Cholera in Ethiopia in the 1990s: Epidemiologic patterns, clonal analysis, and antimicrobial resistance

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Abstract

In 1993, after 6 years of absence, cholera re-emerged in the Horn of Africa. Following its introduction to Djibouti, the disease spread to the central and southern areas of Ethiopia reaching Somalia in 1994. Cholera outbreaks persisted in Ethiopia with a recrudescence of cases in 1998. Twenty-two Vibrio cholerae O1 strains, selected to represent the 1998 history of cholera in Ethiopia, were characterized by random amplified polymorphic DNA patterns, BglI ribotyping and antimicrobial susceptibility. All isolates showed a unique amplified DNA pattern and a prevalent ribotype B8a. All strains were multidrug-resistant and harboured an IncC plasmid which conferred resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim. These findings indicate that a group of closely related V. cholerae O1 strains was responsible for the cholera epidemic in Ethiopia in 1998.

Keywords: Cholera; Clonal analysis; Ethiopia

Introduction

Cholera continues to be an important public health problem among poorer communities, particularly in Africa which accounts for more than 90% of the annual cholera cases notified to the WHO. The modern history of the disease began in 1817 with the onset of the first (1817–1823) of 7 distinct pandemics which have occurred so far. The sixth and, presumably, the fifth were caused by Vibrio cholerae O1 of the classical biotype. The seventh originated on the island of Sulawesi in Indonesia in 1961, and the causative agent was V. cholerae O1 of the El Tor biotype (Kaper et al., 1995).

This seventh cholera pandemic reached the African continent in 1970 where there were at least 2 independent introductions of the infection (Lan and Reeves, 2002): the first one caused outbreaks in the west (Guinea, Sierra Leone, Liberia, Nigeria, and other coastal countries) and the spread of the disease into the interior of the sub-Saharan states. The second route is thought to have originated in the Middle East,
entering Africa from the eastern countries of Djibouti, Ethiopia, and Somalia. Since 1970, parts of Ethiopia have been frequently affected by cholera, acting as sources of infection for the dissemination of the 1985–1986 large-scale epidemic in the Horn of Africa (Maimone et al., 1986; Coppo et al., 1995). From 1993 to 1999, they were again systematically involved as active focuses in the recurrent spreading of the disease in the region. In spite of the critical role of Ethiopia in the epidemic transmission of cholera, no scientific investigations have been carried out.

This paper describes the epidemiologic features of widespread cholera outbreaks we were able to study directly in Ethiopia in the 1990s and the degree of genetic relatedness of 22 \( V. \text{cholerae} \) O1 El Tor strains isolated from 6 outbreaks representative of the 1998 epidemic phases. The El Tor strains were characterized by random amplified polymorphic DNA (RAPD) assay, \( B_{gl}I \) ribotyping, antimicrobial susceptibility testing and PCR detection of phage CTX\( \Phi \) genes and pathogenicity genes.

**Materials and methods**

**Surveillance of the cholera epidemics and characterization of bacterial strains**

Epidemiological data on cholera cases, occurring between 1993 and 1997 in the Harari People state, Ethiopian states of Somali, Oromiya, and Southern Peoples and in the urban administrative regions of Addis Ababa and Dire Dawa were obtained from records kept at the Regional Health Bureau in the Ethiopian city of Harar. No additional epidemiological data were available from other Ethiopian areas.

In 1998, we set up a surveillance system to investigate directly the epidemiological, clinical, and microbiological aspects of severe dehydrating diarrhoeal diseases at the Regional Health Bureau in the Ethiopian city of Harar. In addition to regular surveillance in the Harari People state, other zones where we surveyed outbreaks of acute diarrhoea when cholera broke out in epidemic form were the Ethiopian states of Somali, Oromiya, and Southern Peoples and in the urban administrative regions of Addis Ababa and Dire Dawa (Fig. 1A). After informed consent had been given, rectal swabs in Cary-Blair transport medium for culture of \( V. \text{cholerae} \) were obtained from patients with diarrhoea (> 3 loose or watery stools in a 24 h period) turning up at health facilities for treatment. Uniform information on medical history, symptoms, and demographic background was also taken. No pipe-water networks were present in the areas investigated, and most of the drinking water came from environmental sources such as lakes or streams. Apart from rehydration and electrolyte replacement antibiotic therapy (primarily tetracycline) was also provided.

Attack rates were calculated by using demographic data from the 1994 census and relevant estimates.

The 22 strains characterized in this study were selected from \( V. \text{cholerae} \) O1 isolates we had collected from 6 outbreaks in 5 different Ethiopian regions in 1998 (Table 1 and Fig. 1A). Original stock cultures of isolates had been kept in 20% glycerol Luria-Bertani broth (LB) at \(-70^\circ \text{C}\). Phenotypic strain
characterization was performed as described previously (Scrascia et al., 2003).

Antimicrobial susceptibility was determined by the disc diffusion method as described in the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M2-A9 (CLSI, 2006). The antimicrobial disc contents were ampicillin (AMP; 10 µg), chloramphenicol (CHL; 30 µg), doxycycline (DOX; 30 µg), kanamycin (KAN; 30 µg), streptomycin (STR; 10 µg), spectinomycin (SPT; 10 µg), tetracycline (TET; 30 µg), sulfamethoxazole (SMX; 25 µg), and trimethoprim (TMP; 5 µg) (Oxoid, Milan, Italy). Escherichia coli ATCC 25922 was used as a quality control strain.

### RAPD assay

Genomic DNA extraction and PCR reactions for RAPD assay were performed as described previously (Pazzani et al., 2006). The primers were 6 oligonucleotides selected from entero bacterial repetitive intergenic consensus sequences (ERIC1 and ERIC2), from V. cholerae repetitive sequences (VCR1 and VCR2) and from phage CTXΦ sequences (ATX1 and ATX2). Primers were employed separately and each strain was characterized by an individual RAPD cluster type represented by the combination of the 6 single RAPD patterns. Combinations with 6 identical amplified DNA patterns or with one different amplicon in only one of the 6 patterns were classified in the same specific RAPD cluster type.

All primers were synthesized commercially and purified through a reverse phase chromatography by Invitrogen Life Technologies. PCR reactions were performed in duplicate and results were found to be reproducible. PCR products were separated in 2% (w/v) agarose gel, 45 mM Tris-Borate, 1 mM EDTA buffer, at 60 V and at a temperature of 14 °C. Agarose gels were stained with ethidium bromide at the final concentration of 0.5 µg/ml. Images from gels were converted into digital form by the Gel-Doc 2000 photo documentation system (Bio-Rad, Milan, Italy).

### Bacterial conjugation and plasmid analysis

V. cholerae O1 strains CIRPS1305, a rifampicin-resistant mutant of CIRPS1006, were used as recipients (Maimone et al., 1986; Coppo et al., 1995). Conjugation experiments were performed by mixing equal volumes (100 µl) of overnight cultures of donors and recipient strains. The mixing cultures were applied to a membrane filter of a pore size of 0.22 µm on LB plates and incubated at 37 °C for 6 h. Cells were collected in 1 ml of LB and serial dilutions were spread on plates of LB agar supplemented with rifampicin at 100 µg/ml and concentrations of selected drugs as follows: ampicillin (100 µg/ml), chloramphenicol (30 µg/ml), spectinomycin (200 µg/ml), streptomycin (200 µg/ml), sulfamethoxazole (600 µg/ml), and trimethoprim (100 µg/ml).

The frequency of transfer of a genetic marker was expressed as the number of transconjugants per recipient cell. Incompatibility tests and plasmid detection were performed as described previously (Casalino et al., 1994).

Plasmid DNA extracted from both donors and transconjugants was separated through a Field Inversion Gel Electrophoresis (FIGE Mapper, BioRad), forward voltage 180, reverse voltage 120 V, switch time 0.4–3.5 s linear shape. DNA samples were run in a 0.8% (w/v) agarose gel, 13 mM Tris, 6.6 mM acetate, 0.33 mM EDTA, for 10 h at 14 °C.

### Table 1. RAPD cluster type, antimicrobial resistance pattern, and ribotypes of 22 V. cholerae O1 El Tor strains isolated in Ethiopia in 1998.

<table>
<thead>
<tr>
<th>Region of isolation</th>
<th>No. of strains</th>
<th>RAPD cluster type</th>
<th>Antimicrobial resistance patterna</th>
<th>Ribotype (no. of strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harari People</td>
<td>8</td>
<td>VIII</td>
<td>AMP CHL SMX SPT STR TMP</td>
<td>B8a (8)</td>
</tr>
<tr>
<td>Oromiya</td>
<td>8</td>
<td>VIII</td>
<td>AMP CHL SMX SPT STR TMP</td>
<td>B8a (5), B5a (2), B24a (1)</td>
</tr>
<tr>
<td>Somali</td>
<td>2</td>
<td>VIII</td>
<td>AMP CHL SMX SPT STR TMP</td>
<td>B8a (2)</td>
</tr>
<tr>
<td>Dire Dawa</td>
<td>2</td>
<td>VIII</td>
<td>AMP CHL SMX SPT STR TMP</td>
<td>B5a (1), B8a (1)</td>
</tr>
<tr>
<td>Addis Ababa</td>
<td>2</td>
<td>VIII</td>
<td>AMP CHL SMX SPT STR TMP</td>
<td>B8a (2)</td>
</tr>
</tbody>
</table>

aAMP, ampicillin; CHL, chloramphenicol; SMX, sulfamethoxazole; SPT, spectinomycin; STR, streptomycin; TMP, trimethoprim.
fragments were then used as the molecular size standards.

**Gene detection by PCR**

Detection of genes encoding cholera toxin subunit A (\textit{ctxA}), zonula occludens toxin (\textit{zot}), accessory cholera enterotoxin (\textit{ace}), and toxin-coregulated pilus for intestinal colonization (\textit{tcpA}) was performed by PCR as described previously (Pourshafie et al., 2000; Tamayo et al., 1997). The amplicons were electrophoresed through agarose 0.8% gel (Appligene, Illkirch, France) and then stained with ethidium bromide. A negative control (reaction mixture without template) and a toxin-positive control (\textit{V. cholerae} O1 strain O395) were included in each run.

**Results**

In 1993, after some years of absence, cholera reemerged in Ethiopia: in early October, the first cases were isolated in the city of Dire Dawa from patients with acute diarrhoea and reported to the Regional Health Bureau in the Ethiopian city of Harar. Over the next few months, active focuses were also reported in urban and rural areas of the Ethiopian regional states of Oromiya, Somali, and Southern Peoples and in the Addis Ababa urban region (Fig. 1A). The 1994 institutional reporting was poor and irregular, and the figures on cases and deaths considerably underestimated the real incidence of cholera. Direct investigations were conducted in the town of Jijiga (capital of the Somali regional state) and, for short periods, on the southern Somali zones of Afder and Liban.

The outbreak in Jijiga started in December 1993 and ended in June 1994. The final figures showed 1039 cases (577 in town) with 38 deaths (case-fatality rate 6 per 1000) with a case-fatality rate of 2.2%.

No extensive recrudescence of the disease was observed in 1996 or 1997.

In 1998, when cholera reappeared in the form of an epidemic wave, we set up a surveillance system to investigate the epidemiological and microbiological aspects of severe dehydrating diarrhoeal diseases in the Ethiopian city of Harar. The Ethiopian states of Somali, Oromiya, and Southern Peoples and the urban administrative regions of Addis Ababa and Dire Dawa were also included in our surveillance system when reports of cholera reached us from these areas.

Thousands of cases, distributed in numerous outbreaks, occurred in different areas of the regional states of Oromiya, Somali, and Harari People and in the administrative regions of Addis Ababa and Dire Dawa (Fig. 1A).

The epidemiologic pattern of the major outbreak that occurred in the Harari People state from mid-February to mid-April 1998 was studied in detail. The attack rate was 2 per 1000 population in the 2 months of the epidemic. Of 270 patients seen at the Harar City treatment centres and culture-positive for \textit{V. cholerae} O1, 58% were males and 42% females. The median age of patients was 18 years (mean age 19 years) with a range 1 month to 79 years. Eight percent of the patients were infants, 25% were aged 1–4 years, 11% were aged 5–14 years, 46% were adults 15–44 years of age, and 10% were adults older than 44 years.

Late in 1998 and early in 1999, the epidemic wave declined with a limited, fluctuating number of cases which appeared to decrease progressively.

A group of 22 \textit{V. cholerae} O1 strains was selected from among clinical isolates from 6 outbreaks that had occurred in the regional states of Oromiya (2 outbreaks), Somali, and Harari People, and in the regions of Addis Ababa and Dire Dawa. They were representative of the 1998 epidemic period of the cholera history in Ethiopia. All strains were biotype El Tor and serotype Ogawa.

Genomic DNA fingerprints of the 22 El Tor strains were obtained using PCR to generate RAPD patterns with the 6 oligonucleotides ERIC1, ERIC2, VCR1, VCR2, ATX1, and ATX2. Each strain was characterized by a combination of the 6 single RAPD patterns. All strains produced the same combination E2/E14/V2/V5/A2/A8 (Fig. 1B) and classified within a single RAPD cluster type designated VIII (Table 1). The El Tor strains were also typed by \textit{BglII} restriction patterns of 16S and 23S rRNA genes. Three ribotype patterns were observed. The great majority of strains (18 strains) belonged to ribotype B8a, 3 strains were ribotype B5a, and one strain was ribotype B24a (data not shown).
The 22 strains were resistant to AMP, CHL, SMX, SPT, STR and TMP (Table 1). The genes encoding resistance to AMP, CHL, SMX, STR, and TMP were located on a conjugative plasmid belonging to incompatibility class C. In all the strains, the resistance plasmid had the same molecular size and exhibited identical phenotypic traits such as stability in V. cholerae and frequency of transfer in matings of V. cholerae and V. cholerae (average frequency $3.5 \times 10^{-5}$) (data not shown).

Of 22 strains tested for the presence of genes ctxA, zot, ace, and tcpA, 18 strains were PCR-positive for the 4 genes, 3 strains were negative for the genes ctxA, zot, and ace, and one strain was negative for the gene ace.

Discussion

The history of cholera in Ethiopia has been documented for the 19th and the early 20th centuries (Pankhurst, 1965). At least 5 cholera epidemics, characterized by several outbreaks coming in more than one wave, occurred in 1831–1836, 1856, 1866–1867, 1889–1892, and in 1906. However, with the exception of 850 cases notified in 1970, 16 in 2004 and 54,070 in 2006, no epidemiological studies have been reported for the ongoing 7th pandemic.

This study has investigated the spread of cholera epidemics in Ethiopia in the mid 1990s and the extent of the clonal relationship of V. cholerae O1 strains active in 1998.

The onset of the cholera epidemic in Ethiopia in 1993 can probably be traced back to the spread of V. cholerae O1 strains responsible for the large cholera outbreak which hit Djibouti at the same time (10,055 cases) after 7 years of absence. Following its introduction, cholera spread widely at least through most central and southern Ethiopian areas where it persisted extensively for the following 2 years. In 1994, cholera extended to Somalia causing the largest epidemic (27,904 cases) ever notified to the WHO. Over the following decade, cholera established itself permanently in Somalia with an average of 9000 clinical cases annually.

The attack rate in the Ethiopian urban and rural areas investigated showed no significant difference, ranging from 2–15 per 1000 with an average case-fatality rate of 2.5%.

The recrudescence of the disease in 1998 was characterized by the isolation of multidrug-resistant strains (resistance to ≥3 antimicrobials). Twenty-two V. cholerae O1 strains isolated from the regional states of Oromiya, Somali, and Harari People and in the regions of Addis Ababa and Dire Dawa were selected to represent the re-emergence of cholera outbreaks in these areas. All isolates were resistant to AMP, CHL, SMX, SPT, STR, and TMP conferred by a conjugative IncC plasmid, except for SPT.

Genomic typing by RAPD assay grouped all strains in the same cluster type VIII, while BglI ribotyping identified 3 closely related ribotypes (B5a, B8a and B24a) (Damian et al., 1998), among which B8a was the most frequently identified. Interestingly, V. cholerae O1 strains with indistinguishable antimicrobial resistance pattern and genomic molecular features were widely identified from Somalia and, to a lesser extent, along the south-eastern Kenyan border with Somalia (Garissa). This last group of isolates came very likely from the spread of the V. cholerae O1 strains active in Somalia (Scrascia et al., 2006; Scrascia et al., 2009). Resistance to AMP, CHL, SMX, and TMP increased constantly in Somalia from 1994 to 1996 (Materu et al., 1997) indicating that acquisition of the IncC plasmid AMP–CHL–SMX–STR–TMP can very probably explain the appearance of the multidrug-resistance of the group of clonally related V. cholerae O1 strains which spread in the Horn of Africa in the late 1990s.

To our knowledge, this is the first study on cholera epidemiology in Ethiopia within the 7th pandemic. Data produced from this study have highlighted the role played by Ethiopia in the dissemination of cholera during the large epidemic in the mid 1990s.

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References

