

Vibrio cholerae O1 from Accra, Ghana carrying a class 2 integron and the SXT element

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Objectives: *Vibrio cholerae* O1 from a 2006 outbreak in Accra were commonly resistant to multiple antimicrobials and, in particular, to trimethoprim/sulfamethoxazole, drugs commonly used in the treatment of cholera. We sought to determine the genetic basis for trimethoprim/sulfamethoxazole resistance in outbreak isolates.

Methods: Twenty-seven isolates from the outbreak were screened by PCR and sequencing for class 1 and 2 integrons and for the SXT element.

Results: Twenty-one of the 27 isolates examined, all from the Accra metropolitan area, carried both SXT, an integrated chromosomal element, and a class 2 integron bearing *dfra1*, *sat* and *aadA1* cassettes. All these isolates had identical random amplification of polymorphic DNA profiles and two of them also carried a class 1 integron.

Conclusions: Most strains characterized carried multiple elements conferring resistance to trimethoprim. This suggests that trimethoprim/sulfamethoxazole should not be used empirically in cholera treatment.

Keywords: trimethoprim resistance, antimicrobial resistance, antibiotic resistance, cholera

Introduction

Cholera is an acute diarrhoeal disease caused by *Vibrio cholerae* O1 or O139. Cholera epidemics spread rapidly and, without intervention, can lead to death due to dehydration. In 2006, 98.9% of the cholera cases reported worldwide, and all but 8 of the 6311 deaths, were reported from Africa.¹ Angola, the Democratic Republic of Congo, Ethiopia and Sudan were the worst-hit countries, but most coastal West African countries reported large numbers of cases and case fatality rates of 1% to 6.2%. Between 2 January and 25 June 2006, 1869 cases and 79 deaths (a 4.2% case fatality rate) were reported in Ghana. By the end of the year, the count was 3357 cases and 107 deaths, with an overall case fatality rate of 3.19%.¹

Antimicrobials are not required to manage cholera, but they shorten the duration and reduce the severity of the disease, curbing transmission. Thus, antimicrobial resistance can increase the outbreak size, duration and case fatality rates. Tetracycline

was originally the antimicrobial of choice for cholera and was used widely in Africa until resistance to the drug conferred by incompatibility group C plasmids became common. Subsequently, trimethoprim/sulfamethoxazole, ampicillin and quinolones have been used, but resistance to these and other drugs has been reported (reviewed by Okeke *et al.*²).

Although Africa bears much of the present-day burden of cholera,^{1,3} very little is known about strain susceptibility, particularly in West Africa. In 2000, Dalsgaard *et al.*⁴ described a *V. cholerae* multiresistance plasmid, bearing a class 1 integron from Guinea-Bissau. There have been other reports of antimicrobial-resistant *V. cholerae* from West Africa, but none have undertaken molecular analysis to identify specific resistance genes and dissemination mechanisms.^{5,6} In contrast, multiple studies from outbreaks in southern and eastern Africa elucidate the molecular basis for resistance in *V. cholerae*,^{7–11} where resistance has been increasingly common in recent years and has largely been associated with strains carrying resistance cassettes in class 1

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integrons. Recent data from Zambia have demonstrated that strains carrying a resistance-conferring integrated chromosomal element known as the SXT element have also emerged.¹²

Materials and methods

Strains

We studied antimicrobial resistance in 27 isolates from the January to June 2006 outbreak that occurred in Ghana. The strains were isolated from patients at 14 different locations.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed according to the disc diffusion method described by the CLSI (formerly the NCCLS)¹³ and using *Escherichia coli* NCTC 10418 as a control. Wild-type *V. cholerae* isolates were screened for resistance to trimethoprim/sulfamethoxazole, amikacin, ceftazidime, ceftriaxone, cefotaxime, chloramphenicol, gentamicin, tetracycline, nalidixic acid, cefuroxime and levofloxacin at the University of Ghana Medical School. Recombinant strains, in an *E. coli* background, were tested against ampicillin, trimethoprim, streptomycin, chloramphenicol, sulphonamides, nalidixic acid and ciprofloxacin at the molecular microbiology laboratory at Haverford College. The diameter of inhibition zones was measured in millimetres and interpreted according to the CLSI requirements.¹³

Detection and characterization of resistance genes and elements

V. cholerae genomic DNA was extracted using the Wizard genomic extraction kit (Promega) according to manufacturer's directions and used to create a template of PCR reactions. The oligonucleotide primer pair *dfr1a*, which amplifies six *dfrA* alleles as described by Navia *et al.*,¹⁴ was used to screen the strains for trimethoprim-resistant dihydrofolate reductase genes. Enteroaggregative *E. coli* strain 17-2, which carries *dfrA1*, was used as a positive control. Primers that anneal to the 3' and 5' conserved ends of class 1 and class 2 integrons were to amplify integron-borne cassettes, essentially according to the methods described by Lévesque *et al.*¹⁵ and White *et al.*¹⁶ Integrated cassette amplicons were cloned into pGEMT (Promega) and sequenced, and their *MboI* and *AluI* restriction fragment length polymorphisms (RFLPs) were compared with sequenced amplicons. Enteroaggregative *E. coli* strains 042 and 17-2, which carry an *aadA1*-bearing class 1 integron and a *dfrA1-sat-aadA1*-bearing class 2 integron, respectively, were used as positive controls. The class 1 integron integrase gene (*intI1*) was identified using the primers described by Leverstein-van Hall *et al.*,¹⁷ and the SXT integrase gene was detected by PCR, as described by Dalsgaard *et al.*⁷

Random amplification of polymorphic DNA

Strain relatedness was assessed by random amplification of polymorphic DNA (RAPD), as described by Scarscia *et al.*,¹⁸ employing enterobacterial repetitive intergenic consensus primers (ERIC1 and ERIC2).

Results

Biochemical and serological verification revealed that all the outbreak isolates were *V. cholerae* O1 serotype Ogawa, which is

commonly reported in Africa. As shown in Table 1, only 3 of the 27 isolates were resistant to tetracycline, but all 27 strains were resistant to other antimicrobials, 13 were resistant to 3 or more of the 11 agents tested, and levofloxacin was the only tested drug to which all isolates were susceptible. We observed nalidixic acid resistance in 10 isolates, and 26 of the 27 isolates were resistant to trimethoprim/sulfamethoxazole.

Twenty-five of the 27 isolates produced a 0.47 kb amplicon with the *dfr1a* primer pair that amplifies *dfrA1*, *dfrA5*, *dfrA15*, *dfrA15b*, *dfrA16* or *dfrA16b* cassettes, but we did not amplify cassette regions from class 1 integrons in any of the strains. The class 1 integron integrase gene was, however, detected in two strains, suggesting that these strains had very large variable regions and/or a genetic modification in the 3' or 5' conserved ends recognized by the cassette-region primers of Lévesque *et al.*¹⁵ Importantly, the two class 1 integron-positive strains were two of the three strains that were resistant to seven or more of the antimicrobials tested (Table 1). However, although class 1 integrons were associated with multiple resistance, they could not account for most of the *dfr* cassettes detected. Screening for class 2 integron-borne cassettes produced a 2.2 kb product from control strain 17-2 as well as from 22 *V. cholerae* isolates that produced an amplicon with the *dfr1a* primers. The class 2 cassette-region amplicon from strains V34 and V47 was directionally cloned into pGEMT. The resulting clones were screened for resistance to eight antimicrobials. Both were resistant to ampicillin (encoded on the vector), trimethoprim and streptomycin, but susceptible to chloramphenicol, sulphonamides, nalidixic acid and ciprofloxacin. We sequenced the cloned amplicon from the strain V34 and found that it contained three resistance gene cassettes, in the commonly recovered context that is identical to that in strain 17-2: *dfrA1-sat-aadA1*. Strains that could, produced amplicons of similar size; *MboI* and *AluI* RFLP patterns were also identical to the patterns from strains V34, V47 and EAEC strain 17-2.

Primers for the SXT integrase gene were used to screen for the SXT element, as described by Dalsgaard *et al.*⁷ We obtained a 0.6 kb amplicon, consistent with the expected size of 592 bp produced by strains bearing the SXT element, in 24 strains. As we did not have a positive control strain, we cloned the amplicon from the Ghanaian strain V34 into the vector pGEMT (Promega) and sequenced it. The sequence obtained was 99% identical to the SXT integrase in the GenBank database (accession number AB114188.1).

Of the 27 isolates screened, 3 carried the SXT element alone, one bore a class 2 integron with *dfrA1-sat-aadA* cassettes but no SXT, and 21 strains possessed both elements. All strains harbouring one or both elements showed high-level resistance to trimethoprim/sulfamethoxazole and produced an amplicon with the *dfr1a* primers. Of the two strains that were negative for both elements, strain V111 was susceptible to trimethoprim/sulfamethoxazole and strain V95 exhibited low-level resistance by an unknown mechanism. All the strains that had neither or only one of the two trimethoprim resistance-conferring elements were recovered from patients from Awoshie, Agona Swedru, Ga West and Tema, all of which are away from the Accra Metropolitan area (Table 1). We additionally observed that although most of the strains generated an identical RAPD profile with ERIC2 primers, strains V95 and V111, both of which lacked the class 2 integron and the SXT element and were isolated from outside the Accra Metropolitan area, produced distinctly different profiles (Figure 1).

Vibrio cholerae O1 from a 2006 Accra outbreak

Table 1. Antimicrobial resistance profiles and conferring genetic elements in 27 2006 *V. cholerae* O1 isolates from Accra

Strain	Sex	Age	Location	Antimicrobial resistance profile	SXT element	Class 1 integron (<i>intI1</i> gene)	Class 2 integron	Class 2 integron cassettes
V112	m	3	Tema	SXT	+	–	+	<i>dfrA1-sat-aadA1</i>
V42	m	47	Zongo	SXT	+	–	+	<i>dfrA1-sat-aadA1</i>
V47	m	19	Nungua	SXT	+	–	+	<i>dfrA1-sat-aadA1</i>
V51	m	6	Abuofu	SXT	+	–	+	<i>dfrA1-sat-aadA1</i>
V52	m	34	Ayalolu	SXT	+	–	+	<i>dfrA1-sat-aadA1</i>
V86	f	23	Agbogbloshie	SXT	+	–	+	<i>dfrA1-sat-aadA1</i>
V97	m	60	Dangbe West	SXT	–	–	+	<i>dfrA1-sat-aadA1</i>
V34	f	13	Accra	SXT, NAL	+	–	+	<i>dfrA1-sat-aadA1</i>
V35	f	36	Accra	SXT, NAL	+	–	+	<i>dfrA1-sat-aadA1</i>
V40	m	35	Accra	SXT, NAL	+	–	+	<i>dfrA1-sat-aadA1</i>
V87	f	24	Adabraka	SXT, CRO	+	–	+	<i>dfrA1-sat-aadA1</i>
V95	m	2	Awoshie	SXT, CRO	–	–	–	–
V107	f	37	Agona Swedru	SXT, AMP	+	–	–	–
V98	m	8	Dangbe West	SXT, AMP	+	–	+	<i>dfrA1-sat-aadA1</i>
V1388	f	8	Ga West	SXT, CTX, CXM	+	–	+	<i>dfrA1-sat-aadA1</i>
V1433	m	6	Accra	SXT, CTX, CXM	+	–	+	<i>dfrA1-sat-aadA1</i>
V32	m	29	Ga West	SXT, AMP, NAL	+	–	–	–
V33	m	21	Ga West	SXT, AMP, NAL	+	–	–	–
V53	m	5	Agbogbloshie	SXT, AMP, NAL	+	–	+	<i>dfrA1-sat-aadA1</i>
V45	m	28	Agbogbloshie	SXT, AMP, CHL	+	–	+	<i>dfrA1-sat-aadA1</i>
V111	f	6	Tema	AMK, GEN, NAL	–	–	–	–
V84	f	25	Madina	SXT, AMP, TET, CXM	+	–	+	<i>dfrA1-sat-aadA1</i>
V79	m	9	Weija	SXT, AMK, AMP, CHL	+	–	+	<i>dfrA1-sat-aadA1</i>
V89	m	27	Tema	SXT, AMK, CAZ, CRO, GEN, NAL	+	–	+	<i>dfrA1-sat-aadA1</i>
V85	f	21	Adabraka	SXT, AMP, CRO, CTX, CHL, TET, CXM	+	–	+	<i>dfrA1-sat-aadA1</i>
V78	m	28	Agbogbloshie	SXT, AMP, CAZ, CRO, CHL, NAL, CXM	+	+	+	<i>dfrA1-sat-aadA1</i>
V90	m	7	Agbogbloshie	SXT, AMK, CAZ, CRO, CTX, CHL, GEN, TET, NAL, CXM	+	+	+	<i>dfrA1-sat-aadA1</i>

SXT, trimethoprim/sulfamethoxazole; AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; CHL, chloramphenicol; GEN, gentamicin; TET, tetracycline; NAL, nalidixic acid; CXM, cefuroxime. Also tested: levofloxacin.

Discussion

Recently, resistant *V. cholerae* epidemics in Africa have implicated class 1 integrons;^{4,7,8} however, class 1 integrons were detected in only two strains characterized in this study, both of which were resistant to seven or more of the tested antimicrobials. In *V. cholerae*, resistant dihydrofolate reductase (*dfr*) genes may also be part of a 62 kb transmissible integrated chromosomal element, known as SXT.¹⁹ In *E. coli* and closely related organisms, resistant *dfr* cassettes are commonly associated with class 1 or class 2 integrons. Class 2 integrons have only been recently reported from *V. cholerae*.²⁰ We elected to screen the isolates for both class 2 integrons and the SXT-integrated chromosomal element and found that most of the isolates carried both elements.

Class 2 integrons identified in this study contained similar cassettes as Tn7, a transposon commonly found in enteric organisms, where it may be located on conjugative plasmids or

chromosomally integrated at a specific attachment site between the *pstS* and *glmS* genes. Tn7-like elements have been found in non-O1/O139 *V. cholerae* and other *Vibrio* spp., in which they have been shown to be chromosomally integrated.²⁰ In this study, we report the presence of a class 2 integron in *V. cholerae* O1 Ogawa outbreak isolates, which have not been previously reported in Africa.

The earliest reports of trimethoprim resistance in epidemic *V. cholerae* O1 from Africa were associated with plasmid-borne *dfrA1* genes, most probably acquired from the gut microflora.^{11,21} Laboratory studies and strain characterization in successive outbreaks demonstrated that plasmid-borne resistance genes are sometimes poorly expressed in *V. cholerae* and that the plasmids were often not stably maintained in the absence of resistance or that strains carrying them were less fit and easily displaced by susceptible strains.^{10,22} In contrast, we found that trimethoprim resistance is conferred by integrated chromosomal elements, most strains evaluated in this study carried more than

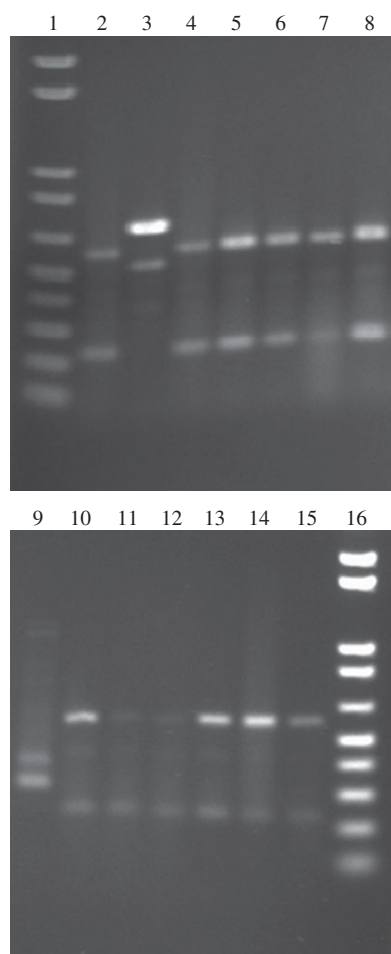


Figure 1. RAPD profiles of *V. cholerae* isolates generated with ERIC2 primers. Lanes 2–8, *V. cholerae* isolates V112, V42, V47, V51, V52, V86 and V97, respectively; lanes 9–15 *V. cholerae* isolates V111, V84, V79, V89, V85, V78 and V90, respectively. All other isolates produced profiles identical to V112. Lanes 1 and 16, 1 kb plus ladder (Invitrogen).

one element, and therefore, resistance is likely to be stable. Data demonstrate that multiple elements conferred trimethoprim resistance on isolates from this 2006 outbreak and that strains bearing such elements were disseminated through the Accra metropolis area. Consequently, trimethoprim/sulfamethoxazole should not be used in cholera treatment in this region. Tetracycline may be a possible alternative in this regard because even though studies in the 1970s and 1980s reported resistance, in this and some other recent studies, susceptibility has been unexpectedly common.^{2,23}

Ten of the isolates evaluated were resistant to nalidixic acid. Quinolones have, until recently, been a fail-safe alternative in cholera and other diarrhoeal disease epidemics. Our data suggest that quinolone susceptibility cannot be taken for granted and therefore this class of drugs is no longer a fail-safe empirical treatment. Stable trimethoprim resistance coupled with the appearance of quinolone resistance is worrisome, in that it further narrows options for empirical antimicrobial therapy and epidemic control. There is a need to monitor resistance profiles throughout present-day outbreaks and to encourage the implementation of non-antimicrobial strategies for transmission control, such as vaccination.

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Transparency declarations

None to declare.

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Vibrio cholerae O1 from a 2006 Accra outbreak

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