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Antimicrobial Resistance and Molecular Characterization of *Vibrio cholerae* O1 During the 2004 and 2005 Outbreak of Cholera in Cameroon

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Abstract

There was an outbreak of cholera in Cameroon during 2004 and 2005; the epidemic began in Douala in January 2004 and spread throughout the south of the country. The World Health Organization (WHO) reported 8005 cases in 2004 and 2847 cases in 2005. Five hundred eighty-nine stool samples were received in the Pasteur Centre of Cameroon and 352 were microbiologically confirmed to be positive for *Vibrio cholerae* O1. Isolated strains were tested for their antimicrobial susceptibilities. All the strains were multidrug resistant and predominantly showed a common resistance pattern at the beginning of the outbreak. Tetracycline, recommended by the WHO for treating cholera in adults, was effective against all the strains tested. Cotrimoxazole (trimethoprim/sulfamethoxazole), previously a first-line treatment in children, was ineffective *in vitro* for all the clinical isolates and was quickly replaced by amoxicillin. Ampicillin resistance emerged at the end of 2004 and was the leading resistance pattern observed in the second half of 2005. This therefore represented the second major resistance pattern. These two major resistance profiles were not associated with patient characteristics (sex and age) or to the geographic origin of strains. However, there was a highly significant relationship between resistance patterns and the year of isolation ($p < 0.001$). The strains possessed genes *ctxA* and *ctxB* encoding the two cholera toxin subunits and were very closely related, irrespective of their antimicrobial resistance patterns. They were not differentiated by molecular typing methods and gave similar ribotyping and pulsed-field gel electrophoresis patterns.

Introduction

Cholera is an acute intestinal infection caused by toxigenic *Vibrio cholerae* of the O1 and O139 serogroups spread by fecally contaminated water and food. These Gram-negative bacteria produce an enterotoxin that causes abundant, painless, watery diarrhea (associated very often with vomiting) that can quickly lead to severe life-threatening dehydration if treatment is not promptly administered (WHO, 2001). Rehydration is the mainstay of cholera treatment. Antimicrobial drugs are a useful adjunctive therapy, decreasing the duration of both diarrhea and bacterial shedding and consequently diminishing the volume of fluid replacement needed for treatment (WHO, 2001). Inexpensive antimicrobials traditionally and widely used include doxycycline or tetracycline for adults, cotrimoxazole (trimethoprim/sulfamethoxazole) for children, and furazolidone for pregnant women (WHO, 2001). Nowadays, multiple antimicrobial-resistant *V. cholerae* strains have been described in many

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areas (Garg *et al.*, 2000; Rakoto Alson *et al.*, 2001; Iwanaga *et al.*, 2004; Faruque *et al.*, 2006) thanks to the regular surveillance of drug resistance. Their progressive increase is becoming a problem of great importance especially in developing countries; a leaflet published in 2004 by the World Health Organization (WHO) Global Task Force on Cholera Control, which was aimed at guiding healthcare workers through the very first days of an outbreak, proposed treatment of young children with liquid erythromycin, due to the increasing resistance to cotrimoxazole (WHO, 2004b).

Worldwide, outbreaks of cholera are reported to WHO annually. The recent outbreak in Cameroon covered the years 2004 and 2005 and extended throughout the south of the country. Eight thousand five persons were infected in 2004 (WHO, 2005) and 2847 in 2005 (WHO, 2006); the case fatality rate was 1.7% in 2004 and 3.9% in 2005. Cases were microbiologically confirmed in the Pasteur Centre of Cameroon (PCC). Infected adults were treated with a single dose of 300-mg doxycycline; children and pregnant women were given a 50 mg/kg weight of amoxicillin, three times daily for 3 days.

This study was aimed at describing and analyzing the antimicrobial resistance patterns of *V. cholerae* O1 isolated at PCC during the 2004–2005 outbreak of cholera and determining the genetic characteristics of strains.

Materials and Methods

Sample collection and bacteriological analysis

During the outbreak, suspected cholera cases were identified according to the WHO recommendation (WHO, 2004a). Briefly, in the area where the disease had not been known, a case of cholera was suspected when a patient aged at least 5 years presented with severe dehydration or died from acute watery diarrhea; in areas where cases had been declared, cholera was suspected if a patient of at least 5 years had acute watery diarrhea with or without vomiting. Stool specimens collected from these patients (one per patient) in sterile dishes were sent with patient data (name, sex, age, and locality) to the PCC for bacteriological analysis. Stools were sometimes collected with sterile swabs into transport medium (Carry Blair agar) and sent to PCC.

Upon arrival, stool samples or swabs were processed immediately or kept in refrigerated conditions and processed within 24 hours. The rapid microbiological cholera diagnosis was obtained by direct observation of highly motile rods on a wet mount of stool specimen examined microscopically. Following this, stools were enriched in alkaline peptone water for 3 to 6 hours at 37°C, and plated on thiosulfate–citrate–bile salts–sucrose agar (TCBS) and Mueller Hinton agar (MH) for 18 to 24 hours at 37°C. Nonluminescent yellow colonies on TCBS and bluish translucent colonies on MH characteristic of *V. cholerae* were subsequently examined for the positive oxidase reaction (only strains on MH). Isolated colonies on MH were serogrouped with polyvalent O1 antiserum, the identification of *V. cholerae* strains was further confirmed based on biochemical characters (API 20E system, bioMérieux, Marcy L'étoile, France). Strains were stored on conservative agar (Bio-Rad Laboratories, Marnes-la-Coquette, France) at room temperature. Stools were also cultured to check for other enteric pathogens.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *V. cholerae* isolates was performed by a disk diffusion method on MH agar to the following antimicrobials: ampicillin (AM, 10 μg), amoxicillin/clavulanic acid (AMC, 20/10 μg), cefotaxim (CTX, 30 μg), streptomycin (S, 10 μg), gentamicin (GEN, 15 μg), tetracycline (TE, 30 μg), chloramphenicol (C, 30 μg), colistin (COL, 50 μg), nalidixic acid (NA, 30 μg) ciprofloxacin (CIP, 5 μg), sulfonamids (SSS, 200 μg), trimethoprim/sulfamethoxazole (TSU, 1.25/23.75 μg) (Bio-Rad Laboratories). Since interpretative criteria have not been established for *V. cholerae*, the disk strength and zone size interpretation were used in accordance with Antibiogram Committee of the French Microbiology Society (CA-SFM, 2005) susceptibility criteria standardized for *Enterobacteriaceae*. The reference strain *Escherichia coli* ATCC 25922 was used as a quality control strain. Strains showing intermediate zones of resistance for clinical reasons were interpreted as resistant. Isolates that were resistant or had decreased susceptibility to three or more antimicrobials were defined as multidrug resistant (MDR).

Determination of minimum inhibitory concentrations (MIC)

Twenty strains with reduced susceptibility to AM (diameter size $\leq$ 14 mm) and 20 other strains fully susceptible were randomly selected for MIC determination of AM and doxycycline (DO) using twofold dilutions. Ten dilutions were tested for both antimicrobials (64 to 0.125 μg/mL). Resistance categories were determined according to the CA-SFM standards for *Enterobacteriaceae*. The recorded MICs were the lowest concentrations of antimicrobials that completely inhibited visible growth of the tested strains in standardized conditions.

Molecular analysis

Detection of genes encoding the cholera toxin and molecular typing of *V. cholerae* O1 strains by ribotyping and pulsed-field gel electrophoresis (PFGE) were carried out in the National Reference Centre for Vibrios and Cholera in the Pasteur Institute in Paris.

DNA extraction. Chromosomal DNA was extracted by a miniprep procedure adapted from the method of Brenner *et al.* (1982) and microdialyzed for 2 hours on VS 0.025 μm nitrocellulose filter (Millipore SA, Molsheim, France), against distilled water. The amount and the purity of the DNA were measured by spectrophotometry (230, 260, and 280 nm) using the programmed dsDNA method of the Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany).

Polymerase chain reaction (PCR) amplification of cholera toxin genes. Genes encoding the two cholera toxin A and B subunits were sought by PCR assays on purified DNA as previously described (Fields *et al.*, 1992; Olsvik *et al.*, 1993). All the primers were synthesized by Proligo (Paris, France).

Ribotyping. Ribotyping was performed as described by Popovic *et al.* (1993); DNA (5 μg) was digested overnight with 10 units/μg of *Bgl*I restriction endonuclease (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C. Restriction fragments were separated on a 0.8% (w/v) agarose gel (SIGMA Type II, St. Louis, MO), in 1×Tris-borate-EDTA buffer (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.3), alkali-blotted onto a positively charged nylon membrane (Roche Diagnostics) and immobilized by incubation for 1 hour at 80°C. Digoxigenin (DIG)-cDNA generated from 16 + 23S ribosomal RNA from *Escherichia coli* (Roche Diagnostics) was used as a probe. *Mlu*I (Roche Diagnostics) restriction fragments of *Citrobacter koseri* strain 32 (Collection de l'Institut Pasteur CIP 1066-70), were used as molecular weight markers. Hybridization was performed overnight at 65°C. Detection of hybridized fragments was performed by anti-digoxigenin alkaline phosphatase–labeled antibodies and the appropriate colorimetric reagents according to the manufacturer's specifications (Roche Diagnostics).

PFGE. PFGE was performed as described by Cameron *et al.* (1994). Electrophoresis of *Not*I restriction fragments was carried out using the Master CHEF DR III system in a 1.2% pulsed-field certified agarose gel (Bio-Rad Laboratories) in 0.5×Tris-borate-EDTA electrophoresis buffer at 6 V/cm, with the following pulse parameters: 5 to 50 seconds (22 hours) and 3 to 4 seconds (12 hours). Phage lambda DNA ladders (Bio-Rad Laboratories) were used as molecular size markers. Gels were stained in ethidium bromide (0.5 μg/mL; Eurobio, Les Ulis, France), and visualized with a Bio-Rad Gel Doc (EQ 170-8066).

Data analysis

The chi square test or Fisher exact test was used as appropriate to compare different *V. cholerae* antimicrobial resistance patterns according to geographic origin, period of stool collection (2004 or 2005), and patient parameters (sex and age). Epi-Info version 6.04 (CDC, Atlanta, GA) was used for all statistical analyses with a significant level of 5%.

Results

Sample characteristics and V. cholerae *O1 isolation*

From January 2004 through December 2005, 589 patients' stool samples and swabs were received at the PCC for cholera diagnoses and antimicrobial susceptibility testing. A total of 352 *V. cholerae* serogroup O1 strains (60% of

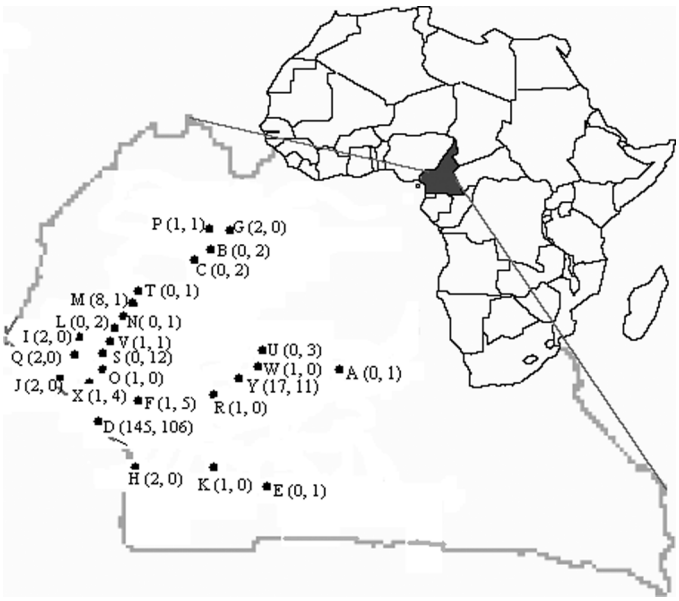

FIG. 1. A representation of the south of Cameroon showing the number of *Vibrio cholerae* O1 strains isolated in each site of sampling: Ayos (A), Bafoussam (B), Bamendjou (C), Douala (D), Ebolowa (E), Edea (F), Foumbot (G), Kribi (H), Kumba (I), Limbe (J), Lolodorf (K), Loum (L), Manjo (M), Mantem (N), Mbanga (O), Mbouda (P), Muyuka (Q), Ngoumou (R), Njombe (S), Nkongsamba (T), Obala (U), Penja (V), Soa (W), Tiko (X), Yaounde (Y). The first number in brackets refers to the number of strains isolated in 2004, and the second to the number of strains isolated in 2005.

specimen analyzed) were detected (188/316 received in 2004 and 164/273 in 2005). The different sites of sampling and their corresponding number of *V. cholerae* O1 isolated are presented in Fig. 1. Almost all the south of Cameroon was affected by the outbreak. The strains were collected from samples of 170 males and 168 females. The median age of the infected patients was 29.5 years (range 4 months to 80 years). Of the stools received, 45.5% were rice watery ($n = 268$), 15.6% colorless watery ($n = 92$), 11.4% yellowish watery ($n = 67$), 9.5% brown watery ($n = 56$), 6.8% greenish watery ($n = 40$), and 3.1% glaireous ($n = 18$); 8.1% ($n = 48$) were swabs. Samples positive for *V. cholerae* O1 included 70.9% of the rice watery stools, 55.4% of colorless, 65.7% of yellowish, 25% of greenish, 33.9% of brown, 38.9% of glaireous, and 64.6% of swabs. Although glaireous stools generally denote *Shigella* infection, in this study, 7 of the 18 collected stools were infected with *V. cholerae*. The isolation rate was significantly higher in rice watery stools than in others ($p < 0.01$).

Antimicrobial resistance patterns

All the *V. cholerae* O1 strains tested were resistant to COL, S, SSS, and TSU. The percentage of resistance to C was also very high (98.9%) during the both years; four strains (1.1%) isolated in 2005 were susceptible to C. Decrease in AM susceptibility was observed in 4.3% of strains in 2004 and 37.8% in 2005; complete resistance was observed only in 2005 and concerned 6.1% of strains. All *V. cholerae* strains were uniformly susceptible to CTX, TE, GEN, NA, and CIP.

The MIC of AM for the 20 strains with resistance or decreased susceptibility to β-lactams varied from 16 to 2 μg/mL with a CI 50 (concentration that inhibited 50% of the strains) of 4 μg/mL, corresponding to the limit of sensitivity of AM (MIC ≥ 4 μg/mL was considered reduced susceptibility). The MIC values of AM for the AM-susceptible strains were in the susceptible zone (CI 50 value of 0.5 μg/mL). MIC values of DO for all the strains analyzed varied from 2 to 0.125 μg/mL with a CI 50 value of 0.5 μg/mL; these results confirmed the higher efficiency of DO and the reduction of AM susceptibility as observed by disk diffusion method.

All *V. cholerae* strains were MDR and showed almost similar patterns, with the exception of β-lactam antimicrobials, for which decreased susceptibility occurred mostly in the second year of the outbreak (2005), and chloramphenicol. Table 1 presents the different resistance patterns of the strains isolated. In all, three resistance patterns were observed. The most common resistance phenotype, COL-S-C-SSS-TSU (pattern A), included 77.3% of all isolates (95.7% of strains in 2004 and 56.1% in 2005). The second important phenotype showed an additional resistance to AM and included 21.6% of all strains (4.3% in 2004 and 41.5% in 2005). This second

Table 1. Antimicrobial Resistance Patterns of *Vibrio cholerae* O1 Strains Isolated in Cameroon in 2004 and 2005

	No. (%) of V. cholerae *O1*		
	2004	*2005*	*Total*
C-COL-S-SSS-TSU[a]	180 (95.7)	92 (56.1)	272 (77.3)
AM-C-COL-S-SSS-TSU[b]	8 (4.3)	68 (41.5)	76 (21.6)
AM-COL-S-SSS-TSU[c]	0	4 (2.4)	4 (1.1)

The antimicrobial susceptibility test was performed by disk diffusion method.
[a]Strains resistant to chloramphenicol (C), colistin (COL), streptomycin (S), sulfonamids (SSS) and trimethoprim/sulfamethoxazole (TSU): Pattern A.
[b]Strains resistant to ampicillin (AM), C, COL, S, SSS and TSU: Pattern B.
[c]Strains resistant to AM, COL, S, SSS, TSU and susceptible to C: Pattern C.

phenotype, AM-COL-S-C-SSS-TSU was called pattern B. Another resistance pattern, showing susceptibility to C and reduced susceptibility to AM, was observed in 2005, and included 1.1% of strains, but this minor pattern (pattern C) was not considered for analysis.

Relation of V. cholerae *O1 resistance patterns with period of sample collection, geographic area and patient parameters*

Only the two major resistance patterns (A and B) have been considered. There was no association between the resistance patterns and the geographical origin of strains. However, the resistance pattern was significantly related to the year of isolation ($p < 0.001$); pattern B was mostly observed in 2005 (41.5%) when compared with 2004 results (4.3%) in which pattern A was observed at 95.7%. The distribution of the A and B patterns during the both years of the outbreak is shown in Fig. 2. From January to April 2004, eight strains showed pattern B; these strains were isolated in the areas of Douala (2), Kumba (1), Limbe (2), Manjo (2), and Muyuka (1). This pattern B re-emerged in March 2005 and remained alone at the end stage of the outbreak. The trend of resistance observed in these *V. cholerae* O1 strains was through acquisition of resistance to β-lactams. There was no significant association between resistance patterns, and sex and age of patients.

Results of molecular characterization

The *ctxA* and *ctxB* genes were detected in all the *V. cholerae* O1 strains tested by PCR. A unique *Bgl*I rRNA gene restriction pattern was observed among all the isolates (Fig. 3A). Moreover a unique PFGE pattern was also observed in all strains studied, irrespective of their AM resistance pattern (Fig. 3B).

Discussion

Increasing antimicrobial resistance affects the selection and use of antimicrobial agents; consequently, updating of antimicrobial resistance data is crucial to ensure effective treatment of patients. We report here antimicrobial-resistant phenotypes of *V. cholerae* O1 strains isolated during an outbreak that occurred in Cameroon from 2004 to 2005. A total of 589 stools were received at PCC and examined for cholera, from which 352 *V. cholerae* O1 strains (60%) were isolated. This percentage of 60% of stool samples positive for *V. cholerae* O1 corroborates the WHO clinical case definition based on watery diarrhea for persons aged 5 years or older in epidemic

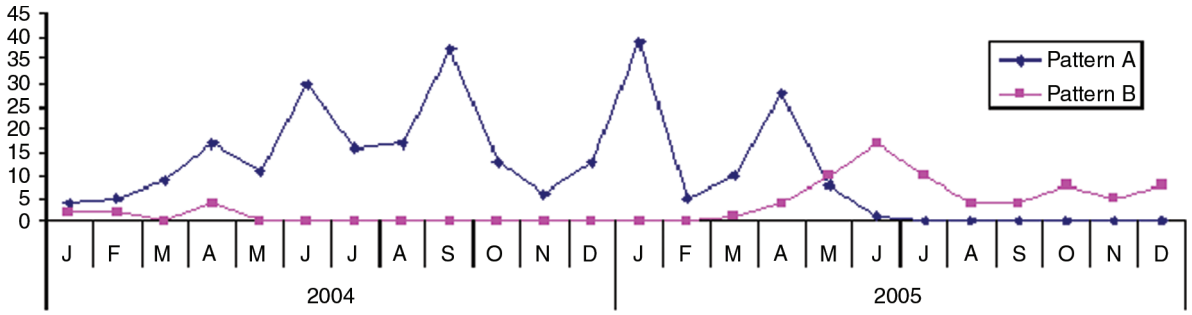

FIG. 2. Evolution of *Vibrio cholerae* O1 resistance patterns in Cameroon in 2004 and 2005. Pattern A represents strains resistant to chloramphenicol, colistin, streptomycin, sulfonamids, and trimethoprim/sulfamethoxazole (cotrimoxazole). Pattern B represents strains with an additional resistance to ampicillin. Letters along the *x*-axis correspond to months January to December of each year.

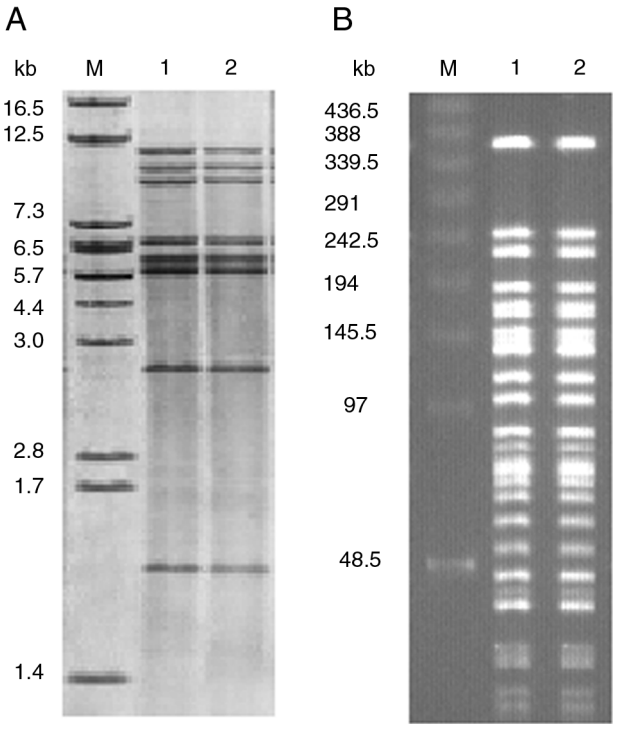

FIG. 3. Representative *Bgl*I ribotype pattern (**A**) and *Not*I PFGE pattern (**B**) of the *Vibrio cholerae* O1 strains susceptible (1) and resistant (2) to ampicillin during the 2004 and 2005 outbreak in Cameroon. The molecular weight marker (M) used in ribotyping (**A**) was the *Mlu*I (Roche Diagnostics) restriction fragments of *Citrobacter koseri* strain 32 (Collection de l'Institut Pasteur CIP 1066-70). In PFGE (**B**), the molecular weight marker used was Phage lambda DNA ladder (Bio-Rad).

settings. A percentage of 70.9% of stools positive for *V. cholerae* O1 culture were rice watery, but this could not be strictly considered in case definition because the color of stool can't clearly predict the presence of a particular bacterial species as shown by these results.

Antimicrobial susceptibility data were generated using a panel of 12 antimicrobial agents. There is yet no *in vitro* method that can determine accurately the susceptibility to erythromycin, although the drug has been recommended for treatment by WHO (2003); therefore, susceptibility to erythromycin was not tested in this study. All the isolates were MDR, with a general pattern of COL-S-C-SSS-TSU. A number of studies have reported high prevalence of *V. cholerae* O1 strains resistant to C, S, and TSU (Hochhut *et al.*, 2001; Iwanaga *et al.*, 2004; Ahmed *et al.*, 2005). The MDR strains appeared in Cameroon after the mass prophylaxis with sulfadoxine in a large outbreak that occurred in Douala in 1983 (Garrigue *et al.*, 1986). Similar results were obtained in Madagascar where multidrug resistance was observed only a few months after the beginning of the antimicrobial treatment. Multidrug resistance of strains may lead to serious cases. In Guinée Bissau, for example, mortality increased from 1% to 5.3% after the occurrence of MDR *V. cholerae* O1 strains in 1996–1997 (Dalsgaard *et al.*, 2000).

An interesting finding in this study was that no cycline resistance was recovered *in vitro*, despite the common use of this drug in Cameroon, and especially during this cholera outbreak. Clinically, DO remained effective in therapy (Guevart *et al.*, 2006) and mass prophylaxis (Guevart *et al.*, 2005). *V. cholerae* O1 susceptibility to DO has also been reported in Senegal (Aidara *et al.*, 1998) despite the common use of this drug during outbreaks.

Resistance to AM was reported in many studies presenting MDR strains. However, in our investigation, decreased susceptibility to AM was mostly observed in the second year of the outbreak. The resistance rate to AM increased significantly from 4.3% in 2004 to 43.9% (including strains susceptible to C) in 2005. The results showed that strains susceptible or resistant to AM coexisted in the same area. Resistance to TSU without AM at the early stage of the outbreak might have been selected by the use of TSU in children and pregnant women. Once the PCC strains had presented *in vitro* resistance to TSU, amoxicillin was introduced in the treatment; this aspect, combined with the extensive use of amoxicillin in Cameroon for the treatment of many other infections, might have contributed to the re-emergence and the expansion of the resistance to AM. Some authors proposed the restricted use of antimicrobials to severe cases of *V. cholerae* O1 infections, to avoid rapid change in susceptibility (Materu *et al.*, 1997). Third generation cephalosporins and quinolones remained active on these strains; however, resistance to quinolones is now being reported in many countries (Tarantola and Quilici, 2007).

This study was intended to present the current resistance patterns of *V. cholerae* O1 isolated in Cameroon and to investigate whether the WHO recommended drugs are still effective against Cameroonian strains. Our results showed that

DO remained active in all strains; on the other hand, the efficiency of TSU and AM was too low. *V. cholerae* O1 resistance to antimicrobials should be considered with more attention in order to improve case management. However, the most effective way to prevent cholera is to promote health education based on the improvement of hygiene. Due to the fact that strains isolated in 2004 and 2005 could not be differentiated by the two DNA-based typing methods used in this study, it can be stated that strains with both resistance patterns cohabitated in the same environment and emerged according to the selection pressure applied. This reinforces our interest in performing antimicrobial susceptibility tests, which can permit the differentiation of strains that are genetically similar. The β-lactams resistance might have been a result of the production of β-lactamases without important genomic variation of the strain. It would be interesting in the future to study the molecular basis of the multidrug resistance of these *V. cholerae* O1 strains.

Disclosure Statement

No competing financial interests exist.

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