Chapter 6

Summary
Shigella dysenteriae, the causative agent of bacillary dysentery, is one of the growing battery of antibiotic-resistant bacteria posing a major threat to mankind. Beta-lactam antibiotics were once the frontline drugs used in the chemotherapy of shigellosis. However, almost all over the world, these bacteria have become resistant to ampicillin, once the drug of choice for treating bacillary dysentery. Multiple resistance to ampicillin-trimethoprim sulfamethoxazole (TMP-SMZ) and nalidixic acid is well documented. This has necessitated the use of the newer quinolones. Although effective, the use of quinolones is limited by the adverse side effects of these drugs, particularly among children, who represent a large percentage of the population afflicted by the disease. Effective reemployment of newer beta-lactam antibiotics as a strategy of combatting shigellosis, needs to be given attention. Understanding the mechanisms involved in development of resistance to beta-lactam antibiotics, is essential towards achieving this goal. No serious attempts have so far been made in this area. The elaboration of beta-lactamases is one of the common means used by Gram-negative bacteria to thwart the challenge of beta-lactam antibiotics. The present study has not attempted to evaluate the role of these beta-lactam hydrolyzing enzymes in beta-lactam resistance of S. dysenteriae. Other possible mechanisms of beta-lactam resistance have been evaluated in this study. Three groups of laboratory mutants resistant to a number of antibiotics have been chosen as models to study the factors involved in resistance to beta-lactams.

In the first study, a cefoxitin-resistant mutant of S. dysenteriae was isolated by mutation with N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Of particular interest
was the fact that resistance towards the PBP1-specific antibiotic cefsulodin, and also towards cefoxitin and amoxicillin occurred simultaneously with the decreased affinities of two new high molecular mass PBPs \(1'\) and \(1''\) compared to PBP1 (of the parent strain) towards these beta-lactams. The increased IC\(_{50}\) values of the beta-lactams tested for PBP1' and 1'' could be correlated with increased MICs of these antibiotics in the case of strain M19. The decreased \(k_{+2}/K\) values of the beta-lactams tested with reference to PBP1' and 1'', suggested that the reduced acylation efficiencies represented one of the factors contributing to the higher IC\(_{50}\) values of these antibiotics. In addition to the markedly reduced affinities of PBP1'' for beta-lactams, the increase in relative amounts of PBP1 (1' +1'') in the mutant could also possibly contribute towards beta-lactam resistance of M19. Liposome swelling assays of permeability ruled out the possibility of resistance arising due to altered rates of diffusion of these antibiotics across the outer membrane. The similarities in the susceptibilities towards structurally unrelated antibiotics argued against the contribution of efflux mechanisms in beta-lactam resistance, since efflux pumps are usually of broad substrate specificity. The very low beta-lactamase activities and the absence of an inducible beta-lactamase, ruled out the involvement of any beta-lactamase in the resistance mechanism. These studies therefore provide evidence that beta-lactam resistance in \(S.\ dysenteriae\) may arise through appearance of altered high molecular mass PBPs with decreased affinities for beta-lactams.

The second study has attempted to evaluate the mechanisms of development of carbapenem resistance in \(S.\ dysenteriae\). We have previously shown in a
clinical isolate of *S. dysenteriae*, the manifestation of beta-lactam resistance although beta-lactamase activity was barely detectable (Kar *et al.* 1997). This isolate lacking a 43 kDa non-specific porin still remained sensitive to imipenem, necessitating understanding of the possible mechanisms of imipenem resistance in *S. dysenteriae*. The present study focuses on imipenem-resistant mutants derived from a susceptible strain by exposure to progressively increasing concentrations of imipenem. The mutants did not show any obvious alterations in the pattern of the OMPs. However, there were alterations in LPS in all the resistant strains. Estimation of 2-keto-3-deoxyoctonate (KDO) showed a progressive decrease with increasing imipenem resistance, and the band patterns in the core region were also different from that of the parent C152. One of the mutants, IRM16, was chosen for studying the permeation of the sugar arabinose and imipenem. Both the test solutes showed lower rates of permeation across the outer membrane of IRM16. Reduced permeation of imipenem therefore appeared to be the major contributing factor in imipenem resistance, at least in the case of IRM16. LPS has been identified as an important outer membrane component required for the assembly of the trimeric PhoE porin of *E. coli* (de Cock and Tommassen, 1996). The assembly of OmpF has been reported to be less efficient in a mutant of *E. coli* with a defective core region (Sen and Nikaido, 1991). In the present instance there is no direct evidence of defective assembly of porins in the imipenem-resistant mutants. In *P. aeruginosa*, it has been proposed that interaction of LPS with porins may
influence the conformation of the porins and the number of open, functional pores (Angus et al. 1982). Imipenem channels (protein D2 or Opr D2) of Pseudomonas aeruginosa are mostly closed in the LPS-free membrane (Ishii and Nakae, 1993). Data from investigations carried out in this laboratory provide evidence that the 38 kDa OMP of S. dysenteriae can function as a channel for imipenem (unpublished observations). This would be in agreement with the fact that the lack of the 43 kDa porin is not sufficient to confer imipenem resistance (Kar et al., 1997). It may be speculated that the altered LPS pattern as well as reduced amounts of LPS in the resistant mutants may influence the number of available open pores, particularly in the case of the 38 kDa porin.

Alterations in LPS have in a number of instances been linked to antibiotic-susceptibility of bacteria (Kropinski et al. 1982; Leying et al., 1991; Tzouvelekis et al. 1994). Our studies provide evidence that alterations in LPS may be crucial to the development of imipenem resistance of S. dysenteriae.

Nalidixic acid was used for the treatment of children with shigellosis caused by strains of S. dysenteriae that are resistant to ampicillin and cotrimoxazole (Bennish and Salam, 1992; Bennish et al., 1992). However, in 1990, 58% of 585 S. dysenteriae type I isolates in Bangladesh were resistant to nalidixic acid (Bennish et al., 1992). In China, upto 50% of S. dysenteriae, S. sonnei and S. boydii isolates are resistant to fluoroquinolones (Acar and Goldstein, 1997). Moreover, the simultaneous resistance to unrelated chemotherapeutic drugs arising during treatment with a particular antibiotic and attributable to multidrug resistance (MDR) efflux systems widely distributed among pathogenic bacteria,
is another cause of growing concern. Fluoroquinolones are now widely employed to treat *S. dysenteriae* infections. The purpose of the third part of the study was to investigate how exposure to fluoroquinolones may generate resistance in *S. dysenteriae* to this antibiotic, whether resistance to unrelated antibiotics, particularly the beta-lactams, also occurs simultaneously, and if so, what mechanisms may be involved.

The results obtained indicate that quinolone resistance arising from exposure of *S. dysenteriae* to the quinolone norfloxacin may involve a mechanism other than a mutation in the *gyrA* gene. The decreased accumulation of quinolones in one mutant, NRM16, compared to the parent strain and the effect of carbonyl cyanide m-chlorophenylhydrazone (CCCP) in bringing accumulation to similar levels in C152 and NRM16, obviously suggested a role of a proton motive force (pmf)-dependent efflux pump in quinolone resistance of NRM16. Since efflux pumps confer resistance to a structurally diverse array of antibacterials simultaneously, this aspect was also investigated. Among the beta-lactams tested, resistance was associated with only ampicillin. In harmony with this observation, the accumulation of ampicillin was lower in NRM16 than in C152, and CCCP showed an effect similar to that in the case of the quinolones. At this point of time it is not possible to infer why ampicillin alone, among the beta-lactams tested, appeared to be affected. A common efflux pump may operate to pump out both the quinolones and ampicillin. Among other drugs tested, there was only a small but consistent increase in MICs of tetracycline and chloramphenicol in NRM16. This is, to our knowledge, the first report of
quinolone resistance of *S. dysenteriae* involving a pmf-dependent efflux system
and in the absence of a *gyrA* mutation. The mutant, however, remained
susceptible to several beta-lactams, with the exception of ampicillin. A small
increase in resistance to tetracycline and chloramphenicol also occurred.

In conclusion, the present investigation has focused on the mechanisms of
antibiotic (particularly beta-lactam) resistance in laboratory mutants of *S.
dysenteriae*. The studies have been carried out in a virtually beta-lactamase-
negative background, in order to rule out the involvement of beta-lactamases in
development of resistance. These studies constitute one of the first steps
towards understanding drug resistance mechanisms in *S. dysenteriae*. The
evaluation of the role of these resistance mechanisms in clinical isolates of *S.
dysenteriae*, is the obvious next step. This has already been initiated.