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Plasmid mediated antibiotic resistance of *Vibrio cholerae* O1 biotype El Tor serotype Ogawa associated with an outbreak in Kolkata, India

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ABSTRACT

Objective: To determine the antibiotic resistance of *Vibrio cholerae (V. cholerae)* O1 biotype El Tor serotype Ogawa isolates involved in an outbreak of watery diarrhea in Kolkata, and to explore the role of plasmid in mediating antibiotic resistance. **Methods:** Antibiotic susceptibility and minimum inhibitory concentration (MIC) values of antibiotics for the isolated *V. cholerae* O1 Ogawa (n=12) were determined by disk diffusion and agar dilution methods, respectively, using ampicillin (Am), chloramphenicol (C), trimethoprim (Tm), tetracycline (T), erythromycine (Er), nalidixic acid (Nx), ciprofloxacin (Cp), amikacin (Ak) and cefotaxime (Cf). Plasmid curing of multidrug resistant (MDR) *V. cholerae* O1 Ogawa strains was done following ethidium bromide treatment. Following electrophoresis, the plasmid DNAs, extracted from the isolated MDR *V. cholerae* O1 Ogawa strains and their cured derivatives, were visualized and documented in 'gel doc' system. **Results:** The outbreak causing *V. cholerae* O1 Ogawa isolates were MDR as determined by disk diffusion susceptibility test, and MIC determination. The isolates showed three different drug resistance patterns: AmTmTErNx (for 6 isolates), TmTErCp (for 5 isolates), and AmTmNx (for one isolate), and showed uniform sensitivity to C, Ak and Cf. The loss of plasmids with the concomitant loss of resistance to Am, Tm, T and Er of the isolates occurred following ethidium bromide treatment. **Conclusions:** The current findings suggest that the *V. cholerae* O1 Ogawa associated with the cholera outbreak were MDR, and resistance to Am, Tm, T and Er among the isolates were plasmid mediated.

1. Introduction

Cholera, caused by *Vibrio cholerae (V. cholerae)* O1 or O139, is one of the most severe forms of infectious diarrheas occurring globally. The etiological agent of the disease *V. cholerae* that belongs to serogroup O1 biotype El Tor has two major serotypes, Ogawa and Inaba. Both the serotypes *V. cholerae* O1 biotype El Tor Ogawa and *V. cholerae* O1 biotype El Tor Inaba, showing resistance to several antibiotics, have been reported to be involved in many cholera outbreaks in different parts of the globe[1-3], including India[4,5]. The incidence of cholera outbreaks is not uncommon in the West Bengal state, India, and Kolkata also faced several outbreaks due to the infection of *V. cholerae* Ogawa and *V. cholerae* Inaba with different resistance patterns to antibiotics[6,7]. Das *et al* reported cholera from North India due to *V. cholerae* O1 Ogawa showing resistance to nalidixic acid (Nx), furazolidone (Fz), cotrimoxazole (Co), tetracycline (T) and chloramphenicol (C)[8], which was in agreement with studies from Chandigarh and Kolkata, India[9]. Karki *et al* reported, from Nepal, the incidence of cholera caused by *V. cholerae* O1 Ogawa that were resistant to ampicillin (Am), Co, Nx and Fz[10]. The *V. cholerae* O1 Ogawa isolates from Mozambique showed resistance to Am, C, Co, T and Nx[11]. As has been reported by Chander *et al*, the all isolates obtained over a period of nine years (1999-2007) from Chandigarh, India, were *V. cholerae* O1 Ogawa, and the isolates showed changing pattern of antibiotic resistance[12].

The multidrug resistances in *V. cholerae* have been reported to be plasmid encoded by earlier authors[13]; however, such phenomenon has been overlooked in our part of the globe. The emergence of multidrug resistant (MDR) *V. cholerae*, their changing antibiogram patterns and involvement of resistance-plasmid among the isolates are the cause of increased concern of treating cholera with antibiotics or reconsidering the role of antibiotics in

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cholera outbreaks. The present study has, therefore, been undertaken to assess the antibiotic susceptibility pattern of *V. cholerae* O1 biotype El Tor serotype Ogawa isolates associated with a recent outbreak of cholera in Kolkata, India, and to explore the role of plasmid in mediating antibiotic resistance among them.

2. Materials and methods

2. 1. Isolation and identification of bacteria

The rectal swab samples (n=12) were collected in Cary-Blair transport medium from patients in connection with an outbreak of watery diarrhea, occurred during middle of the June 2007, in Bally Municipality (Howrah district) Kolkata, India. The samples were inoculated, within 4-5 h of collection, into alkaline peptone water for enrichment, and then sub-cultured on thiosulphate citrate bile salt sucrose (TCBS) agar, McConkey agar and salmonella-shigella agar at the Department of Microbiology, Calcutta School of Tropical Medicine, India. Sucrose fermenting colonies on TCBS agar and non lactose fermenting colonies on the other two media were picked up, and tested biochemically following standard techniques[14]. Identity of *V. cholerae* isolates were further confirmed by serological tests using polyvalent O1 and monovalent Ogawa and Inaba antisera obtained from Difco, USA. The isolated *V. cholerae* were biotyped by sheep red blood cell haemolysis, Voges-Proskauer test and susceptibility to Polymixin B (50 units)[14].

2.2. Antibiotic susceptibility test

Antibiotic susceptibility testing for the isolated bacteria was done by disk diffusion technique as described earlier[15], on Mueller-Hinton agar (Hi-Media, Mumbai, India) using commercially available discs of antibiotics (Hi-Media, Mumbai, India): Am (10 μg), C (30 μg), trimethoprim (Tm, 5 μg), T (30 μg), erythromycin (Er, 15 μg), Nx (30 μg), ciprofloxacin (Cp, 5 μg), amikacin (Ak, 10 μg) and cefotaxime (Cf, 30 μg). *Escherichia coli (E. coli)* ATCC 25922 was used as the control strain. The plates were read after 18 h incubation at 35 ℃. Zone diameter of inhibition (ZDI) for each antibiotic was interpreted as per the Clinical and Laboratory Standards Institute (previously National Committee for Clinical Laboratory Standards; NCCLS) guidelines[16].

2.3. Minimum inhibitory concentration determination

The minimum inhibitory concentration (MIC) values of the antibiotics mentioned above for the isolated *V. cholerae* O1 Ogawa were determined following the NCCLS guidelines[17]. The antibiotic concentrations used in this study ranged 0.5 – 12 μg/mL for C, Cp, Ak and Cf; 0.5 – 150 μg/mL for Er and T; 2–75 μg/mL for Nx; 0.5 – 250 and 50 – 250 μg/mL for Am and Tm, respectively. *E. coli* ATCC 25922 was used as the control strain.

2. 4. Curing experiment

The isolated multidrug drug resistant (MDR) bacteria were subjected to plasmid curing at 40 ℃ to check the loss of resistance trait following the protocol of Anjanappa *et al*[18], with slight modification, and percent curing was calculated. The methodology in details has been mentioned elsewhere[15]. In this study the curing agent used was ethidium bromide (0.1 μg/mL).

2.5. Plasmid DNA isolation and agarose gel electrophoresis

The isolated MDR *V. cholerae* O1 Ogawa strains and their cured derivatives were subjected to plasmid DNA preparation following the protocols published earlier [19, 20], with some modification described elsewhere[15].

The isolated plasmid DNAs were electrophoresed through 0.8% agarose gel (Sigma, USA) for 4 h in tris-borate buffer system in horizontal gel apparatus. The gel was stained with ethidium bromide (Sigma, USA) solution (0.5 μg/mL of double distilled water) for 30 min, and documented with Gel-Doc system. The plasmid DNA of *E. coli* V517 (53.7 kb) was employed as a molecular size marker. Plasmid sizes were estimated from the migration distance in the agarose gel relative to the migration distance of reference *E. coli* V517 plasmid, by the method of Rochelle *et al*[21]. Repeated extraction of plasmid DNA was carried out for all isolates.

3. Results

The all clinical bacterial isolates studied showed typical biochemical and serological reactions of *V. cholerae* and were identified as *V. cholerae* O1 biotype El Tor serotype Ogawa. Thus, a total of 12 *V. cholerae* O1 Ogawa were isolated, one from each of the 12 rectal swab samples, which were collected (from 12 patients involved in the cholera outbreak) and processed microbiologically.

The all 12 (100%) *V. cholerae* O1 Ogawa isolates were sensitive to C, Ak and Cf having ZDI 19–24 mm, 20–26 mm and 22–27 mm, respectively, and resistant to Tm with no ZDI for 6 (50.00%) isolates (6 mm), and ZDI as 8–9 mm for the remaining 6 (50.00%) isolates. Of 12 isolates, 11 (91.66%) showed resistance to T and Er having no ZDI (6 mm) to 12 mm, and to 10 mm ZDI, respectively, while the sensitive one had ZDI as 20 mm and 26 mm, respectively. The Am ZDIs of 8–10 mm for 7 (58.33%) and 19–23 mm for 5 (41.67%) isolates, respectively, indicate their resistance and susceptibility to the antibiotic. To the quinolone antibiotics, Nx and Cp, respectively 7 (58.33%) and 5 (41.67%) resistant isolates had ZDI≤10 mm and ≤11 mm, respectively; the sensitive isolates had Nx ZDI 19–24 mm and Cp ZDI 20–23 mm.

All the isolated *V. cholerae* O1 Ogawa (12, 100.00%) showed susceptibility to C and Cf having MICs 2–8 μg/mL, and Ak (MICs 2–4 μg/mL), and resistance to Tm (MICs 75–200 μg/mL). Most of the isolates (11, 91.66%) were resistant to T (MICs 75–100 μg/mL) and Er (MICs 64–128 μg/mL), while the sensitive one had Er MIC of 2 μg/mL and T MIC of 4 μg/mL. Among the isolates, 7 (58.33%) were resistant

to Am and Nx with MICs 75–200 μg/mL and 32–64 μg/mL, respectively, and the sensitive isolates (n=5, 41.67%) had MICs 2–4 μg/mL and 4–8 μg/mL, respectively. For 7 (58.33%) and 5 (41.67%) of the isolates, the Cp MICs were 0.66 μg/mL (sensitive isolates) and 10 μg/mL (resistant isolates), respectively.

Based upon the MICs and disc diffusion test results, the *V. cholerae* O1 Ogawa isolates shared multiple antibiotic resistance showing three different patterns: AmTmTErNx (for 6 isolates), TmTErCp (for 5 isolates), and AmTmNx for one isolate. Following ethidium bromide treatment the MDR isolates having resistance patterns: AmTmTErNx, TmTErCp and AmTmNx, respectively lost AmTmTEr–, TmTEr– and AmTm–resistances with 79%, 83% and 72% curing, respectively; the isolates retained Nx and Cp resistances.

It showed the plasmid DNA bands, in the gel, isolated from *V. cholerae* O1 Ogawa having different antibiotic resistance patterns, as well as the 53.7 kb molecular marker plasmid from *E. coli* V517 strain. The plasmid DNA isolated from *V. cholerae* O1 Ogawa strain having AmTmTErNx–resistance showed single plasmid band co–migrated with the 53.7 kb plasmid from *E. coli* V517 strain, while the *V. cholerae* O1 Ogawa strains having resistance patterns TmTErCp and AmTmNx showed single plasmid bands, co–migrated with each other, of approximately 50 kb. The cured derivatives of the isolated *V. cholerae* O1 Ogawa strains losing AmTmTEr–, TmTEr– and AmTm– resistances did not show any plasmid band in the gel (Figure 1).

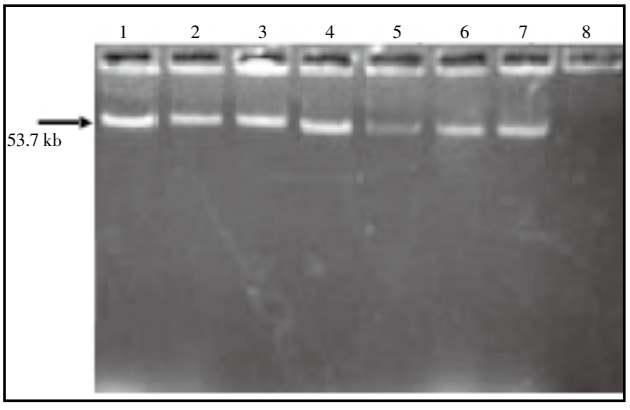

Figure 1. Agarose gel electrophoresis of plasmid DNAs extracted from *V. cholerae* O1 Ogawa strains.
Lane 1: plasmid size marker of 53.7 kb from *E. coli* V517, Lane 2 & 3: *V. cholerae* O1 Ogawa S2 strain (resistance pattern AmTmTErNx), Lane 4 & 5: *V. cholerae* O1 Ogawa S19 strain (resistance psttern TmTErCp), Lane 6 & 7: *V. cholerae* O1 Ogawa S11 strain (resistance pattern AmTmNx), Lane 8: Cured derivative of *V. cholerae* O1 Ogawa S2 strain (Nx resistant). Plasmid DNAs of Lane 2, 4 and 6 were isolated by Kado and Liu method [ref: 27], and for others Birnboim and Doly [ref: 28] method was followed.

4. Discussion

The all *V. cholerae* isolates obtained from the cases involved in an out break of watery diarrhea in Kolkata, India in 2007 were confirmed as *V. cholerae* O1 biotype El Tor serotype Ogawa. The *V. cholerae* O1 Ogawa associated cholera outbreak has been reported earlier from Kolkata[22], wherein during the last decade, the dominance of *V. cholerae* O1 Ogawa was observed. Outbreaks causing *V. cholerae* O1 Ogawa isolates have been reported from Chandigarh, India[4,12]. *V. cholerae* O1 Ogawa were isolated from 24 out of 48 stool samples obtained from patients in an explosive cholera outbreak in Gujrat, India, giving a positivity rate of 50%[23]. The *V. cholerae* O1 El Tor isolates from Mumbai, India were of Ogawa serotype during 2007[24] and 2004–2005[25]. All El Tor *V. cholerae* isolates from patients involved in an outbreak of cholera in the Sangli, Maharashtra were of Ogawa serotype[26]. During cholera outbreaks (2000–2006) in Iran, *V. cholerae* O1 Ogawa were isolated, as has been reported by Tavana *et al*[3]. Thus, the *V. cholerae* O1 biotype El Tor serotype Ogawa has been reported from different parts of the world including India, and has firmly been established in Kolkata, West Bengal state, the homeland of the disease. However, incidence of the *V. cholerae* O1 biotype El Tor serotype Inaba has been described in many parts of India[5,27,28], and the *V. cholerae* O1 strains have been demonstrated to interconvert and to undergo serotype switching between Ogawa and Inaba. In Kolkata, India, *V. cholerae* O1 Ogawa serotype predominated the Inaba serotype during 2004; the reverse was true for 2005[6]. During 2004 and 2005, two diarrheal outbreaks caused by *V. cholerae* O1 serotypes Ogawa and Inaba in two differrrrent areas of the West Bengal state, India, have been reported[7]. The present communication reports a focal outbreak of cholera in Kolkata, India, where the all 12 (100%) *V. cholerae* O1 were of Ogawa serotype; no other serotypes were traced. Regular surveillance of cholera endemic regions for *V. cholerae* infection may prevent occurrence of such outbreaks in the future.

Excessive and imprudent antibiotic use is generally considered to be the principal driving force behind the growing prevalence of antibiotic resistance among bacterial pathogens, and the previous use of antibiotics in earlier outbreaks may be in part responsible, as well, for the extensive increase in antibiotic resistance. The earlier authors from different parts of the world including India reported various patterns of resistance among the *V. cholerae* O1 strains to antibiotics generally used in the treatment of cholera. The *V. cholerae* O1 Ogawa and Inaba isolates from Iran showed resistance to Co, Nx and Fz, intermediately susceptible to C, and susceptible to T, Cp and Er[1]. High incidences of antimicrobial resistance among *V. cholerae* O1 isolates from Mozambique were found for C (57.9%), Co (96.6%) and T (97.3%), while quinolone resistance remained low (4.2%)[11]. Jesudasan[29] reported from Vellore, India T–resistance among the Inaba serotype, and T–sensitivity among the Ogawa strains; the isolates showing resistance to Nx and Co has also been reported earlier from the same place among both Ogawa and Inaba strains, and TmNx–resistance and sensitivity to T, C, Cf and Cp have been reported too[28]. Pal *et al* reported that among the *V. cholerae* O1 isolates from different outbreak regions of Orissa, India, both Ogawa and Inaba strains uniformly showed sensitivity to Am, C, gentamycin (G), norfloxacin (Nf), Cp, and T, while Fz, neomycin (Nm) and Nx resistances were found only among

Ogawa strains[5]. Sharma *et al* reported from Delhi, India that in *V. cholerae* O1 Inaba, multidrug resistance was 98.6% and in *V. cholerae* O1 Ogawa 98.3%; the isolates showed 100% resistances to Fz and Nx, and 100% sensitivity to T[30]. The all *V. cholerae* O1 isolates involved in the two outbreaks during 2004 – 2005 in West Bengal state, India were sensitive to T, G, azithromycin (Az) and Cp (but with reduced susceptibility to Cp for the *V. cholerae* isolated from the Garulia outbreak), and resistant to Am, Co, Nx and Fz[7]. The *V. cholerae* O1 Ogawa and Inaba strains from Kolkata (2004–2005) showed multidrug resistance to Am (76.0–100.0% isolates), Co (67.5–100.0% isolates), Fz (100.0% isolates), Nx (90.9–100.0% isolates), and Str (97.3–100.0% isolates); the resistance to Cf and T were 5.3–36.7% and 27.3%, respectively for Ogawa, and 12.5% and 15.0%, respectively for Inaba isolates[6]. In the present study, the outbreak causing *V. cholerae* O1 biotype El Tor serotype Ogawa isolates also showed various resistance patterns, viz., AmTmTErNx, TmTErCp and AmTmNx. The different pattern of antibiotic resistance was due to both temporal and spatial differences in the antibiotic treatment regimens, and thus continuous surveillance of cholera outbreaks is necessary with particular reference to resistance pattern of the isolates.

In the present communication, we determined MICs of the antibiotics used in the study, and the susceptibility test results from the disk diffusion method corroborated the results from the MICs of the antibiotics for the *V. cholerae* O1 Ogawa isolates. Most of the earlier authors did not report antibiotic MICs, for the test isolates, which is one of the essential criteria in determining susceptibility in order to make antibiotic treatment policy. All the *V. cholerae* Ogawa isolates, which were moderately sensitive to T by disc diffusion method had an MIC for T of 8 μg/mL, while the T sensitive strains had MICs in between 4 mg/mL and 1 μg/mL[26]. Jesudasan[29] showed T-sensitivity among *V. cholerae* O1 Ogawa and Inaba strains by MIC that ranged 8–16 μg/mL. The *V. cholerae* Inaba isolates from North India showed high MICs of Nx (>128 μg/mL) and Tm (64 μg/mL), while the low MICs for the isolates indicated sensitivity to Cf and T (0.25–2 μg/mL), Cp (0.25–0.5 μg/mL), Ak and G (0.25–4 μg/mL), and moderate sensitivity to C and amoxicillin (Ax) (16 μg/mL)[28]. Das *et al* recoded increasing trend of Cp MICs (2–128 μg/mL) among *V. cholerae* O1 Ogawa and Inaba strains from east Delhi, India during 2004–2006[31]. The high range of MICs among the current *V. cholerae* O1 Ogawa isolates, extending from very low level for some antibiotics to very high level for others, is probably due to judicious use of some antibiotics or withdrawal of some indiscriminately used antibiotics from the treatment protocol against enteric bacterial infection, for certain period of time, or rampant application of some extensively used antibiotics in the treatment of all enteropathogenic including *V. cholerae* infections, as we have seen earlier related to *Salmonella enterica serovar* Typhi and *S. enterica* serovar Paratyphi A infection[32,33].

The overall evolution of antibiotic resistance can be attributed to the spread of R-plasmid to various bacterial pathogens mainly because of the selective forces imposed by humans due to the overuse of antibiotics. But, inspire of a marked increase of MDR cholera outbreaks in different parts of India including Kolkata, no report has appeared of any outbreak due to MDR *V. cholerae* carrying R-plasmids. However, plasmid encoded drug resistance are not uncommon among the current sporadic as well as outbreak causing *V. cholerae* strains in the globe. Plasmid mediated resistance to Am, C, Str, sulfamethoxazole (Smz) and Tr has been reported among *V. cholerae* O1 isolates from Ethiopia, Somalia and Kenya[13]. The presence of a plasmid of about 150 kb in *V. cholerae* O1 isolates showing resistance to Am, C, T, Tm, Str, kanamycin and extended spectrum betalactams has been reported by Petroni *et al*[34]. To the best of our knowledge this is the first report of isolation of plasmid DNAs (approximately 53.7 kb encoding AmTmTEr-resistance, and approximately 50 kb conferring TmTEr- and AmTm-resistances) among *V. cholerae* O1 biotype El Tor serotype Ogawa isolates involved in a cholera out break in Kolkata, India during 2007. Thus, the present study highlights the necessity for vigilance of R-plasmid among the *V. cholerae* strains currently emerged in and around Kolkata, India and other parts of the world in order to combat plasmid mediated multidrug-resistance.

The cholera outbreaks that occur seasonally, in dry (March-April) to rainy (September-October) seasons, are a regular feature in Kolkata, and the West Bengal state is known as the homeland of *V. cholerae*. In this study, although the number of isolates is low, 100% isolation of *V. cholerae* was made, and the isolates were resistant to multiple antibiotics with different patterns of resistances, which were plasmid mediated too. Hence, such type of outbreak should not be ignored; rather, continuous monitoring of the changing trends in antimicrobial resistance patterns and vigilance of R-plasmid is a must, because the emergence of such resistance amongst *V. cholerae* may significantly influence the control strategies in future outbreaks. Over all, the long-term control of cholera depends on good personal hygiene, an uncontaminated water supply and appropriate sewage disposal.

Conflict of interest statement

We declare that we have no conflict of interest.

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