

Multidrug-resistant *Vibrio cholerae* O1 in Belgaum, south India

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An outbreak of acute diarrhoea occurred in the Belgundi area (population 3896) of Belgaum Taluka (population 815 581) in Karnataka, South India, in June 2010. An estimated 16.22% of people were affected and 0.16% deaths were reported. *Vibrio cholerae* O1 El Tor was isolated from 18 of the 147 stool samples cultured. Seven out of eight drinking water samples collected from different sources were found to be grossly contaminated with faecal coliforms. All isolates were multidrug resistant, with some showing resistance to quinolones, gentamicin and cephalosporins in addition to co-trimoxazole and tetracycline, the drugs that were being used by the state health authorities for empirical treatment. Two serotypes and at least eight genotypes of *V. cholerae* were observed among the isolates. Cholera was confirmed as one, if not the only, cause of the outbreak, which, to our belief, is the first report of cholera from this region. It might have occurred due to a 'flare up' in the number of endemic strains triggered by shortage of portable water, onset of monsoon rains and breakdown of sanitation systems, rather than being a *de novo* outbreak arising out of new exogenous infectious sources. A change in the empirical treatment, coupled with chlorination, improvement in sanitation measures and extensive Information Education Communication activities, resulted in decline of the outbreak and prevention of further deaths.

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INTRODUCTION

Cholera continues to be an important health problem in India and in many other developing countries. *Vibrio cholerae* serotype O1 belonging to the El Tor biotype is the most common in the country while the frequency of serogroup O139 has declined considerably over the past few years. Although not as common in the south as it is in the deltaic parts of India (Seal, 1960), it has been reported from various southern states over the years (Goel *et al.*, 2011; Jain *et al.*, 2011). During a period of 10 years between 1997 and 2006, out of the 78 cholera epidemics affecting more than 0.2 million people and causing 823 deaths that India reported to the World Health Organization, 12 occurred in the southern states of Karnataka, Kerala, Tamil Nadu and Andhra Pradesh (Kanungo *et al.*, 2010). In Karnataka, outbreaks occurred in 2000, 2002 and 2005 while endemic cholera was reported in 2003 (Kanungo *et al.*, 2010). Although clinical cases of

cholera have been known to be common in Belgaum in north-west Karnataka for a long time, occurrence of outbreaks has not been documented as is evident by the lack of literature from this region. This communication reports the occurrence of an outbreak of diarrhoea in the outskirts of Belgaum city in 2010 from which *V. cholerae* was isolated, which, to the best of our knowledge, is the first microbiological and epidemiological report of cholera from this region. It also raises concerns about emerging resistance to newer antibiotics in *V. cholerae*.

METHODS

Study area and demography. Belgaum is a district comprising 10 'Talukas' with a population of 4 214 505, in north-west Karnataka, bordering Maharashtra and Goa (Government of India, 2001). Belgaum Taluka (province) has a population of 815 581, with 444 371 people living in Belgaum city, the administrative headquarters (Belgaum City Corporation, 2001). An outbreak of severe watery diarrhoea was reported from Ganeshpur and Jyotinagar villages on the outskirts of Belgaum city under the Belgundi Primary Health Centre, which caters for a population of approximately 3896 people (Fig. 1). Most of them belong to the low-income group referred to by

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Abbreviations: MDR, multidrug resistant; RAPD, random amplified polymorphic DNA.



Fig. 1. Map of Belgaum in north-west Karnataka, south India, showing the location of the outbreak (Courtesy: Map Data © 2012 Google).

the administration as ‘below poverty line’ card holders. This area is relatively poor in basic amenities such as roads, water supply and sanitation. Open air defecation and rearing of domestic animals including pigs are common in this area.

Case definition. A person reporting with the passage of three or more ‘rice-water’ stools on or after 12 June 2010 was considered as suspected case of cholera. A suspected case where the patient’s stool samples/rectal swab yielded cultures of *V. cholerae* was considered to be a confirmed case of cholera.

Outbreak and data acquisition. The outbreak was detected on 12 June 2010 (Fig. 2), when 52 cases of diarrhoea were reported, of which 25 were treated in mobile clinics and 27 at the District Civil Hospital. The concerned authorities were notified immediately regarding the outbreak. On 13 June, 77 cases of diarrhoea were reported with one death. A team comprising the district epidemiologist, medical personnel from Belgaum Institute of Medical Sciences and scientists from the Regional Medical Research Center conducted a house-to-house survey in the area and collected information about the illness and water samples from available drinking water sources to understand the aetiology and epidemiology of the outbreak, identify potential sources of contamination, review treatment and suggest control measures.

Clinical specimens. Stool samples were collected from 147 consecutive patients admitted to the District Civil Hospital after 12 June 2010.

Isolation and identification of the aetiological agent. Routine microscopy was carried out on all stool samples for detection of parasites and/or pus cells. All stool samples collected were processed for isolation of bacterial enteric pathogens, which included *Shigella*, *Salmonella*, *Vibrio* and diarrhoeagenic *Escherichia coli*, by standard techniques following World Health Organization guidelines (WHO, 1987). Colonies with the characteristic appearance of *V. cholerae* were confirmed by biochemical and serological tests using polyvalent O1 and monospecific Ogawa and Inaba antisera (Denka Seiken). PCR was employed to rule out the presence of diarrhoeagenic *E. coli*

following Orlandi *et al.* (2006). No attempt was made to isolate viral agents of diarrhoea.

Antibiotic sensitivity tests. Antibiotic sensitivity tests were carried out using the Kirby–Bauer disc diffusion method (Bauer *et al.*, 1966) with Müller–Hinton agar plates following CLSI (2007) guidelines. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were included in each test for internal quality control (Bhattacharya *et al.*, 2011).

PCR assays. Multiplex PCR was performed with *ctxA*-*tcpA* primers developed by exploiting biotype-specific variation of nucleotide sequences of *tcpA*, responsible for the expression of the major subunit protein (TcpA) of the toxin co-regulated pilus of *V. cholerae* (Keasler & Hall, 1993). Amplification of *ctxA* and *tcpA* El Tor

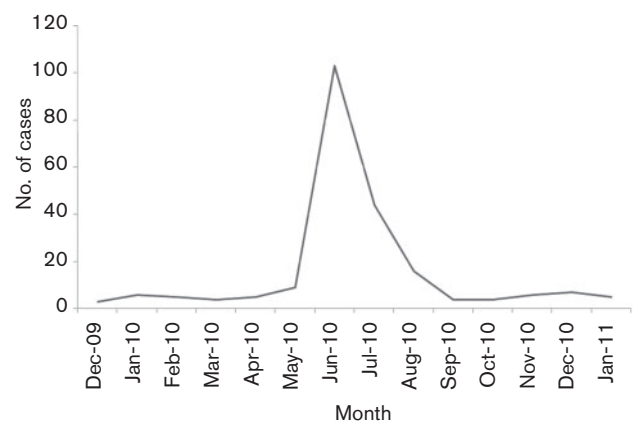


Fig. 2. Epidemic curve of the Belgaum outbreak in 2010. Diarrhoea patients admitted to the District Civil Hospital Belgaum month-wise from December 2009 to January 2011.

produces bands of 301 and 472 bp, respectively. PCR was performed in a Bio-Rad iCycler in 25 µl reaction volumes. A quantity of 2.5 µl template DNA in the form of heat-treated rapid lysates from 18 h cultures was used in PCR with 1 µM each primer, 250 µM each dNTP, 1.5 mM MgCl₂ and 0.5 U *Taq* DNA polymerase in 10 mM Tris/HCl (pH 9.0) and 50 mM KCl. The temperature programme consisted of an initial denaturation of 5 min at 94 °C followed by 29 cycles of 1.5 min at 94 °C, 1.5 min at 60 °C and 1.5 min at 72 °C, and a final cycle of 1.5 min at 94 °C, 1.5 min at 60 °C and a final extension of 7 min at 72 °C.

Random amplified polymorphic DNA (RAPD) fingerprinting assay. Purified genomic DNA was isolated from cultures of *V. cholerae* grown overnight in Luria broth (BD) following the cetyltrimethylammonium bromide method (Ausubel *et al.*, 1999). PB1 primer (5'-GCG CTG GCT CAG-3') was employed in a RAPD fingerprinting assay with all isolates (Roy *et al.*, 2005). PCR was performed at least three times by different individuals in a Bio-Rad iCycler in 50 µl reaction volumes. A quantity of 50 ng DNA extracted from 18 h cultures was used in PCR with 2 µM primer, 250 µM each dNTP, 1.5 mM MgCl₂ and 0.5 U *Taq* DNA polymerase in 10 mM Tris/HCl (pH 9.0) and 50 mM KCl. The temperature programme consisted of 1 cycle of 3 min at 97 °C, 1 min at 40 °C and 1 min at 72 °C; 4 cycles of 1 min at 97 °C, 1 min at 40 °C and 1 min at 72 °C; 24 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C; and 1 cycle of 1 min at 95 °C, 1 min at 55 °C and 7 min at 72 °C. PCR products were electrophoresed onto a 1% agarose gel, with gel red dye, viewed under UV light and documented in a gel documentation system (AlphaImager).

Collection and testing for water samples. Eight water samples were also collected aseptically from different drinking water sources and transported immediately to the Regional Medical Research Center for determination of the contamination levels by a rapid H₂S strip test (Hi-Media Laboratories) and by using the multiple-tube most probable number method as carried out previously (Sugunan *et al.*, 2007).

Control measures. All drinking water sources were superchlorinated. Public awareness and Information Education Communication activities were initiated. The Primary Health Centre staff was instructed to refer all cases of diarrhoea to the District Hospital and to start early rehydration.

RESULTS

Aetiology of the outbreak

Although the outbreak was reported on 12 June 2010, records at the health centres showed that cases fitting into the case definition started increasing from May 2010. A total of 632 cases of diarrhoea were reported after declaration of the outbreak (from 12 June 2010 to 28 August 2010). About 16.22% of the people were affected. The epidemic curve is shown in Fig. 2. *V. cholerae* was isolated from 18 of the 147 stool samples obtained from admitted patients (70 females, 77 males; 39 patients were in the paediatric age group, i.e. <14 years; Table 1). No *Shigella*, *Salmonella* or diarrhoea-genic *E. coli* was isolated from these samples. All the 18 *V. cholerae* isolates were found to belong to the O1 serogroup. While one isolate was found to be of the Inaba serotype, the remaining 17 belonged to the Ogawa serotype. Identities of the representative isolates were also confirmed by the World Health Organization Reference Centre for Diarrhoeal

Diseases at the National Institute of Cholera and Enteric Diseases, Kolkata.

Antibiotic sensitivity

Antibiotic sensitivity tests for the isolates revealed the presence of diverse patterns, and all isolates were multidrug resistant (MDR). All 18 isolates showed resistance to cotrimoxazole, while four showed complete resistance to tetracycline and another eight showed intermediate resistance to tetracycline, the antibiotics that were used in empirical treatment by the state authorities (Table 2). All 18 isolates were resistant to nalidixic acid while some showed resistance to fluoroquinolones such as ofloxacin, ciprofloxacin and gatifloxacin also. Interestingly, as many as eight isolates showed either complete ($n=5$) or intermediate ($n=3$) resistance to the cephalothin disc used as representative of the cephalosporins. Resistance to nitrofurantoin ($n=14$), carbenicillin ($n=5$), chloramphenicol ($n=3$), azithromycin ($n=1$) and gentamicin ($n=1$) was also found. Among all the antibiotics tested, only amikacin and imipenem were found to be effective against all the isolates.

PCR assay

All isolates showed the presence of a 564 bp amplicon marker for the very potent cholera toxin gene *ctxA* (Fields *et al.*, 1992) and a 472 bp amplicon marker for the toxin-co-regulated pilus gene *tcpA* for the El Tor biotype (Keasler & Hall, 1993), indicating high virulence potential. All 18 isolates showed the presence of a 192 bp amplicon marker for the O1*rfb* gene (Hoshino *et al.*, 1998).

RAPD fingerprinting

The RAPD fingerprinting assay, carried out at least three times, by different technicians, revealed the presence of eight different patterns among the 18 isolates of *V. cholerae* (Fig. 3). This indicated that the strains that caused the outbreak were of diverse genotypes.

Water sample analysis

Analysis of water samples both by a rapid H₂S production kit as well as a presumptive coliform test (most probable number method) revealed that seven of the eight water samples collected from various drinking water sources, including a hand pump, dig well, stored drinking water and tap water at Ganeshpur school, were heavily contaminated with faecal coliforms, indicating gross faecal contamination. Only one water sample that was reportedly boiled by a resident was free of faecal contamination.

DISCUSSION

Our study confirmed the presence of cholera during the outbreak in the Belgundi area of Belgaum. MDR strains of

Table 1. Distribution of 147 suspected and 18 confirmed cases of cholera by sex and age group

Age group	Suspected cases			Microbiologically confirmed cases		
	Total	Male	Female	Total	Male	Female
0–14	41	18	23	5	3	2
15–24	18	10	8	2	1	1
25–34	18	6	12	3	1	2
35–44	17	7	10	4	3	1
45–54	21	11	10	1	1	0
55–64	15	6	9	1	0	1
>65	17	11	6	2	1	1
Total	147	69	78	18	10	8

V. cholerae O1 Ogawa and Inaba serotypes belonging to the El Tor biotype were isolated. No other pathogenic bacteria were found to be associated. Although as many as 16.22 % of people were affected, the isolation rate was relatively low at 12.24 % because isolation was attempted on inpatients at the referral hospital, most of whom had already been given antibiotics. Since no attempt was made to test the samples for viral aetiology, chances of viruses such as rotavirus also playing a role in the outbreak cannot be entirely ruled out. However, the clinical presentation of patients, rice-water stools obtained and isolation of *V. cholerae* as the sole bacterial pathogen suggest that *V. cholerae* was the major, if not the only, cause of the outbreak.

All the *V. cholerae* strains isolated during the outbreak were highly pathogenic with the possession of the common

virulence markers *ctx* and *tcp*. MDR profiles indicated the presence of multiple types of the aetiological agent, which was subsequently confirmed by a RAPD fingerprinting assay. On most occasions, outbreaks are caused by single strains/clones and it is generally not necessary to attempt isolations/tests on all case patients. However, in this outbreak, the presence of two serotypes and eight different genetic types of *V. cholerae* is noteworthy. It indicates that the outbreak was probably not from a single source. The existence of multiple serotypes and genotypes and a higher attack rate in children below 14 years of age indicate the occurrence of cholera in the region in the past. Since clinical cases of cholera are common in the region, although not supported by microbiological evidence in literature, it is likely that cholera was very much endemic in

Table 2. Antibiotic sensitivity pattern of the isolates ($n=18$)

AMK, Amikacin; GEN, gentamicin; IMP, imipenem; AZM, azithromycin; CEP, cephalothin; CFX, cefixime; AMP, ampicillin; CAR, carbenicillin; CIP, ciprofloxacin; GAT, gatfloxacin; NAL, nalidixic acid; NOR, norfloxacin; OFL, ofloxacin; COT, co-trimoxazole; TET, tetracycline; CHL, chloramphenicol; NIT, nitrofurantoin; CFT, ceftriaxone. R, Resistant; S, sensitive; I, intermediate.

Sl no.	Isolate ID no.	AMK	GEN	IMP	AZM	CEP	CFX	AMP	CAR	CIP	GAT	NAL	NOR	OFL	COT	TET	CHL	NIT	CFT
1	DCHIP-1	S	S	S	S	I	S	R	S	S	S	R	S	S	R	I	S	R	S
2	DCHIP-2	S	S	S	S	I	S	R	S	S	S	R	S	S	R	I	S	R	S
3	DCHIP-3	S	S	S	S	I	S	R	R	I	S	R	I	S	R	R	R	R	S
4	DCHIP-4	S	S	S	S	S	S	R	S	S	S	R	S	S	R	I	S	R	S
5	DCHIP-5	S	S	S	S	R	S	R	R	I	S	R	I	I	R	R	R	R	S
6	DCHIP-8	S	S	S	S	S	S	R	S	S	S	R	S	S	R	I	S	I	S
7	DCHIP-12	S	S	S	S	R	S	R	R	S	S	R	S	S	S	S	S	S	S
8	DCHIP-18	S	S	S	R	R	R	R	I	S	S	R	R	S	R	R	R	R	R
9	DCHIP-20	S	R	S	I	R	I	R	R	R	R	R	R	R	R	R	S	S	S
10	DCHIP-28	S	S	S	I	S	R	R	S	S	S	R	S	S	R	I	S	R	S
11	DCHIP-32	S	S	S	S	R	R	R	R	S	S	R	S	S	R	I	S	S	I
12	DCHIP-45	S	S	S	S	S	S	R	S	S	S	R	S	S	R	S	S	R	S
13	DCHIP-54	S	S	S	S	S	S	R	S	I	S	R	S	S	R	S	S	R	S
14	DCHIP-55	S	S	S	S	S	S	R	S	S	S	R	S	S	R	I	S	R	S
15	DCHIP-56	S	S	S	S	S	S	R	S	S	S	R	S	S	R	S	S	R	S
16	DCHIP-57	S	S	S	S	S	S	R	S	S	S	R	S	S	R	I	S	R	S
17	DCHIP-60	S	S	S	S	S	S	R	S	S	S	R	S	S	R	S	S	R	S
18	DCHIP-61	S	S	S	S	S	S	R	S	S	S	R	S	S	R	S	S	R	S

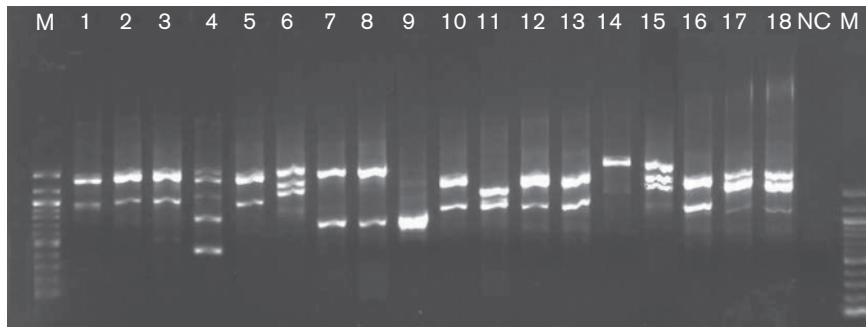


Fig. 3. RAPD fingerprinting PCR of 18 *V. cholerae* isolates. Lanes: M, 100 bp marker; 1–18, isolates 1–18; NC, negative control.

the region, and the outbreak might have been a 'flare up' in the number of endemic strains triggered by shortage of portable water, onset of monsoon rains and breakdown of sanitation systems, rather than a *de novo* outbreak arising out of new exogenous infectious sources. A study on the water samples collected from the area supported this conclusion, with evidence of gross breakdown of water supply and sanitation systems in an existing unhygienic environment. *V. cholerae* has been reported to have a permanent existence in the environment and may remain quiescent for some time when its survival in water bodies allows dissipation of resistance properties to different serotypes or strains (Das *et al.*, 2008). *V. cholerae* O1 strains are known to interconvert between the Ogawa and Inaba forms (Colwell *et al.*, 1995; Garg *et al.*, 2000) and the *wbeT* gene of the highly conserved *wbe* region is responsible for the serotype conversion (Garg *et al.*, 2000). The frequency of conversion of Ogawa to Inaba is approximately 10^{-5} (Bhaskaran & Gorrill, 1957), whereas conversion from Inaba to Ogawa is much rarer and may be dependent on the strain (Manning *et al.*, 1994). Isolation of both Ogawa and Inaba serotypes during this outbreak is suggestive of the same phenomenon.

Although antibiotics are not required to resolve cholera symptoms and cannot be used as a sole treatment for the disease, the use of antimicrobial agents is generally accepted as a method of reducing the duration and volume of diarrhoea as well as decreasing the period of *V. cholerae* excretion in stools. The fact that all 18 isolates were MDR, with several isolates showing resistance to relatively new antibiotics such as fluoroquinolones, cephalosporins and carbenicillin, along with nitrofurantoin, gentamicin and azithromycin, in addition to complete resistance to ampicillin, co-trimoxazole and nalidixic acid, raises serious concern about the effectiveness of these antimicrobials in dealing with cholera in future. Among all the antibiotics tested, only amikacin and imipenem were found to be effective against all the isolates. Although MDR cholera is being reported from India and elsewhere in the neighbourhood (Mishra *et al.*, 2004; Akond *et al.*, 2008; Das *et al.*, 2008; Jain *et al.*, 2011; Ghosh & Ramamurthy, 2011;

Bhattacharya *et al.*, 2012), the wide spectrum of resistance found in *V. cholerae* from this outbreak is not very common and a cause for serious concern. *V. cholerae* possesses a number of mechanisms to evade the effects of antimicrobial drugs and a stage may come when the commonly used antimicrobial drugs are no longer effective (Ghosh & Ramamurthy, 2011). *V. cholerae* becomes drug resistant by exporting drugs through efflux pumps, chromosomal mutations or developing genetic resistance via the exchange of conjugative plasmids, conjugative transposons, integrons or self-transmissible chromosomally integrating SXT elements. However, additional studies are required to determine the role of these factors in the development of resistance among the *V. cholerae* isolates in this region.

Large-scale usage of antibiotics can also generate various patterns of resistance that fluctuate on a short timescale with changes in antibiotic retail sales (Sun *et al.*, 2012), and this may also have contributed to the wide spectrum and various patterns of resistance found in the isolates. Resistance to commonly used antimicrobial drugs is becoming a major public health concern because it complicates treatment and may result in longer hospital stays for patients. Spread of antimicrobial drug resistance has been recognized by the World Health Organization as an extremely serious problem. During the present outbreak, prompt sharing of the results of antibiotic sensitivity with concerned authorities led to a change in treatment regimen in the Government hospital from co-trimoxazole and tetracycline to amikacin, which perhaps helped in prevention of further deaths. Information Education Communication activities and superchlorination of existing water sources were carried out, which were monitored from time to time. As a result of these activities, the outbreak was controlled.

Our study, which, to the best of our knowledge, is the first detailed microbiological communication of a cholera epidemic in north-west Karnataka, underlines the importance of laboratory support to health authorities in an outbreak situation to reduce mortality and devise intervention strategies. Our study also warrants further investigations into the molecular epidemiology of cholera

in the region and institution of a networked system of laboratories for real-time monitoring of emerging resistance to antimicrobials for better implementation of antibiotic usage policies.

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