

Effect of Sulphaphenazole on Pathogenic Microorganism *Klebsiella aerogenes*

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Abstract

The inhibitory action of Sulphaphenazole on pure culture of *Klebsiella aerogenes* was studied with emphasis on the mode of action of the drug. Sensitivity was studied by broth dilution method and by Hi-Comb Method. The Minimum Inhibitory Concentration (MIC) of Sulphaphenazole determined by broth dilution method and Hi-Comb Method against *Klebsiella aerogenes* was 30 µg/ml. Sulphaphenazole induced changes in bacteria which interfered with cell wall synthesis revealing morphological alterations that was shown by Scanning Electron Microscopy (SEM). The present studies were undertaken to investigate the presence and characteristic alterations in surface morphology of cells resulting from the action of Sulphaphenazole known to interfere with intracellular protein synthesis. The morphological abnormalities observed may be surface reflections of specific abnormalities of intracellular protein synthesis or may represent a final common pathway of drug-induced injury at many sites within or on bacterial cells. As the activity of dehydrogenases was inhibited by Sulphaphenazole, cells were incapable of oxidizing substrate. It resulted in limited supply of energy rich compounds such as ATP that affected the synthesis of macromolecules. Ultimately multiplication and growth of the organism got ceased.

Keywords: Sulphaphenazole, *Klebsiella aerogenes*, MIC, Scanning electron microscope, Dehydrogenases, Hi-comb method

1. Introduction

Opportunistic infections are becoming common because of the growing number of immunocompromized individuals. The vast majority of *Klebsiella* infections however, are associated with hospitalization. Opportunistic pathogens *Klebsiella* spp. primarily attack immunocompromized individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus or chronic pulmonary obstruction. The urinary tract is the most common site of infection. *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, e.g., patients with neuropathic bladders or with diabetes mellitus. (Bennett *et al.*, 1995, Lye *et al.*, 1992)

Sulfa drugs are metabolic inhibitors of folic acid in microorganisms. Sulfanilamide and its relatives that soon followed were said to have “dethroned the caption of the men of death”, such was their effectiveness in treating pneumonias (Steinert, 2000).

In present investigation, susceptibility of *K. aerogenes* against sulfa drug Sulphaphenazole was found by broth dilution method and Hi-Comb method (Hi-media) and MIC was determined. After determination of MIC, site of action of drug on cell wall synthesis of *K. aerogenes* was found by SEM revealing morphological alterations which correlated well with their mechanism of action. Emphasis was also given on effect of drug on synthesis of dehydrogenases enzymes.

2. Materials and Methods

2.1 Organisms and Culture media

In present study, bacterial strain of *Klebsiella aerogenes* NCIM 2239 was obtained from the National Collection of Industrial Microorganisms (NCIM), Pune, India. Organism was grown at 37°C in Nutrient Broth medium (Himedia, India) and maintained at 5°C. Culture medium was autoclaved at 121°C for 20 minutes and organism was subcultured in Nutrient broth and Nutrient agar plate and after 24 hour incubation used as an inoculum.

2.2 Determination of MIC

K. aerogenes was treated with Sulphaphenazole (Sigma chemicals, U.S.A.) in broth dilution method (NCCLS, 2001) and MIC was also found by Hi-comb method using Sulphaphenazole Hi-comb (Himedia, India). In broth dilution method, 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 35°C overnight. MIC was read visually following 24 hours of incubation and was defined as the lowest concentration that produced no visible turbidity. 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 4-5 ml of broth. Turbidity was compared with 0.5 Macfarland standards and adjusted with sterile saline or broth if required at OD 600nm. Organism was spread on MRS plates by spread plate method and Hi-comb strip was placed on medium in sterile condition. Plate was incubated for 24 hours at 37°C and zone of inhibition formed in an ellipse 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).

2.3 Effect of Sulphaphenazole on morphological of *K. aerogenes*

After determination of MIC, surface morphology of *K. aerogenes* was studied by Scanning Electron Microscopy (SEM) on Quanta 200 ESEM system (Icon Analytical Equipment Pvt. Ltd., India). Specified concentration of Sulphaphenazole was added to culture in the logarithmic phase of growth (12 hour culture) at 37°C in nutrient broth medium. Incubation of culture with Sulphaphenazole was carried out for 3 hours. After incubation, treated and untreated cells 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 Russell, 1974).

2.4 Effect of Sulphaphenazole on Dehydrogenase activity

Effect of Sulphaphenazole on enzymes of TCA cycle was seen by the procedure followed by Guha and Mookerjee (Guha and Mookerjee, 1979). After the cells were grown for 48 hours at 37°C in Nutrient broth medium, they were used as samples in duplicates. According to the procedure, chloramphenicol was added to disrupt cell wall and MIC concentration of Sulphaphenazole was added to one set where as same amount of distilled water to control tubes. 0.005M Substrates of TCA cycle α -Ketoglutaric acid, Succinic acid, Isocitric acid and Glutamic acid was added to each tube. 0.5M Potassium phosphate buffer at pH 7.0, 0.3M MgCl₂ and Triphenyl Tetrazolium Chloride solutions (9 mg/ml) were finally added and OD was measured at 540 nm. The OD values were compared with the OD values of the control tubes and percentage inhibition of the activity of enzymes was calculated.

3. Results

Based on broth dilution and Hi-Comb methods, the MIC was determined at 30 µg/ml. The inhibition in the growth of *K. aerogenes* was read visually by broth dilution method and in Hi-Comb method, zone of inhibition was seen after 24 hour incubation (Figure 1).

The morphological changes induced in organism subjected to Sulphaphenazole treatment are shown in Figures 2 and 3. Images of control organism which was not treated by Sulphaphenazole (Figure 2) and test organism which was treated by 30 µg/ml of Sulphaphenazole (Figure 3) were taken by SEM. Normal control cells were seen to be in rod shape while cells treated with Sulphaphenazole resembled spheroplasts induced by drug inhibiting cell wall synthesis.

When exposed to Sulphaphenazole, cells of *K. aerogenes* showed inhibition of dehydrogenase enzymes. Percentage inhibition of dehydrogenases activity was Glutamic 45%, Succinic 44%, α -ketoglutaric 48% and Isocitric dehydrogenases 47% (Table 1). The percentage inhibition of the activity of enzymes was calculated by comparing the O.D. values of the control tubes and the tubes containing sulphaphenazole.

4. Discussion

Nosocomial *Klebsiella* infections most commonly involve the urinary and respiratory tracts. Since these two-body sites differ considerably with respect to the host defense mechanisms, it should be expected that the pattern of virulence factors found in UTI-causing strains of *Klebsiella* will differ from that observed in strains isolated from pulmonary sources of patients with pneumonia. *Klebsiella* infections are acquired during hospital stays and account for 5 to 7.5% of all nosocomial infections (Podschun and Ullmann, 1998).

In present study, Sulphaphenazole effect was studied against *Klebsiella aerogenes* and MIC was determined by broth dilution method and was found to be 30 µg/ml (NCCLS, 2001). MICs of the antimicrobials for the isolates were cross-checked by the Hi-Comb MIC test (Himedia) performed and was found to be 30 µg/ml. Menezes *et al.* (2008) found MIC by Hi-Comb test in *S. hemolyticus* and *S. aureus* against vancomycin. Hi-Comb test is useful for determining the antimicrobial susceptibility of aerobes and anaerobes, non-fastidious and fastidious organisms. Fairly accurate MIC values for critical cases including sepsis, endocarditis, meningitis etc. can assist immediate patient management.

Sulfa drugs act as competitive antagonist of p-aminobenzoic acid (PABA) which is an integral component of the folic acid structure (Hanafy *et al.*, 2007). Inhibitory action of various diphenyl sulphones and sulphonamides on dihydropteroate synthetase from *E. coli*, *Toxoplasma gondii*, *Pneumocystis carinii*, *Plasmodium falciparum* etc., was studied (McCullough and Maren, 1973; Pashley *et al.*, 1997; Hong *et al.*, 1995; Triglia and Cowman, 1994). Sulfa drugs act by inhibiting the enzyme dihydropteroate synthetase (DHPS) the folate biosynthesis enzyme that catalyzes the linkage of 7, 8-dihydro-6-hydroxymethylpterin pyrophosphate (H_2PtCH_2OPP) with p-aminobenzoic acid (PABA) to form dihydropteroate (Hong *et al.*, 1995).

In present study, effect of Sulphaphenazole was seen on surface morphology of *Klebsiella aerogenes* by Scanning Electron Microscopy. These latter observations have been substantiated by the morphological changes noted by Klainer and Perkins (1972). The SEM data presented here are in substantial agreement with the previous reports of surface disruption of antibiotic-treated *E. coli* (Klainer and Perkins, 1972). The mechanism by which the various aberrant forms seen by SEM arise is unclear but would seem in general to be a consequence of unbalanced cellular metabolism, a supposition that is supported by the fact that not all cells respond in the same manner and the development of abnormal forms is extremely dependent on the physiological state of the culture at the time of inhibition, the growth medium used and the completeness of the inhibition (Klainer and Perkins, 1972; Allison *et al.*, 1962; Greenwood and O'Grady, 1970; Pulvertaft, 1952).

The four dehydrogenases involved in the TCA cycle, glutamic, succinic, α -ketoglutaric and isocitric dehydrogenases were inhibited to a greater extent, when bacterial cells were exposed to Sulphaphenazole. Due to this the supply of energy rich compounds like ATP got considerably reduced and thereby synthesis of macromolecules like protein, DNA and RNA declined and subsequently the growth of the cells got ceased (Surve and Bagde, 2009; Surve and Bagde, 2010). Surve and Bagde (2009, 2010) reported similar inhibition effects of silver and arsenic on dehydrogenases activity of pathogenic microorganisms.

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Table 1. Effect of Sulphaphenazole on the activity of dehydrogenases of *Klebsiella aerogenes*. The percentage inhibition of the activity of enzymes was calculated by comparing the OD values of control tubes and the tubes containing Sulphaphenazole.

Dehydrogenases enzymes	Control			Activity with Sulphaphenazole		
	O.D.	Activity %	Inhibition %	O.D.	Activity (%)	Inhibition (%)
Glutamic	0.18	100	0	0.1	55	45
Succinic	0.16	100	0	0.09	56	44
α Ketoglutaric	0.19	100	0	0.1	52	48
Isocitric	0.15	100	0	0.08	53	47



Figure-1

Figure 1. Determination of the MIC of Sulphaphenazole towards *K. aerogenes* using HiComb method. The clear area indicated the growth inhibition zone of the bacterium.

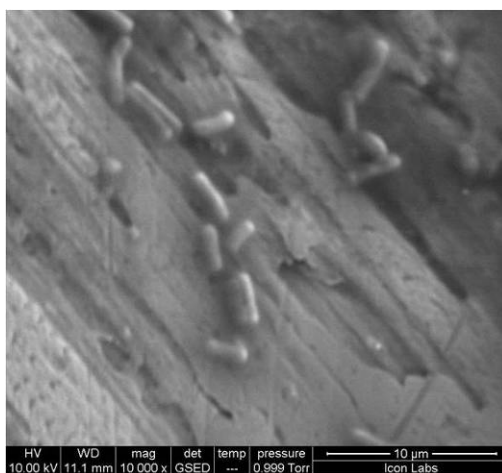


Figure-2

Figure 2. *Klebsiella aerogenes* under the untreated condition are rod shaped.

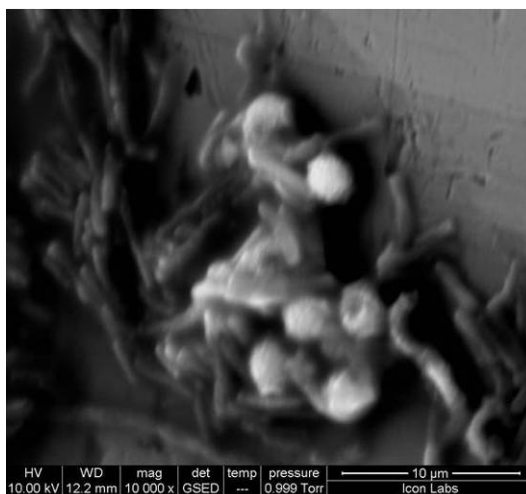


Figure-3

Figure 3. *Klebsiella aerogenes* following treatment with Sulphaphenazole.