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Original Article

Epidemic waves of cholera in the last two decades in Mozambique

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Abstract

Introduction: Africa is increasingly affected by cholera. In Mozambique, cholera appeared in the early 1970s when the seventh pandemic entered Africa from the Indian subcontinent. In the following decades, several epidemics were registered in the country, the 1997–1999 epidemic being the most extended. Since then, Mozambique has been considered an endemic area for cholera, characterized by yearly outbreaks occurring with a seasonal pattern.. At least three pandemic variants are thought to have originated in the Indian subcontinent and spread worldwide at different times. To understand the epidemiology of cholera in Mozambique, whether the disease re-emerges periodically or is imported by different routes of transmission, we investigated clinical *V. cholerae* O1 isolated during 1997–1999 and 2012–2014 epidemics.

Methodology: By detecting and characterizing seven genetic elements, the mobilome profile of each isolate was obtained. By comparing it to known seventh pandemic reference strains, it was possible to discern among different *V. cholerae* O1 variants active in the country.

Results: During 1997–1999, epidemic strains showed two different genetic profiles, both related to a pandemic clone that originated from India and was reported in other African countries in the 1990s. Isolates from 2012–2014 outbreaks showed a genetic background related to the pandemic strains currently active as the prevalent causative agent of cholera worldwide.

Conclusions: Despite cholera being endemic in Mozambique, the epidemiology of the disease in the past 20 years has been strongly influenced by the cholera seventh pandemic waves that originated in the Indian subcontinent.

Key words: cholera; seventh pandemic wave; *V. cholera*; Mozambique.

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Introduction

Cholera, an acute waterborne diarrheal disease, continues to be a significant health threat worldwide. Cholera-endemic regions include coastal parts of the Bengal Delta and sub-Saharan Africa, including Mozambique. Since its first appearance in the 1970, the disease represented a major public health issue for the country, especially in 1997–1999. Since then, the number of cases has decreased; however, in 2009, reported cases of cholera in Mozambique accounted for one-third to one-fifth of all cases reported in Africa.

Even though cholera in Mozambique is endemic, the epidemiology data are sparse. In particular, little is known about which of the different *V. cholerae* epidemic variants caused cholera in the country in the last two decades. Therefore, this study was designed to understand the identity of the strains isolated during various epidemics and to establish how they are related to pandemic strains circulating globally. Using a molecular and comparative approach, we studied informative genomic signatures in our strains able to identify distinct *V. cholerae* clades. This article describes a major shift between different variants occurring during the decade of 2000. The major finding of our work is that, despite endemicity, cholera in Mozambique is heavily influenced by pandemic waves originating in the Indian subcontinent.

Africa is considered to be a new homeland of cholera, accounting in 2006 for 99% of the officially reported cholera cases worldwide [1]. Most of the cholera outbreaks in the African continent originated in coastal regions of sub-Saharan countries. The epidemiology of African cholera is still not completely uncovered, since most of our understanding of cholera on a global scale is based on data from studies that have been concentrated on Bengal Delta epidemics, or on single local outbreaks. However, cholera in Africa has different dynamics than Bengal cholera [1].

The causative agent of this severe, life-threatening diarrheal disease belongs to the species *V. cholerae* [2]. The extreme genome plasticity of this pathogen

has generated a significant heterogeneous group of strains capable of inhabiting water and marine environment worldwide and originating pathogenic variants in the human host [2,3]. The species *V. cholerae* continually presents newly emerging pathogenic clones carrying diverse combinations of phenotypic and genotypic features and thereby significantly affects public health efforts to control the disease. Specifically, the current seventh cholera pandemic is caused by different pathotypes belonging to a single phyletic lineage, exemplified by the *V. cholerae* O139 and *V. cholerae* O1 El Tor hybrid variants [3]. This group includes the Matlab types I, II, and III [4], altered El Tor [5], Mozambique El Tor [6], and hybrid El Tor strains [7]. Recent comparative genomics studies demonstrated that these variants originated as a result of horizontal genetic transfer (HGT) from a common recent ancestor, characterized by a highly conserved genome background and differentiated by their content of mobile genetic elements (the mobilome), such as integrative conjugative elements (ICEs), genomic islands (GIs), and prophages [8].

ICEs of the SXT/R391 family take part in the evolutionary process of *V. cholera*e O1 [3]. These genetic elements are self-transmissible, able to integrate into the host bacterial chromosome and to transfer by conjugation. They are acknowledged for their important role in bacterial genome plasticity and as vectors of antibiotic resistance and alternative metabolic pathways [9]. VSP-II is a polymorphic GI, originally considered a molecular tag for the seventh pandemic *V. cholerae* strains. However, a novel rearrangement of VSP-II GI, with a 14.4 Kb deletion, was found in *V. cholerae* O1 El Tor strain CIRS 101 and in recent isolates from Bangladesh [10]. GI-12, GI-14, and GI-15 were recognized as novel GIs able to discriminate, with their presence or absence, closely related atypical El Tor Matlab strains such as *V. cholerae* O1 MJ1236 from Bangladesh and the Mozambique El Tor strain *V. cholerae* O1 B33 [3]. Prophages Kappa and TLC are typically found in the genomes of *V. cholerae* O1 epidemic strains; the latter was recently recognized as an important player in *V. cholerae* toxigenic conversion.

The seventh pandemic reached Mozambique in 1973; an epidemic phase ended in 1975 and was followed by an endemic phase up to 1985 [11]. The disease re-emerged in 1989 in the central province of Tete and reached the capital (Maputo) in 1991 with over 3,600 cases reported in the following two years [11,12]. The spread of cholera was facilitated by the civil war that lasted 16 years and devastated much of the Mozambican infrastructure, including the healthcare system [11]. After a number of years, in 1997–1999, a new epidemic spread throughout Mozambique (and Zimbabwe) and was one of the longest-lasting epidemics in the country, with more than 50,000 reported cases and a case fatality rate of around 3.2% [13,14]. Since then, the number of cases has decreased while remaining linked to environmental and social conditions that characterize Mozambique. Cases of cholera reported in Mozambique accounted for one-third to one-fifth of all cases reported in Africa in the years 1992, 1993, 1998, 1999, and 2004 [15]. In recent years, from 2009 to 2013, the reported cases dropped significantly, with a fatality rate of around 1% [15].

From a molecular epidemiology standpoint, the first pandemic clone reaching Mozambique in the early 1970s was the prototypical *V. cholerae* O1 El Tor, which likely continued to dominate as the active cholera causative agent until the late 1990s [14]. In 2004, a new *V. cholerae* O1 El Tor variant was isolated in Beira, central Mozambique, genetically related to the Matlab variant causing cholera in Bangladesh at the same time [6]. After that, we have no information about the pandemic clone circulating in the last decade in Mozambique, particularly if the *V. cholerae* O1 El Tor variant of 2004 is still active or was substituted by the new pandemic variant isolated globally [16].

To elucidate recent cholera epidemiology in Mozambique, a collection of Mozambican *V. cholerae* O1 clinical isolates was screened. The strains' collective mobilome was studied to determine which possible epidemiological correlations linked this country to the cholera seventh pandemic waves.

Methodology

V. cholerae strains

Clinical *V. cholerae* O1 isolates collected from distinct outbreaks that occurred in different regions of Mozambique from 1997 to 2014 were analyzed.

Fourteen *V. cholerae* O1 isolated during 1997–1998 epidemics in Mozambique, from Maputo (south), Zambezia (center), and Tete (center-west) provinces (Figure 1), belonging to Professor Colombo's laboratory strains collection, were included in the analysis. These strains were previously studied and genotyped, and mobilome profiles were assessed [14,17]. Four *V. cholerae* O1 isolates from Zimbabwe in 1997–1998, never characterized, were also included

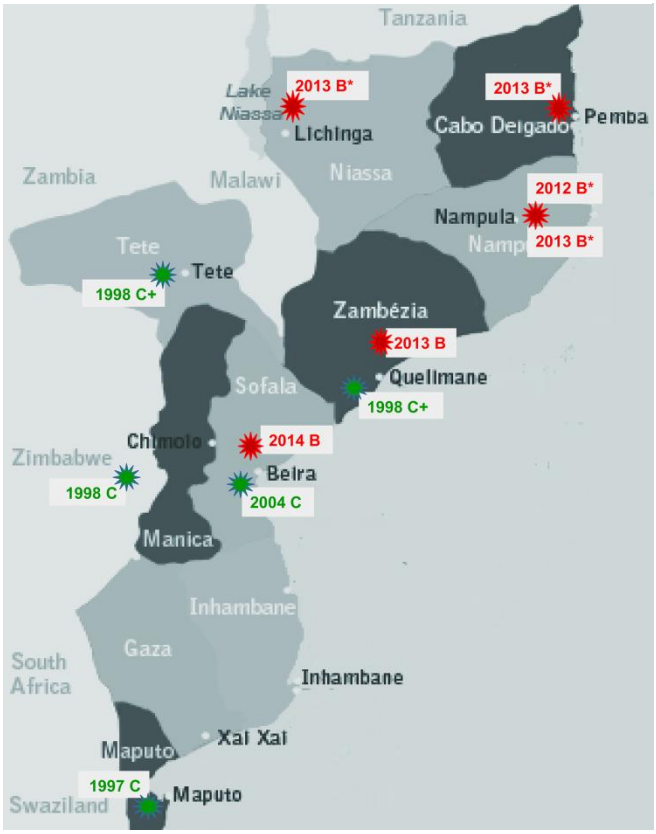
since they belonged to the same epidemic episode and area [11] (Table 1).

A recent cholera surveillance program held by the Mozambican National Institute of Health lead to the isolation of 59 *V. cholerae* O1 during 2012–2014 cholera outbreaks. Cases were registered solely in the center and north of the country, in the provinces of Nampula, Cabo Delgado, and Niassa, with the southernmost affected provinces being Sofala and Zambezia. Southern provinces did not report any cholera cases in 2012–2014 (Figure 1). All *V. cholerae* O1 strains were collected according to Mozambique's national ethical guidelines for health research, within a national surveillance, and not specifically for this study.

Five completely sequenced reference strains were used as positive controls and for comparison to the strains under study (Table 1).

Rectal swabs and archived isolates, which were obtained from provincial health centers and hospitals, were initially subcultured on thiosulphate citrate bile salts sucrose agar (TCBS). Further TCBS subculturing and confirmation of isolate identity was undertaken by the microbiology laboratory of the National Institute of Health of Mozambique. Standard biochemical tests and serology using polyvalent, anti-Ogawa and anti-Inaba antisera (Denka Seiken, Tokyo, Japan) were performed as previously described. Bacterial strains were routinely grown on LB agar at 37°C for 16–18 hours with appropriate antibiotic selection and were maintained at -80°C in LB broth containing 15% (vol/vol) glycerol.

Figure 1. Geographic distribution of mobilome profiles of *V. cholerae* O1 strains isolated in 1997–1998 and 2012–2014 cholera epidemics



C+: profile C with TLC phage; B*: profile B with K phage

Table 1. Mobilome profile of tested genetic elements of *V. cholerae* O1 isolated in Mozambique during 1997–1998 and 2012–2014 epidemics

Isolation		Ref strain/	Mobilome							
Place	Date	# of strains	ICE	VSP-II	TLC	Kappa	GI-12	GI-14	GI-15	profile
India	1975	N16961	–	+	+	–	–	–	–	A
India	1992	MO10	SXT	+	+	+	–	–	–	E
Bangladesh	1994	MJ-1236	ICE*Vch*Ban9	+	–	+	+	+	–	D
India	2002	CIRS101	ICE*Vch*Ind5	+ (tr)	+	–	–	–	–	B
Mozambique	2004	B33	ICE*Vch*Moz10	+	–	+	–	+	+	C
Maputo	1997	5	ICE*Vch*Moz10	+	–	+	–	+	+	C
Tete	1998	3	ICE*Vch*Moz10	+	+	+	–	+	+	C + TLC
Zambezia	1998	6	ICE*Vch*Moz10	+	+	+	–	+	+	C + TLC
Zimbabwe	1998	4	ICE*Vch*Moz10*	+	-	+	–	+	+	C
Nampula	2012	5	ICE*Vch*Ind5	+ (tr)	+	+	–	–	–	B + K
Nampula	2013	4	ICE*Vch*Ind5	+ (tr)	+	+	–	–	–	B + K
Cabo Delgado	2013	15	ICE*Vch*Ind5	+ (tr)	+	+	–	–	–	B + K
Niassa	2013	3	ICE*Vch*Ind5	+ (tr)	+	+	–	–	–	B + K
Zambezia	2013	3	ICE*Vch*Ind5	+ (tr)	+	–	–	–	–	B
Nampula	2014	17	ICE*Vch*Ind5	+ (tr)	+	–	–	–	–	B
Sofala	2014	11	ICE*Vch*Ind5	+ (tr)	+	–	–	–	–	B

DNA, primers, and polymerase chain reaction (PCR)

A detection method developed by Spagnoletti *et al*. was applied with the goal of detecting genomic signatures able to distinguish among the prevalent seventh pandemic *V. cholerae* O1 variants [17]. The method consists of double multiplex PCR assays designed to amplify key seventh pandemic mobile genetic elements: ICE, VSP-II island, TLC and K phages, and GI-12, GI-14, and GI-15 genomic islands.

For the characterization of the CTXΦ cluster, further molecular analysis was carried out. *RstR*, *ctxB*, and *tcpA* genes were screened for different alleles, *i.e*., classical and El Tor, as previously described [18]. Furthermore, in this study, the double-mismatch-amplification mutation assay (DMAMA) devised by Naha and colleagues was used to accurately discriminate the classical, El Tor, and Haitian alleles of the *ctxB* gene through a rapid PCR-based assay [19].

The structure of the CTXΦ array was determined by multiple PCR analyses using primers informative on the physical organization of the different modules that comprise the CTX cluster: RS1, RS2, and the core. The combination of primers tlcF/rstAR, rstCF/rstAR, ctxAF/rstAR, and ctxAF/rtxR were used to detect the presence of CTXΦ on chromosome 1 and to determine the position of the RS1 element. The presence/absence of CTXΦ or RS1 on chromosome two was assessed using primers chr2F/chr2R [18].

Genomic DNA was prepared with a Wizard Genomic DNA Purification Kit (Promega, Madison, USA), according to manufacturer's instructions. PCR was performed in 50 μL reaction mix containing 1 U of GoTaq DNA polymerase (Promega) and 50 ng/μL DNA. Amplification was performed in an automated thermocycler (BioRad MJ-Mini Personal Thermal Cycler, Biorad, Hercules, USA). Oligonucleotides and PCR conditions for the double multiplex PCR were optimized as previously described [17].

Results

Mobilome analysis: 2012–2014 epidemics

Fifty-eight *V. cholerae* O1 El Tor from three different locations in northern Mozambique: Pemba (Cabo Delgado, 15 strains), Nampula (26 strains) and Lichinga (Niassa, 3 strains), and from the central provinces of Sofala (11 strains) and Zambesia (3 strains), were analyzed (Figure 1). The mobilome genotyping analysis showed two profiles for the 58 *V. cholerae* O1 strains. The genomes of 21 isolates harbored key genomic signatures of the altered *V. cholerae* O1 El Tor variant, specifically the truncated VSP-II island and the ICE*Vch*Ind5 multiple antibiotic resistance element found within wave three seventh pandemic isolates. Thirty isolates showed the same profile with the addition of the phage K (Table 1). Furthermore, the absence of GI-12, GI-14, and GI-15 unequivocally confirms that these *V. cholerae* O1 isolates are members of the seventh pandemic *V. cholerae* El Tor clade and originated as part of wave three of the cholera pandemic represented by the hybrid *V. cholerae* El Tor CIRS 101 [16].

Overall, the mobilome comparative analysis of the 2012–2014 *V. cholerae* O1 strains indicated that they are descendants of the current seventh pandemic *V. cholerae* O1 CIRS 101 hybrid variant that originated in India and has been isolated worldwide [20]. However, even though the K phage cannot be considered a reliable genotyping signature, the presence of this element in 30 isolates indicates that the seventh pandemic *V. cholerae* O1 population causing cholera outbreaks in central and northern Mozambique is not strictly clonal.

Mobilome analysis: 1997–1998 epidemics

The mobilome analysis of 14 *V. cholerae* O1 El

Table 2. CTXΦ cluster organization of *V. cholerae* O1 isolated in Mozambique during 1997–1998 and 2012–2014 epidemics

Province	Year	# of strains	CtxB	rstR	tcpA	TLC-RS1	CORE-RTX	TLC-RS2	Chr II
Maputo	1997	5	cla	cla	Et	0	0	0	+
Tete	1998	3	cla	cla	Et	0	0	0	+
Zambezia	1998	6	cla	cla	Et	0	0	0	+
Zimbabwe	1998	4	cla	cla	Et	0	0	0	+
Nampula	2012	5	cla	cla	Et	3000	1542	1740	0
Nampula	2013	4	cla	cla	Et	3000	1542	1740	0
Cabo Delgado	2013	15	cla	cla	Et	3000	1542	1740	0
Niassa	2013	3	cla	cla	Et	3000	1542	1740	0
Zambezia	2013	3	cla	cla	Et	3000	1542	1740	0
Nampula	2014	17	cla	cla	Et	3000	1542	1740	0
Sofala	2014	11	cla	cla	Et	3000	1542	1740	0

Tor strains isolated from the outbreaks that occurred in 1997–1998 in three different regions of Mozambique: Tete (north west), Zambezia (center), and Maputo (south), has previously been reported (Figure 1) [17].

In addition, the analysis of four *V. cholerae* O1 strains isolated in Zimbabwe during a major cholera epidemic that lasted several months between 1997 and 1998 showed interesting results. As for 1997–1998 Mozambican *V. cholerae* O1, all strains had a mobilome profile linked to the one of *V. cholerae* O1 B33 (prototypical VSP-II, the GI-14 and GI-15 genomic islands, uniquely found in the B33 genomic background), but with the remarkable exception of the ICE element. Interestingly, when tested with the mobilome multiplex, none of the Zimbabwean *V. cholerae* O1 strains amplified neither the ICE conserved integrase gene *int* nor any of the specific genetic markers contained in one of the variable insertions of the SXT/R391 ICE family. However, when tested with two different primer sets for the *int* gene, all four strains gave positive amplification. Hence, the ICE element carried by the Zimbabwean *V. cholerae* O1 belongs to the SXT/R391 ICE family, linked but not identical to the ICE*Vch*Moz10 of *V. cholerae* O1 B33 causing cholera epidemics in that geographic area. Furthermore, all four strains were negative for TLC phage, making them closer to the Maputo isolates than to those of geographically closer Tete and Zambezia provinces (Table 1).

Overall, analysis revealed that strains genetically related to the atypical variant *V. cholerae* O1 B33, isolated for the first time in the central province of Beira in 2004, were already present in the area of Maputo and in the northern and central provinces of Mozambique, and spread to the bordering areas of Zimbabwe in 1997–1998.

CTXΦ cluster analysis

Due to the great variability of the CTXΦ genetic cluster and the emergence of atypical *V. cholerae* O1 El Tor variants within the ongoing seventh pandemic [21], the organization of CTXΦ arrays was investigated, along with the presence of different alleles of *ctxB*, *rstR*, and *tcpA* genes of the Mozambican and Zimbabwean isolates.

Combined CTXΦ analysis by multiple PCR confirmed the mobilome profiling and showed the expected CTXΦ arrays found in the two major *V. cholerae* O1 El Tor variants B33 and CIRS 101 [21]. All strains from Mozambique and Zimbabwe 1997–1999 cholera epidemics showed the *V. cholerae* B33 CTXΦ genotype: the classical allele of both the *ctxB* and the *rstR* genes, and the El Tor allele of the *tcpA* gene (Table 2). These strains showed CTXΦ in a tandem array fashion inserted in the small chromosome (Table 2). Furthermore, the five strains isolated from Maputo and four strains from Zimbabwe were also devoid of the RS1 element linked to the CTX prophage, while the nine strains isolated from Tete and Zambezia provinces showed the presence of a RS1-RS1 array linked to the TLC phage on chromosome one, as previously reported by Spagnoletti *et al*. (Table 2) [17].

The *V. cholerae* O1 currently causing cholera epidemics in north central Mozambique confirmed a CTXΦ genotype and a molecular arrangement similar to the *V. cholerae* O1 CIRS 101: the classical allele of both the *ctxB* and the *rstR* genes, and the El Tor allele of the *tcpA* gene (Table 2). The latter also proved to be negative for any CTXΦ integration on the small chromosome and devoid of CTXΦ tandem arrays as detected by primer pairs chr2F/chr2R and ctxAF/cepR, respectively (Table 2).

Discussion

This study was based on a collection of *V. cholerae* O1 clinical strains isolated from 1997–1999 and 2012–2014 in several provinces of Mozambique and bordering areas of Zimbabwe (Figure 1 and Table 1). Despite the work of local national health authorities, it was not possible to recover strains over a broader temporal and geographic scale. Nevertheless, we believe that this study represents an important source of data, depicting the presence of seventh pandemic *V. cholerae* O1 in Mozambique.

From our previous analysis of the mobilome of 1997–1998 Mozambican *V. cholerae* O1, we were able to recover two main profiles, both linked to the reference strain *V. cholerae* O1 B33. The genome of all 14 *V. cholerae* O1 isolates harboured B33 genotype key signatures: the prototypical VSP-II genomic island, the ICE*Vch*Moz10 element, and the GI-14 and GI-15 genomic islands. Further, three and six *V. cholerae* O1 isolated in Tete and Zambezia provinces, respectively, carried the TLC phage in their genome, which was absent in the five strains isolated from Maputo, as in the *V. cholerae* O1 B33 reference genotype. Interestingly, in this study, we confirmed a result revealed by our group while genotyping the strains by *Bgl*I digested enterobacterial repetitive intergenic consensus (ERIC) typing technique, which evidenced a difference between isolates collected from Maputo and the central provinces (Tete and Zambezia) [14].

The epidemiology of *V. cholerae* O1 in Africa can be explained by multiple introductions of cholera from endemic regions of Asia; the first introduction followed the early dissemination of the seventh pandemic in the 1970s, with N16961 prototypical *V. cholerae* O1 El Tor-like being the first biotype having reached the continent [16]. It is reasonable to speculate that descendants of this strain continued to cause cholera epidemics throughout the continent during the 1980s and 1990s. Our analysis of Somalian *V. cholerae* O1 strains from a 1985 epidemic showed that the etiologic agent was a strain related (if not identical) to the prototypical seventh pandemic *V. cholerae* O1 N16961 (unpublished data). Furthermore, our analysis of the Angolan cholera epidemics that occurred between 1992 and 1996 demonstrated that, despite a great variability of molecular profiles, all *V. cholerae* O1 strains isolated were linked to the prototypical seventh pandemic genomic background [18,22].

However, somewhere between the late 1980s and early 1990s, a second wave of pandemic clones originating from the Indian subcontinent landed in East Africa, whose representative strain was *V. cholerae* O1 El Tor B33, isolated in Beira, Mozambique, in 2004 [16]. In the present study, we confirmed that, several years before its first isolation, the main pandemic strain circulating in Mozambique and in adjacent regions of Zimbabwe between 1997 and 1998, was indeed *V. cholerae* O1 B33. This finding is significant because this strain is genetically closely related to *V. cholerae* O1 MJ1236 isolated in Bangladesh in 1994, which occurred at the same time as the Mozambique epidemics, indicating a close correlation between the two cholera epidemic regions, further confirming the finding of Mutreja's comparative study [16].

According to recent comparative genomics of African *V. cholerae* O1 El Tor, a third wave of global transmission of the cholera seventh pandemic reached Africa between 1989 and 1997 [16,23]. However, *V. cholerae* O1 isolations in Mozambique up to 2005 showed the second-wave genotype reference strain as the prevalent causative agent of epidemic cholera [6].

Due to the gap of isolations, it was not possible to reveal when exactly the third pandemic wave entered Mozambique from our results. However, it appeared to have stabilized as the prevalent pandemic clone somewhere between 2005 and 2012. Indeed, current cholera outbreaks in north central Mozambique are caused by a *V. cholerae* O1 no longer related to *V. cholerae* O1 B33, but genetically closer to third pandemic wave *V. cholerae* O1 CIRS 101. The latter was responsible for the 2010 Haiti epidemic and is currently the prevalent causative agent of cholera epidemics in the Indian subcontinent and worldwide [16,20,23].

Unfortunately, as mentioned above, the lack of strains isolated between 2005 and 2012 hampered our ability to calculate the exact timing when the shift between the two genetically distinct variants occurred in the country. Because Mozambique is a cholera-endemic area, with outbreaks occurring every year, a gradual substitution of the CIRS-101-like strains over the B33-like strains may be hypothesized, wherein a geographical gradient of distribution of the two genotypes coexists from the north to the south provinces, culminating in the complete substitution of the current third wave genotype over the old one.

Conclusions

Overall, our study suggested that, despite cholera being endemic in Mozambique, the epidemiology of the disease in the past 20 years has been strongly influenced by the cholera seventh pandemic waves that originated in the Indian subcontinent. In other words, unlike in Angola [22], where the heterogeneity of *V. cholerae* O1 genotypes implies a differentiation of strains driven by local adaptation, major dynamics of cholera in Mozambique appear not to evolve locally, but rather to be periodically re-introduced from distant epidemic regions by, to date, uncertain ways of transmission. Since our data strengthen the epidemiological link of Mozambique cholera cases to South Asia, it is necessary to continue comparative epidemiological studies and ongoing national efforts of surveillance to determine possible human or environmental routes of transmission.

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