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Emergence of Nalidixic Acid Resistant *Vibrio cholerae* O139 in Orissa, India and Identification of its Responsible Protein Component

Hemant Kumar Khuntia, Surya Kanta Samal, Santanu Kumar Kar and Bibhuti Bhusan Pal*

Department of Microbiology, Regional Medical Research Centre, Bhubaneswar - 751 023, India.

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This study reports the emergence of nalidixic acid resistant *Vibrio cholerae* O139 strains associated with diarrhoeal disorders in Orissa, India during 1999 till 2001. During 1999 to 2001, 67 V. cholerae O139 strains were isolated from the hospitalized acute diarrhea patients in Orissa. All 10 strains of O139 isolated during July 1995 to October 1999, were sensitive to nalidixic acid. The emergence of first nalidixic acid resistant V. cholerae O139 strains were observed in November 1999 and all 57 strains isolated thereafter till 2001 were resistant to nalidixic acid. Polyacrylamide gel electrophoresis of whole intracellular protein of nalidixic acid resistant V. cholerae O139 strains exhibited two extra protein bands which may be responsible for development of resistance and this should be confirmed by further analysis.

Key words: *Vibrio cholerae* O139, Antibiotic, Nalidixic acid, Resistance, Protein.

Epidemic and endemic cholera is a major public health problem in many developing countries and continues to be an important cause of morbidity in many areas of Asia, Africa, and Latin America. Among more than 200 serogroups of *V. cholerae* so far identified[1] only O1 and recently developed O139 serogroup[2-3] are capable of causing epidemic cholera. *V. cholerae* O139 was first identified in September 1992 in Southern India[3] and rapidly spread to all cholera endemic areas in India[4] and neighboring countries[5]. During 1994 to 1998 many clones of *V. cholerae* appeared and disappeared in India associated with changes in phenotypic and genotypic characters[6--10]. In cholera endemic area in Orissa, Eastern India, the department of Microbiology, RMRC, Bhubaneswar has been conducting study in cholera since 1995. The emergence of *V. cholerae* O139 was first reported in 1995[11] carrying homologous antibiogram pattern to strains of *V.*

* To whom all correspondence should be addressed.
Tel.: +91-674-2302801(O)
E-mail: bbpal_rmrc@yahoo.co.in

cholerae O139 serogroup prevail in Indian subcontinent since 1992. All the *V. cholerae* O139 strains were sensitive to nalidixicacid till June 1999, whereas in November 1999 the emergence of nalidixic acid resistant O139 was noticed[12] for the first time in Orissa and subsequently in 2000; all the *V. cholerae* O139 were observed resistant to nalidixic acid. Nalidixic acid is considered to be the drug of choice for the empirical treatment of gastroenteritis especially in children when either there were no culture facilities or it is not possible to wait for a culture report. This study took an attempt to examine the minimum inhibition concentration (MIC) of nalidixic acid and the factor responsible for the development of resistance.

MATERIAL AND METHODS

MIC values

The strains of *V. cholerae* O139 serogroup were isolated from the rectal swabs collected from hospitalized diarrhoea patients following standard techniques[13]. Susceptibility testing of the isolates was carried out by the standard disc diffusion method of Kirby and Bauer[14]. The MIC value of nalidixic acid resistant and sensitive O139 strains was estimated by HI Comb (Himedia, Mumbai). The lowest concentration of drug in comb allowing no visible growth around comb tip after 18 hr of incubation at 37°C was taken as the MIC.

Extraction of intracellular Protein

To determine the protein component responsible for the development of resistance to nalidixic acid, total intracellular protein was extracted from eight laboratory nalidixic acid resistant (JP2, JP46, JP25, JP33, JP8, JP31, JP22 and Pocy43), one sensitive (DJ15) and control strain (SG24) of O139 serogroup. Initially the laboratory pure strains of nalidixic acid resistant and sensitive strains of O139 and standard strain of O139 serogroup, SG24 were grown in Luria agar plate. A single bacterial colony from the Luria agar plate was inoculated in 10 ml of Luria Bertani broth and incubated on a shaker (100-150 rpm) for 16-18 h at 37°C to harvest high yield of bacteria. Cells were collected from LB broth by centrifugation at 10, 000 x g for 2 minutes at room temperature. After three washes with phosphate buffer saline (PBS), the young cultures were disrupted with 2X treatment buffer to extract total protein following the method described elsewhere[15]. The treatment buffer consists of 0.125M Tris-HCI, 4% Sodium dodecyl sulfate (SDS), 20% glycerol and 0.02% bromophenol blue. The cells in treatment buffer were boiled for 10 minutes and then centrifuged at 10, 000 x g for 5 minutes at room temperature to pellet the debris and insoluble materials. The supernatant containing total protein extracts were transferred to fresh 1.5 ml tubes. The concentrations of extracted proteins were determined by spectrophotometer at a wavelength 280nm following Lowry's method. Bovine serum albumen (BSA) was used as protein standard.

SDS-polyacrylamide gel electrophoresis (PAGE) of total proteins from the isolates

The extracted whole cell protein from the isolates together with protein markers were diluted in an equal volume of sample buffer and boiled for 5 min at 100^0C in water bath shaker. Total protein extracts of each isolate were subjected to SDS-PAGE using 12% separating gel and 4% stacking gel and 1.5mm thick slab gels with Tris/ Hcl buffer (PH8.3). After electrophoresis (180 min, 60 v) gels were stained with Coomassie Blue R-250. The gel was then destained by soaking in distilled water for 15 min. Finally the whole cell protein profiles of samples were visualized and captured using photographed under Alpha Imager (Alpha Infotech Corporation, USA).

RESULTS AND DISCUSSION

During 1999 to 2001, 67 *V. cholerae* O139 were isolated from the hospitalized acute diarrhea patients in Orissa. All 10 strains of O139 isolated during July 1995 to October 1999, were sensitive to nalidixic acid. The emergence of first nalidixic acid resistant *V. cholerae* O139 strains were observed in November, 1999 and 57 strains isolated thereafter were resistant to nalidixic acid. Control strain using SG24, *V. cholerae* O139 revealed satisfactory result.

All the 30 representative strains of O139 studied, the MIC values of nalidixic acid were above the susceptibility range; which is 5 µg/ml. The MIC values of nalidixic acid resistant O139 strains vary between 8-230 µg/ml. Among these

3, 9 and 18 strains had MIC of 8µg/ml, 120µg/ml and 230µg/ml respectively. The three known *V. cholerae* O139 isolated before 1999 had MIC values of <5 µg/ml. All the nalidixic acid resistant O139 strains were also found susceptible to other fluoroquinolones like norfloxacin and ciprofloxacin.

Total protein as estimated revealed nalidixic acid resistant O139 strains carried more protein in quantity (ranged between 3.8mg/ml to 3.4mg/ml) and two extra of protein bands (Fig 1) in comparison to the sensitive strains. There are many mechanisms including plasmid or chromosomal mediated contributing to drug resistant phenotype in infecting agents is well understood. Spontaneous mutation in chromosomal genes is one of the important pathways for evolution of resistance among drug resistant strains. Evidence of extra protein band and variation in MICs of the nalidixic acid resistant strains of O139 strains in the present study presumes synthesis of new enzyme protein due to single or multiple mutations within the chromosomal gene leading to nalidixic acid resistance and to confirm it the study is under way.

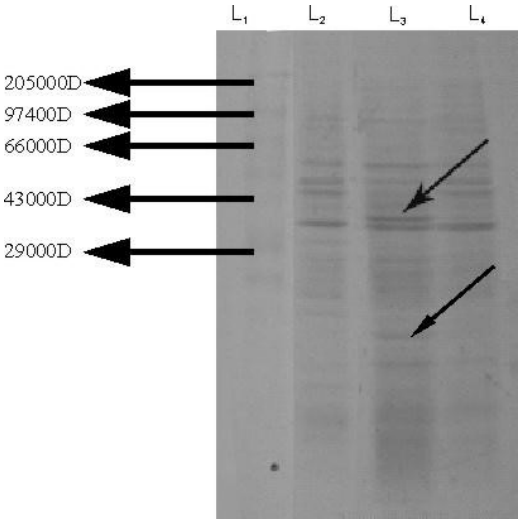

Fig 1. Polyacrylamide gel electrophoresis of whole intracellular protein of nalidixic acid sensitive and resistant strains of *V. cholerae* O139 serogroup isolated from Orissa. Lane 1: Protein marker (Bangalore Genei, India); Lane 2: SG24, nalidixic acid sensitive strain; Lane 3: JP2, nalidixic acid resistant strain of O139 strain and Lane 4: DJ15, nalidixic acid sensitive O139 strain. Two extra proteins are marked by two arrow marks on Lane 3.

Emergence of nalidixic acid resistant O1 has been reported since 1994[7] onwards while O139 serogroup was susceptible since emergence and was observed resistant in 1999 for the first time in Orissa. Few nalidixic acid strains of O139 were also found in Delhi and Kolkatta during 2000[16].

Although almost all the *V. cholerae* O139 strains were resistant to nalidixic acid however susceptible to tetracycline, norfloxacin and ciprofloxacin which are important drug of choice to be prescribed for gastroenteritis including cholera. Nalidixic acid resistance among *V. cholerae* O1 is a pre-requisite for development of fluoroquinolne resistance has been elucidated[17]. Emergence of nalidixic acid resistance among *V. cholerae* O139 in 1999 and subsequently fluoroquinolone resistance in 2000[12] presumes nalidixic acid resistance by O139 serogroup may be the forerunner of the fluoroquinolone resistance. Study of molecular mechanism is essential to address the detail genetic phenomena of evolution of nalidixic acid resistance among *V. cholerae* O139 serogroup.

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