INTRODUCTION

Vibrio spp. and *Shigella* spp. are pathogens that cause diarrhea or dysentery (diarrhea containing blood and mucus). They are common cause of death in developing countries and cause of infant deaths worldwide. The loss of fluids through diarrhea can cause dehydration and electrolyte disturbances such as potassium deficiency or other salt imbalances. For shigellosis, antibiotics are the primary treatment while in cholera primary treatment is oral rehydration therapy and secondary treatment is administration of antibiotics. The increasing occurrence of resistant bacteria gradually renders antibiotics ineffective in treating infections and has enormous health and economic implications worldwide (1, 2). Resistance to antibiotics in microbes has been attributed to genetic factors that could be inherent for the bacteria or acquired by them due to mobile genetic elements. Mobile genetic elements include plasmids, integrons and integrating conjugative elements (ICEs) which are potent vectors for acquisition and dissemination of antibiotic resistance genes among the bacterial populations. Bacteria may be inherently resistant to an antibiotic. For example, an organism may lack the target of the antibiotic molecule (mutations or alterations in target genes) or may export antibacterial agents before they can reach their target sites and exert their effect (Efflux pumps); or porins on the cell envelopes will not let the antibiotic enter the bacteria (mutation in porins).

India has witnessed *Vibrio* and *Shigella* epidemics in different regions like West Bengal, Orissa, Maharashtra, Punjab and Gujarat all the time (3-6) and work has been carried out to reveal various drug resistance mechanisms in these pathogens from various labs in India. The quinolone resistance mechanisms (7), imipenem resistance (8) and resistance to β lactam antibiotics (9) have been studied. Plasmids harbouring quinolone resistance genes, efflux pumps and mutations in topoisomerases have been implicated in quinolone resistance (7, 10-12). Clonality studies to reveal the emergence of *Shigella* strains from outbreaks and sporadic cases have been performed (13, 14). Internationally, work has been carried out to unravel the genetic mechanisms governing multidrug resistance phenotypes in *Vibrio* spp and *Shigella* spp. Integrons have been found with various drug resistance cassettes (15-19). Plasmids have also been characterized in *S. dysenteriae* type 1 strains from widely scattered geographical locations (20, 21). SXT element which encodes resistance to sulfamethoxazole, trimethoprim, chloramphenicol and streptomycin has also been characterized in *Vibrio cholerae* O139 (22). It is well documented that pathogenic bacteria continuously change themselves by the process of horizontal gene transfer through various mobile genetic elements in order to evade the antibiotic pressure.

As antibiotic resistance is the most pressing health threat, continuous surveillance of ever changing pathogenic species including *Shigella* and *Vibrio* could explain the present scenario of multiple drug resistance (MDR) and the complications they cause during treatment of infectious diseases. Understanding

various drug resistance mechanisms such as mobile genetic elements could yield an insight into the dispersal of antibiotic resistance genes in any population of pathogenic bacteria causing outbreak/epidemic/endemic at any geographical location. This is the aim of our present study.

RATIONALE OF THE STUDY

Diarrheal infections caused by *Shigella* and *Vibrio spp* are major health threat in developing countries like India. In addition, there is a problem of antibitotic resistance and emergence of MDR which pose problems for treatment resulting in excessive morbidity and mortality. Therefore, apart from continuous surveillance to monitor drug resistance patterns in a given geographical region/population to control the spread of the disease, it is also of great consequence to understand the basic mechanisms that lead to such MDR phenotypes. Understanding of drug resistance mechanisms could provide new treatment options to disarm potential pathogens and management of these diseases. For example, development of efflux pump inhibitors to use as adjuvants in antibiotic therapy or use of β- lactamase inhibitors like clavulinic acid along with β- lactam antibiotics.

The major genetic factors/mechanisms for MDR have long been recognized, including integrons, transposons, multidrug efflux, hyper-mutability, plasmids and promiscuous drug resistance. Within many individual isolates, the complexity of resistance is increasing, with multiple determinants being gained, amplified and lost. In this scenario, it is very obvious that the clinical aspect of antibiotic resistance is only the tip of the iceberg and many of the aspects in the study of antibiotic resistant bacteria like *Vibrio* spp. and *Shigella* spp. remain unexplored till now. Therefore, understanding role of mobile genetic element in spreading various drug resistance factors could give possible reasons for dissemination and dispersal of antibiotic resistance in any population of pathogenic bacteria causing diarrhea outbreak at any geographical location.

OBJECTIVES OF THE STUDY

The objective of this work was to unravel and understand the role of mobile genetic elements in the acquisition and dissemination of drug resistance genes in the context of molecular epidemiology in *Vibrio* spp. and *Shigella* spp.

- Acquisition and confirmation of bacterial isolates (*Vibrio* spp. and *Shigella* spp.) and study of their clonal relationships.
- Determination of antibiotic susceptibility profiles of these bacterial isolates with appropriate antibiotics.
- Screening of these isolates for the presence of mobile genetic elements [plasmids, integrons and conjugative transposons (SXT elements)] and determination of their transferable resistance traits.
- Detection of inherent/chromosomal borne drug resistance mechanisms like efflux pump and mutation in topoisomerases that could be working in synergy with these mobile genetic elements.

- Sequencing and detailed analysis of genes isolated from integrons and plasmids by tools like BLAST/Alignments/ORF finder and GenBank submissions of these sequences.

RESULTS AND DISCUSSION

The work was carried out with 13 clinical isolates of *V. fluvialis* and 95 clinical isolates of *Shigella spp*. which were procured from National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India in the form of stabs. All the clinical isolates were obtained from the patients with acute diarrhea, admitted to the Infectious Diseases Hospital, Kolkata, India. The bacteria from stabs were regenerated and confirmed for their authenticity on selective media. Subsequently, the cultures were grown in LB broth and saved as glycerol stocks and stabs. Further study was carried out as described below.

Characterization of mobile genetic elements in multidrug resistant *V. fluvialis* isolates

Out of thirteen *V. fluvialis* isolates, twelve were isolated in 2006 while one *V. fluvialis* BD146 was isolated in 2002. The clinical isolates of *V. fluvialis* from 2006 were analysed for their drug resistance profiles. Antibiotic susceptibility tests revealed that all the isolates displayed drug resistance with varying antibiograms. However, resistance to ampicillin and neomycin was common to all of them. Three isolates showed the presence of plasmids. Pulsed Field Gel Electrophoresis (PFGE) analysis of total genomic DNA with *NotI* digests from these isolates revealed that three isolates L13230, L13211 and L12482 had a similar band pattern and therefore could be derived from the same clone. The remaining nine isolates appeared to have different pulsotypes. Three isolates harboured plasmids carrying drug-resistance genes that could be transferred to recipient strains by conjugation and transformation. Class 1, class 2, class 3, class 4 integron and SXT element were found to be absent in these isolates. Mutations in gyraseA ($serine_{83}$→isoleucine), ParC ($serine_{85}$→leucine) and the presence of the *qnrVC* gene were found to contribute towards quinolone resistance. Efflux pump study of two representative isolates for quinolone resistance revealed that efflux pumps were operative for the quinolones but they did not account for difference in quinolone resistance levels in these isolates. These results corroborated the earlier findings by several groups that resistance to quinolones could be chromosome-borne (mutations in topoisomerases and efflux pumps) or plasmid-mediated (*qnr*) (11, 23).

In another study, out of 14 antibiotics used, *V. fluvialis* BD146 (2002) showed complete resistance to twelve drugs and intermediate susceptiblity to the rest of two drugs. The transformation experiments and screening on ampicillin indicated the presence of two types of plasmids: a low copy number plasmid and a high copy number plasmid of 7.5 kb. Two class 1 integrons were present in *V. fluvialis* BD146, one integron carried the putative exporter protein, while the other integron resident on low copy number plasmid carried various gene cassettes responsible for rifampicin, ampicillin, chloramphenicol, gentamicin and kanamycin resistance. The 7.5 kb plasmid of *V. fluvialis* BD146 carried genes like *dfr*6, *qnrVC*, *parE*, a replicase and a novel integrase *BDint*. pBD146 had propensity to transfer to another bacterium through

conjugation, and it was co-transferred with another conjugable plasmid that harbored ampicillin and rifampicin resistance. In addition, BLAST analysis of *V. fluvialis* pBD146 showed 99% identity with *V. cholerae* pVN84 (2004) and *V. parahaemolyticus* V110 (2010) indicating a possible exchange of plasmid between three *Vibrio* spp. To check the presence of integrase *BDint* in other *V. fluvialis* isolates, PCR experiments were carried out using integrase specific primers. Results revealed that this integrase was also present in some of the *V. fluvialis* isolates from 2006. Class 2, class 3, class 4 integrase and SXT element was found to be absent in this isolate.

Therefore, the present study clearly showed that in these *V. fluvialis* clinical isolates from Kolkata, plasmids and class 1 integrons played an important role in dissemination of drug resistance genes within human population.

Characterization of mobile genetic elements in multidrug resistant *Shigella* isolates

The global surveillance data by WHO in 2014 for antibiotic resistance in Shigella and other pathogens report increasing MDR resulting in the treatment failure (1). Ninety five clinical isolates of *Shigella* were analysed to understand the frequency and patterns of antimicrobial resistance. In this population of clinical isolates, *S. flexneri* and *S. sonnei* were found to be more prevalent as compared to the other two species i.e. *S. dysenteriae* and *S. boydii*. Majority of these isolates were resistant to trimethoprim, nalidixic acid, streptomycin, kanamycin, co-trimoxazole, tetracycline, ciprofloxacin and norfloxacin. PFGE analysis of these *Shigella* isolates revealed that *S. sonnei* had more clonally related isolates as compared to other *Shigella* isolates. In the present study, typical class 1 integron was present only in one *S. sonnei* isolate and harboured the dihydrofolate reductase gene cassette responsible for trimethoprim resistance. Forty one isolates comprising of *S. flexneri, S. dysenteriae* and *S. boydii* harboured the atypical class 1 integron. Sequence analysis of variable region of atypical class 1 integrons from the representative isolates revealed that they harbored the genes responsible for trimethoprim resistance (*dfrA*) and aminoglycoside resistance (*aadA1*). Class 2 integron was prevalent in *Shigella* isolates as it was present in 83% of all these isolates. All representative class 2 integrase positive isolates harboured *dfrA1-rimI* or *dfrA1-rimI-aadA* variable region except *S. sonnei* NK4846 which harbored a novel cassette array *InsE-InsO-dfrA1-rimI* on class 2 integron. Plasmid analysis by resistance transfer from representative *Shigella* isolates revealed that each isolate was replete with multiple plasmids. Therefore, multiple plasmids could be the possible source of drug resistance. Sequences of topoisomerases from representative isolates indicated the presence of mutations in QRDR regions of topoisomerases which accounted for the observed nalidixic acid and ciprofloxacin resistance. Hence, in this study, mobile genetic elements such as plasmids, class 1 integron, atypical class 1 integron and class 2 integron seemed to play an important role in dissemination of drug resistance in these *Shigella* isolates.

To summarise, the study pursued here has indicated an interplay of large number of genetic elements/factors such as multiple plasmids, integrons and mutations being responsible for the

prevalence of high drug resistance in pathogens belonging to the genera *Vibrio* and *Shigella* from the region of Kolkata.

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