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Molecular characterization of cholera outbreak isolates in South Africa, 2008-2009

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Abstract

Background

Cholera is an acute diarrhoeal disease that generally presents as abrupt watery diarrhoea and vomiting. For the years 2008 to 2009, South Africa experienced two major outbreaks of cholera. The first outbreak was reported from May to July 2008 (Chapter Three) and the second outbreak from November 2008 to April 2009 (Chapter Four). Within both events, *Vibrio cholerae* (*V. cholerae*) O1 identified at peripheral laboratories displayed resistance to three or more routinely tested antimicrobial agents. The molecular epidemiology and mechanism of antimicrobial resistance of *V. cholerae* O1 isolates was investigated. This was achieved by using various molecular techniques, which included pulsed-field gel electrophoresis (PFGE) analysis, polymerase chain reaction (PCR), nucleotide sequencing, identification of plasmid DNA and Southern blot hybridization analysis.

Methods

As part of routine characterization of *V. cholerae* isolates at the Centre for Enteric Diseases (CED), isolates underwent serological and biochemical confirmatory identification as well as antimicrobial susceptibility testing using the Etest method. PFGE analysis was performed on *V. cholerae* O1 isolates digested with *Not*I restriction enzyme. One-hundred *V. cholerae* O1 isolates, ten isolates characterized in Chapter Three and 90 isolates characterized in Chapter Four were selected for further analysis to ensure that all PFGE banding patterns were represented. Three probable mechanisms of antimicrobial resistance were investigated. Firstly, PCR was used to detect for the presence of class 1 integrons (3'-CS and 5'-CS), class 2 integrons (*intI2*), plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrS*, *qnrC* and *qepA*), quinolone resistance determinant (*qnrVC3*), ESBL producing genes (bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$), genes coding for the quinolone resistance-determining region (QRDR) of DNA gyrase (*gyrA*/*gyrB*) and topoisomerase IV (*parC*/*parE*), SXT element-integrase gene (SXT*int*) and associated SXT resistance genes (*floR*, *sul2*, *dfrA1*, *dfr18*, *strA* and *strB*) and the class A tetracycline resistance determinant (*tetA*).

The presence of resistance plasmids was investigated by isolation of intact bacterial plasmid DNA. Southern blotting and DNA probing was used to investigate the location of resistance genes on the plasmids. Secondly, nucleotide sequencing was used to detect amino acid mutations in the QRDR of DNA gyrase and topoisomerase IV respectively. Thirdly, to determine the role of an active efflux pump in quinolone resistance, susceptibility testing to nalidixic acid was investigated in ten *V. cholerae* O1 isolates characterised in Chapter Three using agar dilution in the presence and absence of two efflux pump inhibitors, reserpine and phenylalanyl arginine-β-naphthylamide. PCR analysis was used to detect for virulence determinants, which included the enzymatic A subunit of the cholera toxin (CT), *ctxA* and the gene encoding for the toxin co-regulated pilus (TCP), *tcpA* respectively. In addition, the complete coding region of the *ctxAB* gene was amplified and sequenced from four *V. cholerae* O1 isolates, two isolates characterized in Chapter Three and two isolates characterized in Chapter Four as several *V. cholerae* O1 atypical El Tor isolates have been described in Africa. Minimum inhibitory concentration (MIC) values for azithromycin were determined for all 100 *V. cholerae* O1 isolates using both the Etest and agar dilution methods (Chapter Five). PCR-analysis was used to determine the presence of seven macrolide resistance determinants (*mefA*, *ereA*, *ereB*, *ermB*, *mphA*, *mphB* and *mphD*) in all 100 *V. cholerae* O1 isolates.

Results

For both cholera outbreaks, a total of 751 isolates were received and available for analysis. All 31 isolates recovered from the first outbreak (Chapter Three) were characterized as *V. cholerae* O1 serotype Ogawa. For the second outbreak (Chapter Four) 708 isolates were characterized as serotype Ogawa, while the remaining 12 isolates were characterized as serotype Inaba. All isolates analyzed from both outbreaks were susceptible to ciprofloxacin and imipenem, but resistant to six or more antimicrobial agents tested for surveillance purposes. All *V. cholerae* O1 isolates were shown to be resistant to nalidixic acid, co-trimoxazole, trimethoprim, sulfamethoxazoleand streptomycin. Extended-spectrum β-lactamase (ESBL) activity was observed in *V. cholerae* O1 isolates (MIC 64 µg/ml) from both outbreaks. In the second outbreak reduced susceptibility to ampicillin, tetracycline, kanamycin, chloramphenicol, erythromycin and furazolidone were observed. Dendrogram analysis produced two main PFGE clusters. PFGE fingerprint patterns from *V. cholerae* O1 isolates recovered from the first outbreak clustered away from *V. cholerae* O1 isolates

recovered from the second outbreak (data not shown in this study). Class 1 integrons, class 2 integrons and PMQR genes were not detected by PCR.All 100 *V. cholerae* O1 isolates were PCR-positive for the SXT*int* gene and five of the six associated SXT resistance genes encoding for chloramphenicol (*floR*), sulfamethoxazole (*sul2*), trimethoprim (*dfrA1*) and streptomycin (*strA* and *strB*). Seventeen *V. cholerae* O1 isolates (ten isolates characterized in Chapter Three and seven isolates characterized in Chapter Four) were PCR-positive for the *tetA* resistance determinant. Nucleotide sequencing of the QRDR, showed that all nalidixic acid-resistant isolates harboured the same mutations in GyrA (S83-I) and ParC (S85-L) but none were observed in GyrB and ParE.There was no involvement of an active efflux pump in quinolone resistance in ten isolates characterised in Chapter Three. Sixteen *V. cholerae* O1 isolates (ten isolates characterized in Chapter Three and six isolates characterized in Chapter Four) harboured a single plasmid of approximately 140 kilobase pairs in size and showed to harbour the bla_{TEM} gene, which produced the TEM-63 β-lactamase.PCR analysis showed that all 100 *V. cholerae* O1 isolates were positive for the CT, and all were PCR-positive for the El Tor variant of the TCP. Nucleotide sequencing of the *ctxAB* gene of the four selected isolates showed that all four isolates expressed the encoded *ctxB* allele for the CT of the classical biotype and were defined as "altered El Tor". A mobilome is characterized by related genome sequences that differ by combinations of genomic islands, prophages and integrative conjugative elements. All four isolates contained an identical mobilome profile pattern, profile B. Comparative analysis using both the Etest and agar dilution methods (Chapter Five) showed that all *V. cholerae* O1 isolates were susceptible to azithromycin provided that the tentative breakpoint of ≤ 16µg/ml is applied. All 100 isolates were PCR-negative for all seven macrolide resistance determinants, which are commonly associated in the family *Enterobacteriaceae* respectively.

Conclusion

This is the first incidence of TEM-63 β-lactamase-producing, antimicrobial-resistant, toxigenic *V. cholerae* O1 altered El Tor isolates in South Africa. This study highlights the need to further analyze antimicrobial resistance and track emerging epidemic isolates of *V. cholerae* O1. The MIC values and PCR results reported in this study for azithromycin provides a foundation for the surveillance of azithromycin susceptibility and to determine MIC breakpoints in *V. cholerae* O1 isolates circulating in South Africa.