerogenes NCIM 2239 was obtained from the National Collection of Industrial Microorganisms (NCIM), Pune, India. Bacterial strain was grown at 37°C in nutrient broth medium (Hi-media, India) and maintained at 5°C. Culture medium was autoclaved at 121°C for 20 min, and the organism was subcultured in nutrient broth and nutrient agar plates and after 24 h incubation used as an inoculum. Drug pefloxacin was obtained from Sigma chemicals (U.S.A.) in the form of pefloxacin mesylate dihydrate.

Determination of minimum inhibitory concentration (MIC)

Sensitivity of pefloxacin against *K. aerogenes* was studied by determining Minimum inhibitory concentration (MIC) by broth dilution method and by Hi-Comb method (Hi-media, India). In Broth dilution method, different concentrations of antibacterial agents were prepared. Inoculums were adjusted to 0.5 Macfarland turbidity standards and an aliquot of 0.1 ml of inoculums was added to each tube of dilution. The tubes were incubated at 37°C overnight. MIC was read visually following 24 h of incubation and was defined as the lowest concentration that produced no visible turbidity (NCCLS, 2001).

In Hi-Comb method, at least 4 to 5 well isolated colonies of same morphological type from agar plate were touched with a wire loop and growth was transferred to tube containing 5 ml of broth. Turbidity was compared with 0.5 Macfarland standards and adjusted with sterile saline or broth if required. Organism was spread on agar plates by spread plate method and Hi-Comb strip was placed on medium in sterile condition. Plate was incubated for 24 h at 37°C and zone of inhibition was observed. According to Hi-Comb MIC test, MIC value is the value at which the zone converges on the comb-like projections of the strips and not at the handle and zone of inhibition below the lowest concentration is to be considered (CLSI, 2008).

Effect of pefloxacin on morphology of *K. aerogenes*

Surface morphology was studied by SEM on Quanta 200 ESEM system (Icon Analytical Equipment Pvt. Ltd., India), after determining the MIC. Specified concentration of Pefloxacin (0.1 µg/ml) was added to culture in the logarithmic phase of growth (12 h culture) at 37°C. After different incubation time period 3, 6 and 24 h with pefloxacin, SEM was performed. Treated and untreated cells after incubation were washed by centrifugation in 0.9% NaCl and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and images were taken by SEM (Klainer and Perkins, 1974).

Effect of pefloxacin on dehydrogenases activity

According to the procedure followed by Guha and Mookerjee (1978), the effect of pefloxacin on inhibition of dehydrogenase enzymes activity of *K. aerogenes* was studied. Cells grown for 48 h at 37°C were used as samples. According to the procedure, chloramphenicol was added to disrupt cell wall and MIC concentration of pefloxacin was added. 0.005 M substrates of TCA cycle, α-Ketoglutaric acid, succinic acid, isocitric acid and glutamic acid was added to each tube. 0.5 M Potassium phosphate buffer at pH 7.0, 0.3M $MgCl_2$ and Triphenyl Tetrazolium Chloride solutions (9 mg/ml) were finally added and OD values of the control tubes were measured and percentage inhibition of the activity of enzymes was calculated.

Effect of pefloxacin on DNA gyrase of K. aerogenes

DNA gyrase was purified from *K. aerogenes* by affinity chromatography (Tabary et al., 1987; Bjornsti and Osheroff, 1999). The work was carried out at National Institute of Research in Reproductive Health, Mumbai, India. *K. aerogenes* (40 g) was suspended in 40 ml of 100 mM Tris hydrochloride (pH 7.6) -20% sucrose. Dithiothreitol, EDTA, phenylmethylsulfonylfluoride were added to 2, 20 and 1 mM, respectively. 16 mg of lysozyme was added after 10 min in ice, and the mixture was frozen at -80°C after an additional 10 min, and thawed at 20°C; Brij 58, $MgCl_2$ and KCl were then added to 0.1%, 5 mM and 750 mM respectively. The lysate was mixed by several inversions and centrifuged at 100,000 × g for 3 h. Dialysis of supernatant was done against buffer A (20 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM Tris hydrochloride, 10 % glycerol [pH 7.8]). It was then loaded onto a 2 ml novobiocin-Sepharose column and washed with buffer A until the optical density at 280 nm was minimal, with 25 ml of 20 mM ATP in buffer A and finally with buffer A until there was no more A_{280}. Elution of DNA gyrase was done with 5 M urea in buffer A. Fractions were dialyzed against buffer A without $MgCl_2$ and glycerol, concentrated with polyethylene glycol 20,000, dialyzed against buffer A without $MgCl_2$ but with 50% glycerol and stored at -20°C. Protein concentrations were estimated to be 400µg/ml by Folin Lowry method. The purity was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis and the specific activity was 2×10^4 U/mg of protein.

Relaxed pBR322 DNA (Topogen Inc., USA) and 1 U of gyrase were incubated in an 18 µl reaction mixture containing 25 mM KCl, 20 mM *N*-2-hydroxyethylpiperazine-*N*^{1}-2-ethanesulfonic acid (HEPES), 4 mM dithiothreitol, 1.7 mM spermidine, 1.7 mM ATP, 6 mM magnesium acetate, 0.5 mM EDTA, 3% ethylene glycol and 2 mM Tris hydrochloride (pH 8). Under these conditions DNA is totally supercoiled in 30 min at 37°C. The mixture was incubated for 30 min at 37°C and the reaction was stopped at 0°C by the addition of 1µl of 1% sodium dodecyl sulfate and 2 µl of 0.4% bromophenol blue in 60% sucrose. The extent of supercoiling were determined by 1% agarose gel electrophoresis.

1% agarose gel was made in EDTA (36 mM), Tris hydrochloride (pH 7.5) by using agarose. Samples applied to the gel and resolved at 70 V for 3 h. The gel was stained with ethidium bromide at room temperature and photographed under UV.

RESULT

MIC of *K. aerogenes* against pefloxacin determined by broth dilution method and Hi-Comb method was found to be 0.1 µg/ml. According to broth dilution method, inhibition in the growth was read visually and by Hi-Comb method, zone of inhibition was seen after 24 h incubation (Figure 1).

In the present report, morphological changes induced by pefloxacin on *K. aerogenes* are shown in Figures 2 and 3. Figure 2 shows images of control organism which were seen to be rod shaped bacilli. In Figure 3, changes on surface of cells were seen after different time interval of pefloxacin treatment; Figure 3a shows elongation and spheroplast formation after 3 h incubation time period; Figure 3b also shows elongation and spheroplast formation after 6 h incubation time period; Figure 3c shows pieces of bursted cells along with elongated and spheroplast cells.

Cells of *K. aerogenes* were exposed to pefloxacin and

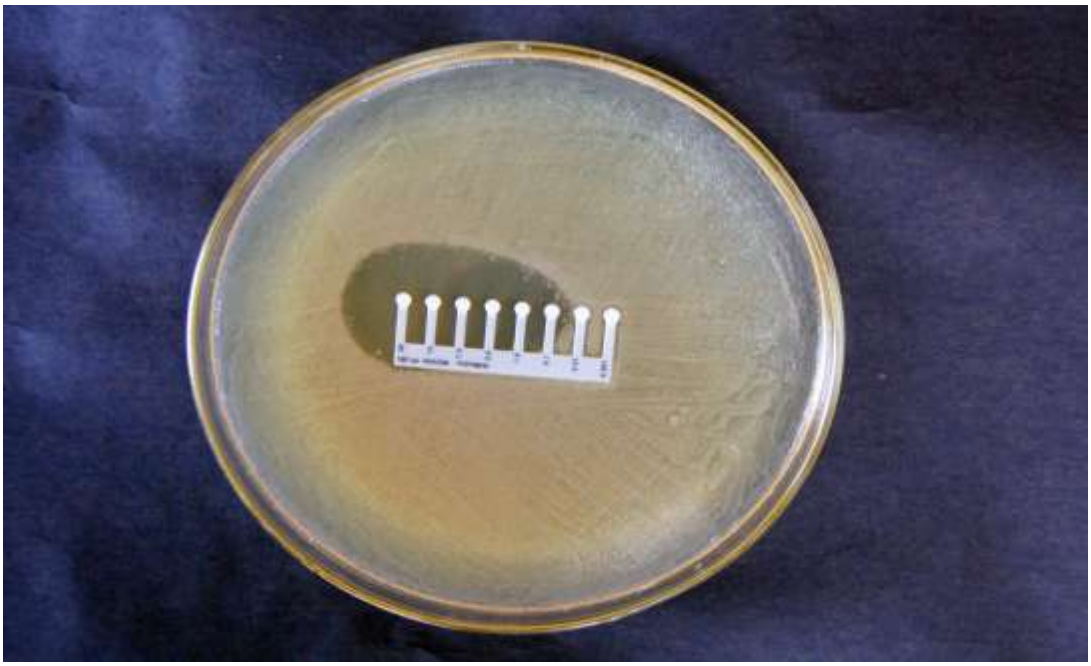


Figure 1. Determination of the MIC of Pefloxacin against *K. aerogenes* using Hi-Comb method. The clear area indicated the growth inhibition zone of the bacterium.

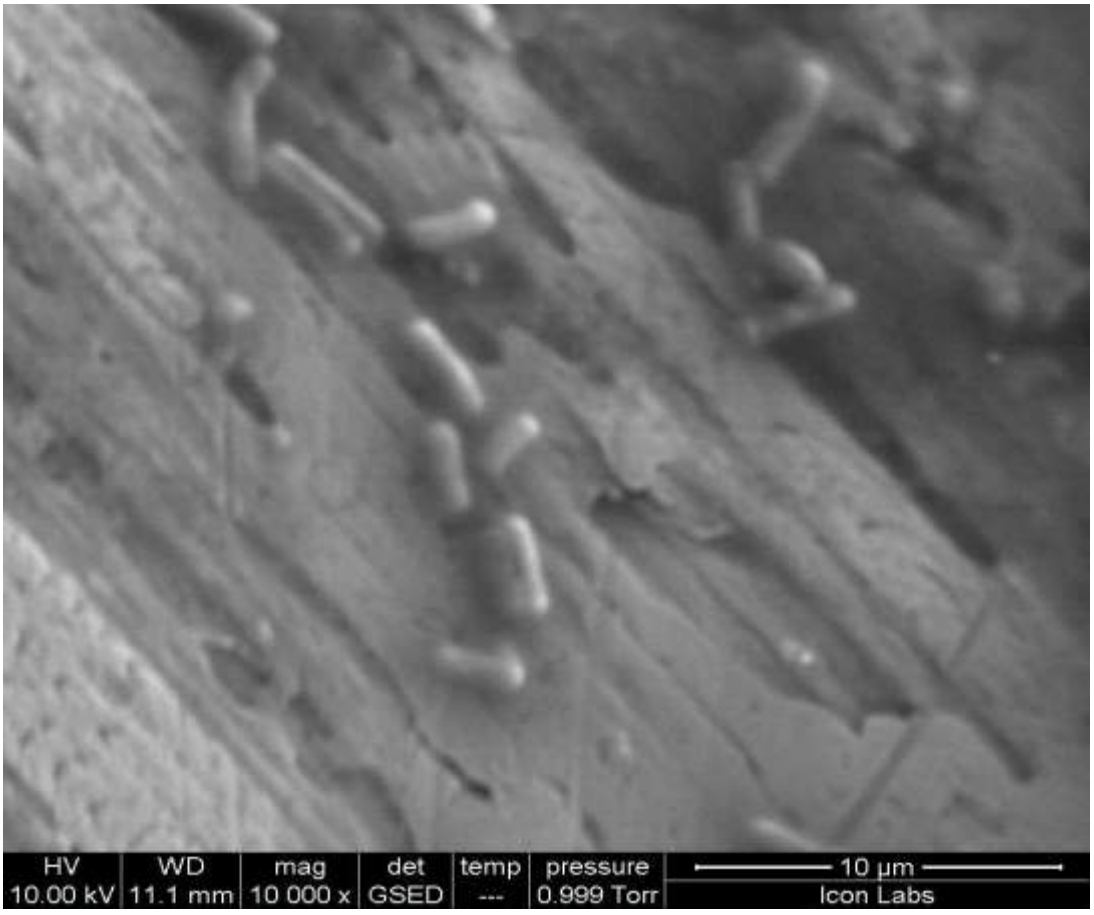
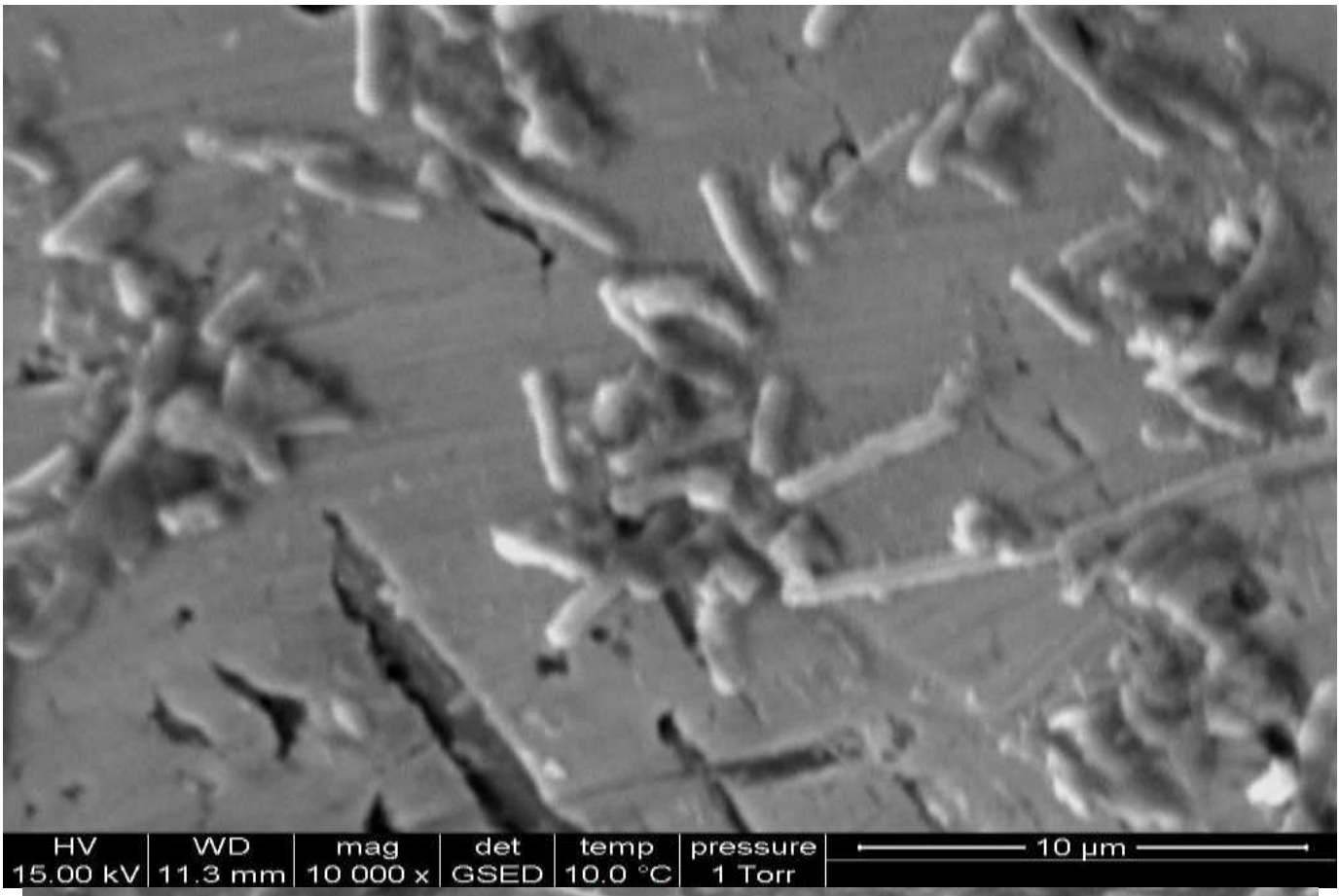


Figure 2. *K. aerogenes* under the untreated condition is a rod-shaped bacilli.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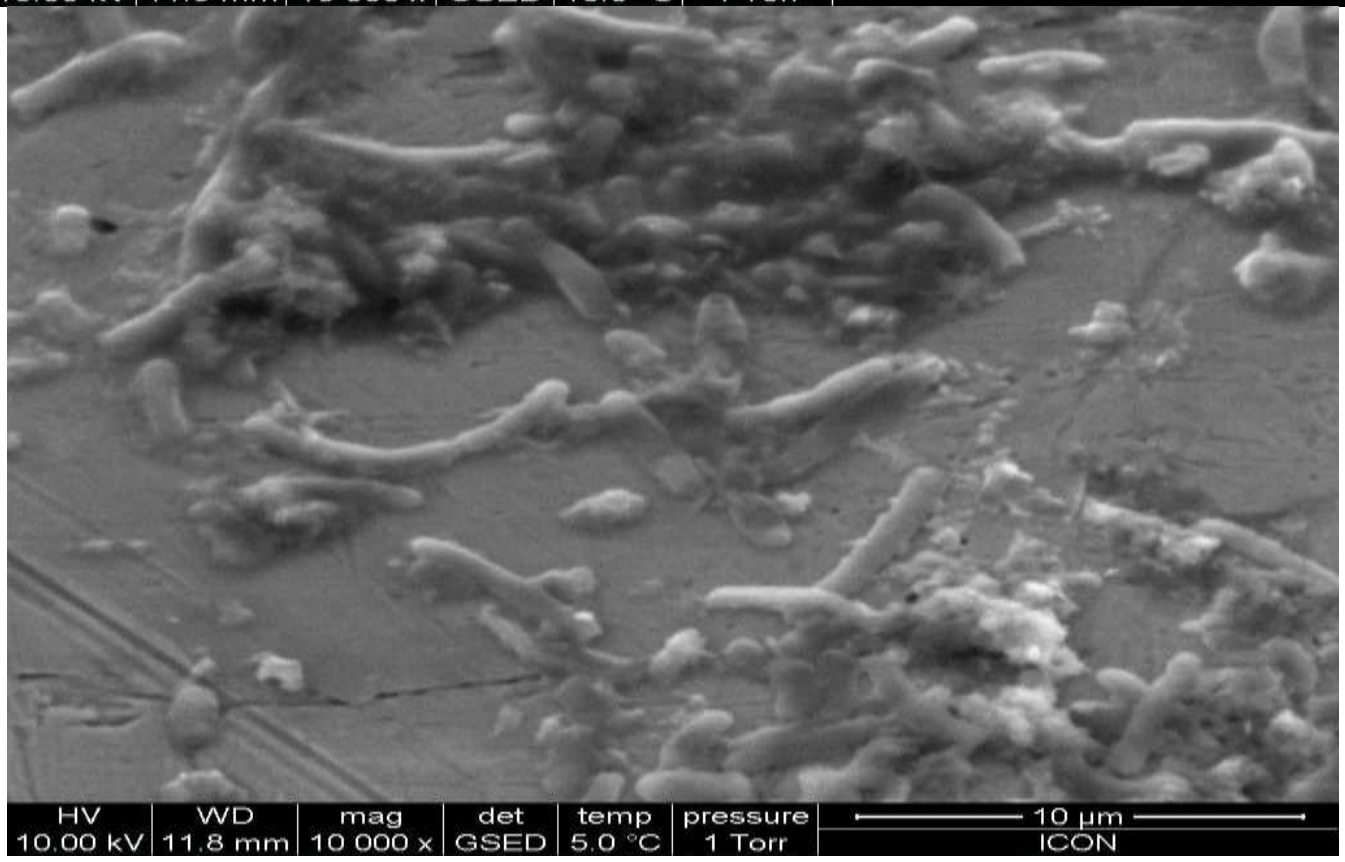
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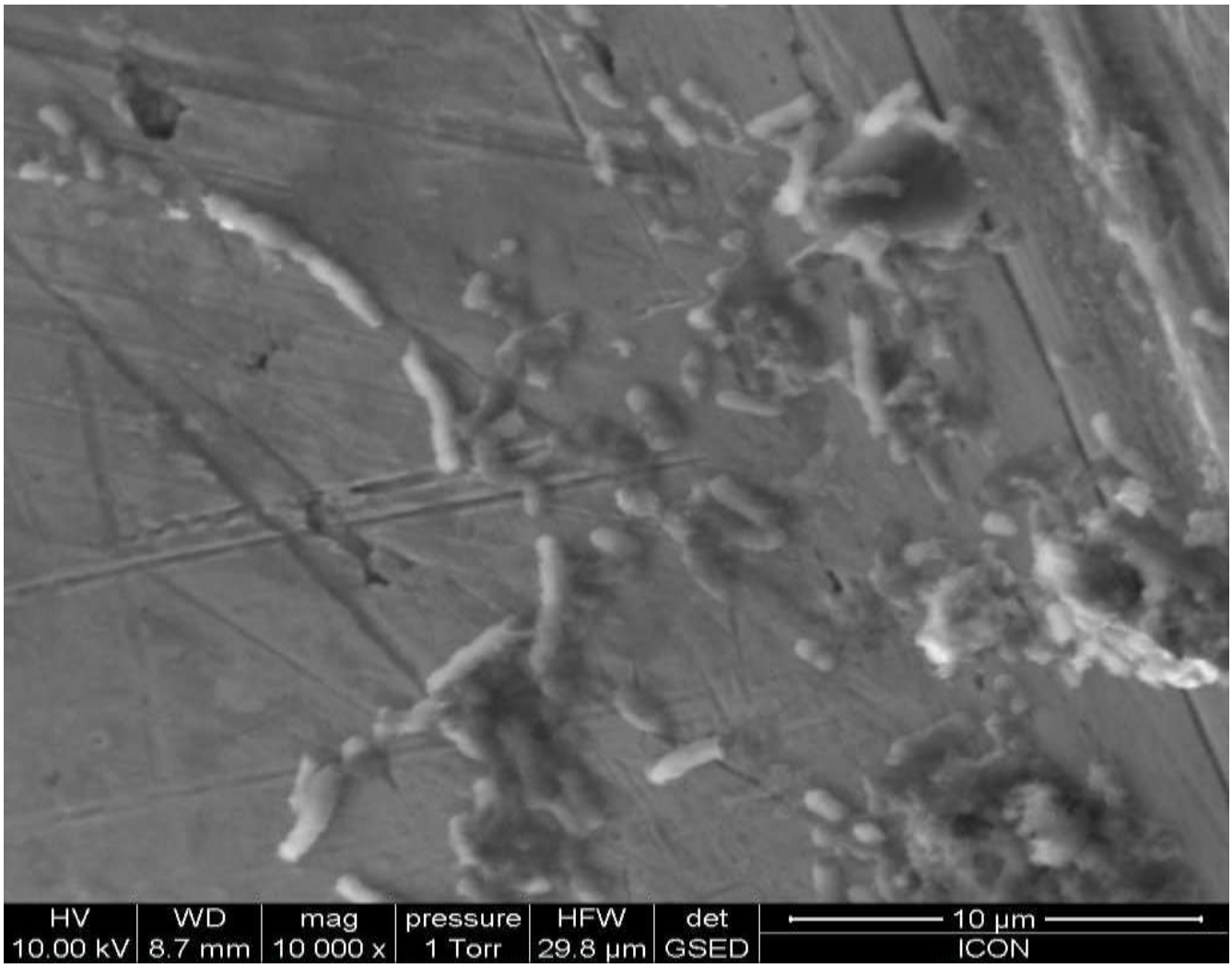

Figure 3. *K. aerogenes* following treatment with Pefloxacin (0.1 µg/ml) at different incubation time period, a. exposed for 3 h, b. exposed for 6 h, c. exposed for 24 h.

inhibition on the activity of dehydrogenases enzymes was studied. Inhibition percentage of dehydrogenases activity was glutamic 45%, succinic 48%, α-ketoglutaric 47% and isocitric dehydrogenases 45% (Table 1). Percentage inhibition of the activity of enzymes was calculated by comparing the O. D. values of the control tubes and the tubes containing pefloxacin.

DNA gyrase was eluted by affinity chromatography and purity was checked by SDS PAGE. SDS-PAGE analysis revealed one band which corresponds to 100 kDa (Figure 4). Finally, DNA supercoiling by DNA gyrase was studied in the presence and absence of pefloxacin (Figure 5). Lane a and c corresponds to the standards of Relaxed and supercoiled pBR322 DNA, respectively. Lane b corresponds to test containing pefloxacin, DNA gyrase and relaxed pBR322 DNA. According to the figure, pefloxacin inhibited DNA gyrase and relaxed DNA was not supercoiled. Lane d shows supercoiling activity of DNA gyrase in absence of Pefloxacin.

DISCUSSION

There has been a recent dramatic increase in information about the fluoroquinolones, a new class of potent orally absorbed antimicrobial agents. The first analog of this class of synthetic agents used clinically was nalidixic acid, a nonfluorinated agent which was released for treatment of urinary tract infections in 1962. In past decade, new fluoroquinolones, also called quinolones, 4-quinolones, carboxyquinolones, or quinolone carboxylic acids, have been developed that include norfloxacin, ciprofloxacin, ofloxacin, pefloxacin, enoxacin and others.

The principal bacterial target of the quinolones is deoxyribonucleic acid (DNA) gyrase (Crumplin et al., 1984; Gellert et al., 1977; Hooper et al., 1987; Sugino et al.,1977), an essential bacterial enzyme (Drlica, 1984; Cozzarelli, 1980; Gellert, 1981). This enzyme is a member of the class of type II topoisomerases and is composed of two A subunits encoded by the gyr A gene

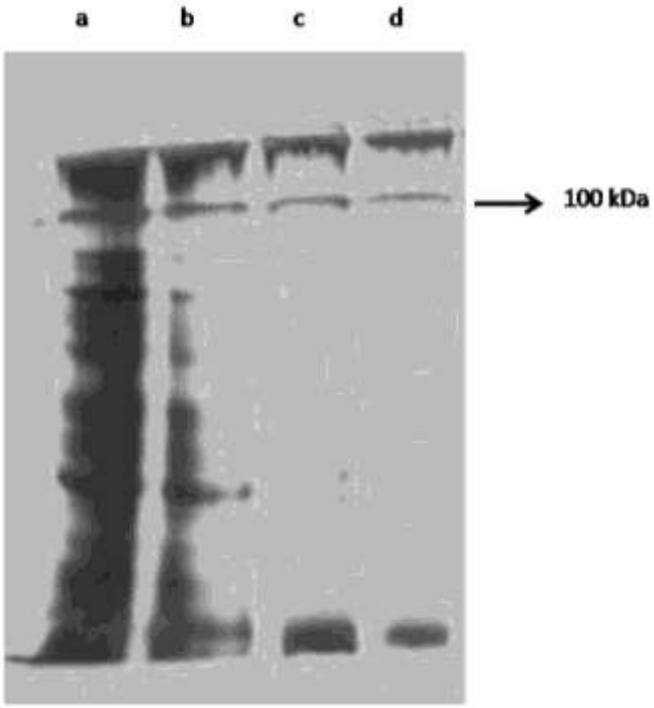

Figure 4. SDS-PAGE analysis of purified *K. aerogenes* DNA gyrase. Lanes a and b: Protein eluted before affinity chromatography of *K. aerogenes*. Lanes c and d: Protein eluted after affinity chromatography of *K. aerogenes* showing DNA gyrase (100kDa).

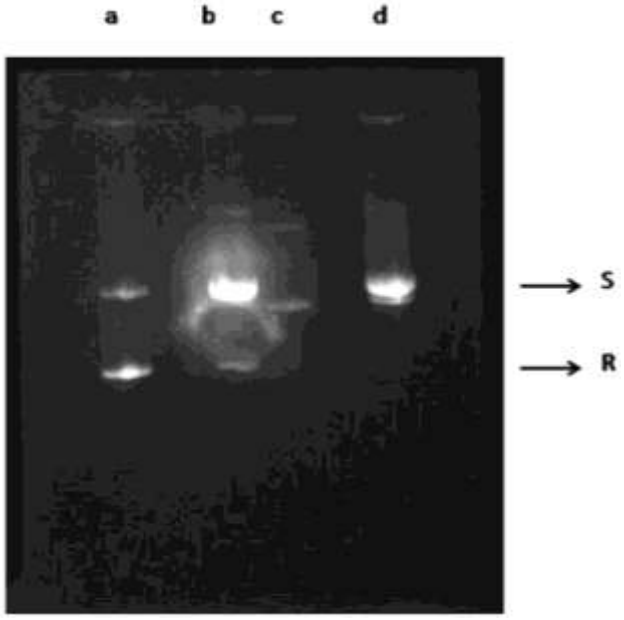

Figure 5. Agarose gel electrophoresis of relaxed pBR322 DNA, DNA gyrase and pefloxacin, showing supercoiling activity. Lane a: Control of Relaxed pBR322 DNA; lane b: Showing effect of pefloxacin on DNA gyrase inhibiting supercoiling activity; lane c: Control of Supercoiled pBR322 DNA; lane d: DNA gyrase supercoiling activity without pefloxacin.

and two B subunits encoded by the gyr B gene. DNA gyrase has been most extensively studied in *Escherichia Coli*, but DNA gyrases have also been purified from *Micrococcus luteus* (Klevan and Wang, 1980; Liu and Wang, 1978), *Bacillus subtilis* (Orr and Staudenbauer, 1982; Sugino and Bott, 1980), and *Pseudomonas aeruginosa* (Inoue et al., 1987; Miller and Scurlock, 1983).

Tabary et al. (1987) studied the effects of DNA gyrase inhibitors pefloxacin and five other quinolones on *E. coli* Topoisomerase I and Pan and Fisher (1999) studied the effects of fluoroquinolones on *Streptococcus pneumoniae* DNA gyrase and Topoisomerase IV. Pan et al. (2009) studied the effects of quinolones and Quinazolinedione PD 0305970 on DNA gyrase and Topoisomerase IV of Gram positive pathogens, including quinolone resistant isolates. Schultz et al. (1996) studied the structure and conformational changes of DNA topoisomerase II by electron microscopy.

DNA gyrase catalyze the supercoiling activity of covalently closed circular DNA relaxed by topoisomerase I. Quinolones inhibit this activity at concentrations near MICs. In the present investigation, the same effect was studied on *K. aerogenes* by pefloxacin with the reference to the prior research done. DNA gyrase was purified by Sepharose-novobiocin affinity chromatography and the effect of pefloxacin was studied. Pefloxacin inhibited the DNA gyrase activity by inhibiting supercoiling of relaxed DNA.

SEM data presented here are in substantial agreement with the previous reports of surface disruption of antibiotic treated with *E. coli* (Klainer and Perkins, 1972, 1974). The present study demonstrates that antimicrobial agent whose site of action is thought to be intracellular may cause morphological alterations which are similar to those induced by cell-wall active drugs. Surve and Bagde (2010b) studied the effects of methicillin on cell surface of *Streptococcus agalactiae* by SEM and reported similar results.

The effect of pefloxacin was also studied on the activity of dehydrogenases enzymes activity of *K. aerogenes*. Inhibition of four dehydrogenases involved in the TCA cycle, glutamic, succinic, α-ketoglutaric and isocitric dehydrogenases was found when bacterial cells were exposed to pefloxacin. Due to this, the supply of energy rich compounds like ATP got considerably reduced, and thereby the synthesis of macromolecules like protein, DNA and RNA declined and subsequently the growth got ceased. Surve and Bagde (2009, 2010a, b) reported similar inhibition effects of silver, arsenic and methicillin On dehydrogenases activity of pathogenic micro-organisms.

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