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Vibrio cholerae O1 serotype Ogawa and serotype Inaba isolates from the cholera epidemic that occurred in 2001 and 2002 in South Africa were compared with isolates of V. cholerae O1 serotype Inaba from the epidemic that occurred between 1980 and 1987. PFGE using NotI digestion was used to compare stored isolates received during the 1980s epidemic with those received during the epidemic in 2001/2002. A selected number of these isolates were then sequenced to compare the sequence of the wbeT gene in the V. cholerae O1 Ogawa strains of 2001/2002 with that in the V. cholerae O1 Inaba strains of the 1980s and 2001/2002. Isolates from the recent epidemic were shown to be related, irrespective of serotype, and had comparable banding patterns on PFGE, using NotI. They were distinctly different from those from the previous epidemic. Sequencing of the wbeT gene showed that the gene was highly conserved between the two epidemics. A single deletional mutation of an adenine residue was observed in the V. cholerae serotype Inaba isolates from the 2001/2002 epidemic, resulting in the serotype switch between the V. cholerae O1 strains from the recent epidemic. The distinct differences in PFGE patterns among isolates from the first and second epidemics exclude the possibility that the Inaba strain from the 1980s became dormant in the environment and mutated to serotype Ogawa, causing the 2001/2002 epidemic, despite the apparent consistency in the site of mutation in the Inaba serotypes between the two epidemics.

INTRODUCTION

Between the years 1997 and 2005, South Africa experienced a cholera epidemic that peaked in 2001. KwaZulu-Natal was the worst affected of the provinces, but cases were identified in the Eastern Cape, Mpumalanga, Limpopo and Gauteng (Department of Health; http://www.doh.gov.za/facts/stat-notes-f.html). Over 100 000 cases were notified based on clinical diagnosis between 2000 and 2002 (Department of Health; http://www.doh.gov.za/facts/stat-notes-f.html). Initially, the causative organism was identified as Vibrio cholerae in the Eastern Cape, Mpumalanga, Limpopo and Gauteng (Department of Health; http://www.doh.gov.za/facts/stat-notes-f.html). Over 100 000 cases were notified based on clinical diagnosis between 2000 and 2002 (Department of Health; http://www.doh.gov.za/facts/stat-notes-f.html). Initially, the causative organism was identified as Vibrio cholerae serogroup O1 serotype Ogawa, but in mid 2001, another serotype emerged in KwaZulu-Natal: V. cholerae O1 Inaba. Subsequently, further isolates of V. cholerae O1 Inaba were identified from other provinces.

A previous cholera epidemic in South Africa occurred in the 1980s, with over 20 000 culture-confirmed cases being documented. Again, KwaZulu-Natal was the worst-affected province, although cases were described in Limpopo and Mpumalanga. This epidemic was primarily due to V. cholerae O1 Inaba (Küstner & Du Plessis, 1991). This followed an outbreak of cholera in Maputo in Mozambique between 1980 and 1981 (Swerdlow & Isaacs, 1994).

Two serogroups, O1 and O139, of V. cholerae have been associated with epidemic disease, although V. cholerae O139 has to date not been identified in epidemics in Africa. Serogroups are defined by the antigenicity of the O-antigen portion of the lipopolysaccharides (Manning et al., 1994). Serotype switching in V. cholerae O1 has been described previously. It is due to a change in the genetic make-up of the wbeT (previously known as rfbT) gene (Reeves et al., 1996), resulting in premature truncation of the gene product (Manning et al., 1994; Stroeher et al., 1992). This genetic change may be due to either a point mutation or a deletion, both resulting in the transcription and translation of a premature stop codon (Stroeher et al., 1992; Ito et al., 1993). The serotype of V. cholerae O1 depends on which of the genes encoding somatic antigens A, B and C are expressed. Serotype Ogawa carries antigens A and B;
serotype Inaba carries antigens A and C. The third serotype, *V. cholerae* O1 Hikojima, expresses antigens A, B and C. Expression of antigens A and B in *V. cholerae* O1 Ogawa would thus change to expression of antigens A and C in *V. cholerae* O1 Inaba (Manning *et al.*, 1994).

*V. cholerae* has been recognized to exist in the environment in a viable but non-culturable form from which it can re-emerge (Colwell & Spira, 1992). The purpose of this study was to identify whether South Africa was affected by a single clone of *V. cholerae* in the recent epidemic, which underwent a serotype switch from Ogawa to Inaba, or whether the epidemic was due to two different clones, resulting from the introduction of *V. cholerae* O1 Ogawa and the re-emergence of the *V. cholerae* O1 Inaba from the 1980s epidemic. To differentiate between the two possibilities, we performed PFGE as well as mutation analysis of the *wbeT* gene of isolates from the recent epidemic and of stored isolates from the previous epidemic.

**METHODS**

Between 2001 and 2002, 189 isolates of *V. cholerae* from diagnostic laboratories around South Africa (RSA) were received by the Enteric Diseases Reference Unit, National Institute for Communicable Diseases (NICD). These included isolates from Mpumalanga and Gauteng that were isolated from Mozambican patients who had acquired the disease in Mozambique and were seeking medical care in South Africa. Serotyping was performed on arrival at the NICD laboratories. All isolates were stored at −70 °C for further analysis. Fifteen *V. cholerae* serotype Inaba isolates from patients admitted in 1980 to Shongwe Hospital, Mpumalanga, were included in the study to be compared with the 2001/2002 epidemic strains (Fig. 1).

All isolates were serotyped before storage and 127 isolates were investigated by PFGE. These included 19 *V. cholerae* Inaba and 93 *V. cholerae* Ogawa from the 2001/2002 epidemic. On the basis of restriction pattern groupings observed, 31 of the 127 isolates (24.4 %) were selected for further analysis by nucleotide sequencing of the *wbeT* gene. This included 19 Ogawa and 12 Inaba serotypes. The nucleotide sequences obtained were aligned with one other and the corresponding protein translations were compared for each of the 31 isolates.

Serotyping. Serotyping was performed in accordance with guidelines prescribed by the Clinical and Laboratory Standards Institute, USA, using commercially available antisera (Murex Biotech).

DNA extraction. Total genomic DNA was extracted from overnight growth of isolates on 10 % blood agar (Diagnostic Media Products). Suspensions containing $10^2$–$10^6$ organisms ml$^{-1}$ in 0.3 ml sterile water were boiled for 10 min at 99 °C. This was followed by centrifugation for 2 min at 12 000 r.p.m. and the DNA-containing supernatant was stored at 4 °C.

PFGE. PFGE was performed according to the protocol of the National Molecular Subtyping Network for Foodborne Disease (Pulse-Net) as set out by the Centers for Disease Control and Prevention, Atlanta, USA, on a CHEF-DR III System (Bio-Rad Laboratories) (Centers for Disease Control and Prevention; http://www.cdc.gov/pulsenet/protocols/vibrio_May2006.pdf). Total genomic DNA was restricted with *NotI* endonuclease (Roche Diagnostics). A strain of *Salmonella enterica* serotype Braenderup digested with *XbaI* was used as reference size marker. The digested DNA was separated on a 1 % agarose gel (Seakem Gold agarose; BioWhittaker Molecular Applications) at 14 °C in 0.5 × Tris-borate-EDTA (TBE). The following electrophoresis conditions were used: block 1, 6 V cm$^{-2}$ for 12 h with 2 and 10 s (initial and final switch times) pulses; block 2, 6 V cm$^{-2}$ for 15 h with 10 and 15 s (initial and final switch times) pulses. The gel was stained with ethidium bromide (1 m g ml$^{-1}$) (BDH Laboratory Supplies), destained in nuclease-free water and viewed by UV illumination. The image was photographed under UV light using a DS34 Polaroid Direct Screen Instant Camera.

**Amplification of the ctxA and wbeT genes.** To establish the toxicity of the isolates, PCR amplification of the cholera toxin gene

![Fig. 1. Geographical distribution of serotypes of *V. cholerae* isolates investigated in this study. Adapted from www.anc.org.za/images/maps/samapo.gif. Information is further clarified in Fig. 3.](http://jmm.sgmjournals.org)
ctxA was carried out using the CTX2/CTX3 primer pair, which produces an amplification product of 563 bp (Mekalanos et al., 1983). A second set of primers was designed to amplify the wbeT gene. This included primers SN3 (5′-AGGTTCTCAAACCTGATCTG-3′) and SN2 (5′-CCCATATATCCTGGTTGC-3′) targeting positions bp 17 113–17 133 and bp 18 003–18 023 of the gene, respectively. These primers amplify a region of 910 bp within the wbeT gene. This primer set was constructed from the nucleotide sequence of a strain of V. cholerae serotype Ogawa, accession number X59554, encoding the wbeT gene was selected for the sequence alignment and comparison.

RESULTS

Serotyping
All 15 isolates from 1980 were confirmed as V. cholerae O1 serotype Inaba. Isolates from the 2001/2002 epidemic included 166 isolates with serotype Ogawa and 23 with serotype Inaba. The geographical distribution of the sample set is shown in Fig. 1.

PFGE
Irrespective of the geographical origin, the PFGE patterns obtained from serotype Inaba isolates from the 2001/2002 epidemic differed from those isolated in 1980 (Fig. 2). Of the 112 isolates from the 2001/2002 epidemic that were available for genotyping, 90 (80.4 %) were indistinguishable and formed the major clone. An additional 11 (9.8 %) showed a pattern closely associated with that of the major clone. Of these, 5 showed an additional band and 6 had a deletion. This family of strains comprised isolates from KwaZulu-Natal as well as from other regions of RSA. Restriction patterns completely different from the most common patterns observed were exhibited by 11 isolates, all of which were Inaba isolates from the 1980 outbreak. The PFGE patterns obtained were analysed using Gel Compare II (www.applied-maths.com) (results not shown). Thirty-one representative strains from each PFGE cluster, based on origin, 100 % identity within each clone and year of isolation, were selected for sequencing analysis by primer set SN3 and SN2.

Fig. 2. PFGE restriction patterns of selected isolates of V. cholerae O1 serotype Inaba. Lanes: 2–6 were isolated in 2001/2002 and strains in lanes 7 and 9–14 were isolated in 1980. Lanes: 2–4, isolates from Gauteng; 5, KwaZulu-Natal isolate; 6, 7 and 9–14, isolates from Mpumalanga province. Strains in lanes 2–6 were isolated in 2001/2002 and strains in lanes 7 and 9–14 were isolated in 1980. Lanes: 1, Salmonella enterica serotype Braenderup; 2, G4222 – serotype Inaba; 3, G4305 – serotype Inaba; 4, G4307 – serotype Inaba; 5, K7377 – serotype Inaba; 6, N8069 – serotype Inaba; 7, S69145 – serotype Inaba; 8, salmonella enterica serotype Braenderup; 9, S69146 – serotype Inaba; 10, S69147 – serotype Inaba; 11, S69148 – serotype Inaba; 12, S69173 – serotype Inaba; 13, S69174 – serotype Inaba; 14, S69175 – serotype Inaba; 15, salmonella enterica serotype Braenderup.
Both the Ogawa and the Inaba isolates from the 2000/2001 epidemic appeared highly clonal on analysis, but showed less than 80% similarity with Inaba isolates from 1980. This is illustrated in the dendrogram portraying the genetic diversity of the 31 isolates selected for sequence analysis (Fig. 3).

**PCR**

The ctxA gene was detected in all of the isolates tested by PCR (results not shown).

**Comparison of nucleotide sequences of \( wbeT \) genes**

The \( wbeT \) gene was detected in all 31 isolates tested. The nucleotide sequences of the amplification product of isolates belonging to the Inaba and the Ogawa serotypes were determined and compared with one another. Two isolates demonstrated identical sequences of the amplicons using either the forward or the reverse primer, which indicated the presence of tandemly repeated positions of primer homology. The remaining 29 isolates could be sequenced by the forward and reverse primers yielding separate products.

Analysis of the peptide sequences was done by comparison with the peptide sequence translated from the nucleotide sequence of the selected reference strain \( V. \) cholerae serotype Ogawa, accession number X59554 (Fig. 4). Relative to the amino acid arrangement of the \( wbeT \) gene in the reference strain, a distinct difference at position 46 was noted in each of the 31 RSA isolates analysed. The result of this change is the replacement of the leucine indicated in the reference strain by tryptophan in the RSA isolates. Additional amino acid changes were identified in four of the Inaba isolates, of which three showed the same pattern. All 12 Inaba isolates (two from 1980 and 10 from 2001/2002) exhibited the presence of a stop codon sequence at the same position (bp 17618) that was not present in the sequence of the selected reference strain of \( V. \) cholerae O1 serotype Ogawa (Stroher et al., 1992) or in any of the other RSA Ogawa isolates. This termination sequence was generated in the Inaba strain by a one-base difference at nucleotide 17610, namely the deletion of a single adenine residue, which resulted in premature termination of translation (IQT – stop instead of NTDI). No other mutations observed resulted in premature stop codons.
DISCUSSION

Cholera is a rare disease in the developed world, although it remains common in parts of the developing world. Over the last decades, South Africa has been affected by two distinct outbreaks of cholera: one in the 1980s and one starting in the late 1990s and continuing until 2004 (Department of Health; http://www.doh.gov.za/facts/stat-notes-f.html; Küstner & Du Plessis, 1991). These affected primarily the rural areas, where piped water as well as sewerage drainage systems may be absent. In both epidemics, the causative organism was \textit{V. cholerae} serogroup O1. Serogroup O139 has not been found on the African continent thus far. That \textit{V. cholerae} isolated from patients was indeed the cause of the epidemic of diarrhoea was confirmed by the presence of the cholera toxin gene \textit{ctx} in a small selection of isolates from the 2001/2002 epidemic (data not shown). It is noteworthy that during

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**Fig. 4.** Comparison of nucleotide sequences and their deduced amino acid sequences of the \textit{wbeT} genes of \textit{V. cholerae} O1 Ogawa and Inaba serotypes and \textit{V. cholerae} serotype Ogawa, accession number X59554. Sequences 1 and 2 represent \textit{V. cholerae} O1 Inaba isolates from 1980, sequences 3–13 represent \textit{V. cholerae} Inaba isolates from 2001/2 and sequences 14–31 represent \textit{V. cholerae} Ogawa isolates from 2001/2 (refer to Fig. 3). The underlined spaces (\_) represent gaps which are the result of frameshift insertion/deletion mutations within the corresponding codon, and are introduced to allow alignment of the \textit{wbeT} sequences with that of the \textit{V. cholerae} serotype Ogawa, accession number X59554.

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the 2001/2002 epidemic the death rate was less than 1%, which is unusually low in developing countries (World Health Organization; http://whqlibdoc.who.int/hq/2003/WHO_CDS_CPE_ZFK_2003.3.pdf).

While the *V. cholerae* O1 isolates from the epidemic in the 1980s belonged to serotype Inaba, the recent epidemic started with serotype Ogawa. However, fairly early in the epidemic, serotype Inaba emerged. Serotype switches do occur in response to selective pressure due to the development of population or individual immunity (Sheehy *et al.*, 1966; Sack & Miller, 1969), and are therefore expected later in an epidemic. We therefore considered that the *V. cholerae* O1 Inaba could be the cause of a concomitant epidemic due to the re-emergence of the strain from the 1980s. Such a re-emergence by a strain dormant in the environment has been reported before (Colwell & Spira, 1992). In order to differentiate between the strains from the current and previous epidemics, we performed PFGE to establish the clonality of the different serotypes and to identify whether the 2001/2002 *V. cholerae* O1 Inaba was related to the strain from the previous epidemic in the 1980s or to the *V. cholerae* O1 Ogawa from the recent epidemic.

PFGE clearly showed a distinct clustering of *V. cholerae* O1 Inaba isolates from 1980 with a pattern that differed significantly from that of the 2001/2002 isolates (Fig. 2, Fig. 3, full data not shown). Although two serotype Ogawa strains from 2000/2001 (U15104 and U15823) appeared to have some similarity with serotype Inaba strains from 1980, they were still more closely related to the current 2000/2001 strains, approaching 80% similarity on analysis of banding patterns. Sequencing results of the *wbeT* genes for these two strains did not show any significant differences from the other strains analysed in that portion of the genome and the mutations that resulted in the PFGE differences must lie elsewhere in the genome. The remaining strains from the recent epidemic were highly clonal, irrespective of serotype, with only minor banding differences on PFGE in some of the strains (Fig. 3). This suggests the occurrence of a serotype switch in the strains from 2001/2002 from serotype Ogawa to serotype Inaba. This was confirmed by sequence analysis of the *wbeT* gene and was due to a deletonal mutation at nucleotide 17610, resulting in a premature stop codon at position 17618. Other investigators have also observed that serotype switching may be the result of either deletional mutations or point mutations resulting in the truncation of the outer-membrane protein during translation (Stroeher *et al.*, 1992; Ito *et al.*, 1993; Manning *et al.*, 1994). The reversal of this switch, Inaba to Ogawa, as occurred in the cholera epidemic in South America in the 1980s and India in the 1990s (Wachsmuth *et al.*, 1993; Garg *et al.*, 2000) would then require a second mutation, removing the premature stop codon from the gene. This has been calculated to occur at a lower frequency of $10^4$ compared with an Ogawa to Inaba switch (Manning *et al.*, 1994).

It is interesting to note that certain amino acids in the strains of *V. cholerae* O1 from both the 1980s as well as 2001 were consistent, but differed from those of the published reference strain, irrespective of serotype (Fig. 4) (Stroeher *et al.*, 1992). Also, despite the temporal difference in the years of isolation, the specific mutation resulting in the premature stop codon in the Inaba strains was conserved (Fig. 4). This may reflect that these strains of *V. cholerae* O1 are prone to mutation at a specific site on the gene, as the PFGE patterns do not support a relationship between the 1980s Inaba and 2001 Inaba isolates.

Significant amino acid changes occurred in two isolates but this did not affect their serotyping. As the remaining sequences of the *wbeT* genes in these two strains were comparable with the other *wbeT* gene sequences, and both of these isolates had restriction patterns similar to the other strains from the current epidemic, these mutations were thus unlikely to reflect an alternative origin of the two isolates and suggested that they were part of the current epidemic. One of these isolates, strain U15098/02 from the Eastern Cape (sequence 29), which was serotype Ogawa appeared to have certain nucleotide sequences more in common with the Inaba strains than with the Ogawa strains from the current epidemic (full data not shown). As the first cases of cholera in that area were found later in the epidemic, in 2002, this may reflect a rare reverse mutation from serotype Inaba to Ogawa (Manning *et al.*, 1994). The second strain, K15854/02, exhibited differences between amino acids 128 and 135, i.e. HSLSSS instead of PLVEFHH.

Other strains also showed point mutations within the *wbeT* gene that did not affect serotyping (Ito *et al.*, 1993). Three further strains, two isolated from Mpumalanga (S69145/80 and S69175/80) and one from KwaZulu-Natal (K1290/01) (neighbouring provinces in South Africa), showed the same amino acid changes at position 157–160 (Fig. 4). Review of the PFGE gels and dendrogram (Fig. 3) confirmed that these isolates were less closely related and that this mutation does not reflect an epidemiological linkage.

Earlier work on the isolates from the most recent epidemic as well as antimicrobial susceptibility testing on the isolates from 2001 to 2002 has shown them to be multidrug-resistant and to carry a transmissible SXT element, an *aadA2* gene cassette and the *tetA* gene (Dalsgaard *et al.*, 2001). Little is known about the epidemiology of the origin of the 2001/2002 epidemic. Some isolates were from cholera cases imported from Mozambique, suggesting that, as in the 1980s, migration from that country could have happened. Ribotyping of South African isolates from the recent epidemic showed these to be related to the Mozambican isolates identified collected in 1997 (Dalsgaard *et al.*, 2001).

This work provides evidence that the emergence of *V. cholerae* O1 Inaba during the 2001/2002 epidemic was due to a serotype switch, rather than to the re-emergence of a previously dormant strain. It has been suggested that serotype switches are the result of selective pressure on the organism due to the acquisition of immunity. This was shown in previous work by Stroeher *et al.* (1992), and has
been borne out by others (Ito et al., 1993; Wachsmuth et al., 1993; Garg et al., 2000).

The early appearance of this switch may have resulted from the strain circulating in the population for a considerable period of time without being recognized, as suggested by Dalsgaard et al. (2001). This could have resulted in a build-up of sufficient population immunity to allow for preferential transmission of the Inaba mutant. Little is known regarding the epidemiology of cholera in a population with a high HIV prevalence, and this was greater than 30% in the antenatal seroprevalence survey during the outbreak period (Department of Health; http://www.doh.gov.za/docs/reports/2000/hivreport.html) and may have impacted on the serotype switch. No studies were done to show that the Inaba strain may have acquired increased colonization capacity, resulting in its early appearance.

Cholera in Mozambique is considered to be an endemic disease, with seasonal variation (Lucas et al., 2005), but in South Africa, this does not appear to be true. The 2001/2002 cholera epidemic was due to the introduction of a new strain of *V. cholerae* O1 that appeared in South Africa in the late 1990s. The serotype switch from Ogawa to Inaba arose *de novo* in KwaZulu-Natal in 2001 and the isolates are not clonally related to the *V. cholerae* O1 serotype Inaba isolates from the 1980s.

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REFERENCES


