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Distribution of phage types of *Vibrio cholerae* 01 biotype El Tor in Nigeria (2007-2013): Implication in cholera mortality

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Nigeria has been plagued with seasonal epidemics of cholera with high mortality impact since 2007. Data to understand the molecular epidemiology of strains for developing country-specific control measures are either not available or incomplete in most of the epidemic states. To bridge this information gap, this study determined the phage type profiles of some selected *Vibrio cholerae* 01 biotype El Tor strains involved in epidemic between 2007 and 2013 in nine states of the country. A total of 52 epidemic strains of *V. cholerae* from nine states: Abia, Bauchi, Borno, Kano, Gombe, Ilorin, Lagos, Ogun and Osun based on viability and positive serogrouping of 122 stock cultures were phage typed using both the old (two groups) and new (10 groups) typing schemes according to World health Organization (WHO) guidelines. Data were descriptively analyzed for variation and predominance of phage types using STATA 8.0. The proportions of strains belonging to the T2 and T4 phage types were 55.7 and 44.3%, respectively (P>0.05). With the new typing scheme, epidemic strains of phage types ranging from 4 to 8 were found per state. Further analysis revealed phage predominance in the following decreasing order: T-27>T-24>T-23. Other phage types seen were T-7, T-10, T-12, T-14, T-16 and T-17. Phage variation analysis further revealed involvement of strains from multiple phage types ≥3 during the 2010 cholera epidemics versus ≤2 in other cholera epidemics since 2007. Findings from this study indicate that multiple phage types of *V. cholerae* 01 biotype El Tor with the predominance of T-27 are common in Nigerian cholera epidemic situation since 2007. This study also revealed phage multiplicity to play a role in the documented higher case fatality of 2010 cholera epidemics compared to recent outbreaks in the country.

Key words: *Vibrio cholerae* 01 biotype El Tor, phage typing, cholera, Nigeria.

INTRODUCTION

In Nigeria, cholera due to *Vibrio cholerae* 01 El Tor has persisted as a public health problem since 1971 when the first epidemic was reported (Wilson, 1971). The pathogenicity of *V. cholerae* is credited to its myriad of virulence factors, including cholera toxin. This pentameric protein mediates loss of water and electrolytes from the gut by signaling intracellular cyclic AMP elevation, leading to dehydration, acute weight loss and death (Muanprasat and Chatsudthipay, 2013). Since 2007, Nigeria has been plagued with yearly episodes of cholera epidemics, which affect many states of the country, causing significant morbidity and mortality. Case fatality

rates due to cholera have been reported to range from 10.4% in 2007 to 3.4% in 2013 (WHO 2009; 2010; 2011). In recent times, the 2010 cholera epidemic has been described as the most devastating outbreaks with the highest case fatality rate of 4.1%.

In response to cholera epidemics, there has been an improvement in the coverage of key interventions in the country. They include improvement in access to good sanitation and use of ORT by mothers at household (UNICEF, 2008; Nwaezuoke et al., 2003). However, information, regarding the characteristics strains responsible for epidemics is grossly lacking in the country. Most of the previous studies could only characterized the epidemic strains serologically for which the Ogawa serotypes have been found to drive most of the recent epidemics more than their Inaba counterpart (Adagbada et al., 2012; Oyedeji et al., 2013; Marine et al., 2013). In cholera endemic settings, proper identification of epidemic strains has been found to be useful in the development of cost-effective and targeted interventions to avert future epidemics and save lives (Ismail et al., 2013).

Phage typing has been established as a valuable strategy for the identification and discrimination of strains during epidemics, pre-epidemics and post-epidemic periods (Bockemul and Meinicke, 1976; Sarkar et al., 1999; Sarkar et al., 2011). It is based on the premise that bacteriophage contribute to the evolution *V. cholerae* via horizontal gene transfer, genomic recombination and predation. This phenomenon results in selection or clonal enrichment of certain strains of *V. cholerae* in a geographical location (Basu and Mukerjee, 1968). In the last 60 years, two phage typing schemes have been developed (Chattopadhay et al., 1993). The first typing scheme developed by Basu and Mukerjee (1968) restricted strains into two clonal groups: phage type-2 and phage type-4, while the second typing scheme developed in 1993 provided an opportunity for better discrimination of strains (Mukerjee, 1978). As there are no phage type information about epidemic strains of *V. cholera* 01 in Nigeria, the present study was planned to determine the distribution and frequency of *V. cholerae* phage types responsible for cholera epidemics in Nigeria since 2007. The contribution of phage type to reported higher case fatality rate of 2010 cholera epidemic was also investigated.

MATERIALS AND METHODS

Study design and *V. cholerae* 01 El Tor strains

This study was a retrospective, exploratory study of a 52 viable *V. cholerae* 01 El Tor strains recovered from total of 122 stock cultures of isolates collected from nine states in Nigeria. The stock cultures served as repositories of strains recovered during cholera epidemics in these states from 2007 to 2013: Borno (27), Bauchi (10), Kano (7), Abia (20), Osun (18), Ogun (10), Gombe (9), Lagos (7), and Ilorin (14). Viability of the stock cultures was assessed by subculturing in alkaline peptone water (APW), PH 8.6 overnight at 37°C, followed by inoculation on thiosulfate citrate bile salt sucrose agar (TCBS) and further incubation at 37°C for 24 h. A total of 58 cultures showed viability as per yellow colony growth on TCBS, indicating sucrose fermentation after incubation. Six colonies were further excluded for phage typing due to negative serogrouping result by slide agglutination that was done in duplicate using serogroup 01 and 0139 specific antisera. Serotypes of the remaining 01 serogroup strains were confirmed by slide agglutination using Inaba and Ogawa serotype specific antisera. The El Tor biotype status of the strains was primarily determined based on non-haemolytic reaction on sheep blood agar, polymoxyin B resistant and chicken erythrocyte agglutination (Ismail et al., 2013) before confir-mation by toxin co-regulated pilli subunit A gene (*tcpA*) genotyping by PCR using *tcpA*-El Tor/Classical gene specific primers (Kaesler and Hall, 1993). On the whole, the recovered 52 viable *V. cholerae* 01 El Tor strains belonged to Inaba (n=12) and Ogawa (n = 40) serotypes, respectively and retrospectively represented strains recovered during the cholera epidemics of 2007 to 2011 and 2013 from the nine states.

Phage typing of *V. cholerae* 01 biotype El Tor

The phage typing study was performed on the basis of the standard methodology adopted by (Sarkar et al., 2011). *V. cholerae* 01 strains were streaked onto a nutrient agar plates and the plates were incubated for 18 h at 37°C. After overnight incubation, a single colony from the nutrient agar plate was inoculated into nutrient broth (2 to 3 ml) and was incubated under stationary conditions for 3 to 4 h. A young broth culture mixed with 3.5 ml of molten soft agar (maintained at 42°C in a water bath) was poured into a nutrient agar plate to prepare a uniform lawn of growth of the bacterial strain on the surface of the agar to provide an adequate substrate for phage action. The plates were then allowed to air dry for about 20 to 30 min. After the plates were dried, drops of phage lysate at the routine test dilution (RTD) were applied onto the plates with a Pasteur pipette aseptically on the agar. The plates were kept at room temperature to air dry and were then incubated at 37°C for 14 to 18 h. *V. cholerae* MAK 757 was used as a control for each set because it was lysed by all of the phages. On the following day, the plates were observed to evaluate the zones of inhibitions based on the degree of lysis. Strong lysis, semiconfluent lysis, or the appearances of at least five plaques were considered positive results. Lesser degrees of lysis or reactions of inhibition were disregarded.

Data analysis

Data were presented as numbers (n) and percentages (%), while difference in frequency of T-2 and T-4 phage types was analyzed using chi-square test. Phage diversity or multiplicity was defined as the number of distinct phage types seen per state or per epidemic year. Phage types detected at a frequency of ≤2 per epidemic years were defined as minor phage types. Variations in the frequency of major phage type seen using the new phage typing scheme were graphically presented. Statistical outcome with P-value < 0.05 was considered to be significant. Data were analyzed using STATA 8.0 statistical software.

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Table 1. Distribution of *V. cholerae* El Tor biotype phage types based on the new phage typing scheme according to state.

Phage type	Borno	Kano	Bauchi	Gombe	Abia	Ilorin	Lagos	Ogun	Osun	Serotype n (%)	
										Inaba	Ogawa
T-7	-	-	-	1	-	-	-	-	-		
T-10	-	1	-	1	-	-	-	1	-		
T-12	-	-	1	-	1	-	-	-	1		
T-13	-	-	-	-	-	-	-	-	1		
T-14	1	-	-	-	-	-	-	-	-		
T-16	1	-	-	-	1	-	-	-	-	12 (23.1)	40 (76.9)[a]
T-17	-	2	-	-	-	-	-	-	-		
T-23	1	2	1	-	1	-	-	-	1		
T-24	1	1	1	2	-	1	1	-	-		
T-27	3	1	5	1	6	2	2	5	1		
Total	7	7	8	5	9	3	3	6	4		

Data are frequencies of phage types.[a]P<0.05 (Inaba vs. Ogawa).

Table 2. Distribution of *V. cholerae* El Tor biotype phage types based on the old typing scheme according to state.

Phage type	Borno	Kano	Bauchi	Gombe	Abia	Ilorin	Lagos	Ogun	Osun	Total N (%)
T-2	4	4	5	4	5	2	0	3	2	29 (55.8)[b]
T-4	3	3	3	1	4	1	3	3	2	23 (44.2)
Total	7	7	8	5	9	3	3	6	4	52 (100)

Data are frequencies of phage types [b]P>0.05 (T-2 vs. T-4).

Table 3. Distribution of *V. cholerae* El Tor biotype minor types according to year.

Phage type	2007	2008	2009	2010	2011	2013
T-7	-	-	-	1	-	-
T-10	1	-	-	1	-	1
T-12	-	-	1	1	1	-
T-13	-	-	1	-	-	-
T-14	-	-	-	1	-	-
T-16	-	-	-	1	1	-
T-17	1	1	-	-	-	-

RESULTS

Results presented in Table 1 show the frequency of occurrence and distribution of phage types of *V. cholerae* 01 biotype El Tor strains by state using the new typing scheme. The predominant phage type was by far T-27 with 26 strains. However, the number of distinct phage types ranged from 4 to 8 strains across the studied sites with Ogun and Borno/Kano having the lowest and highest phage diversity of 2 and 5, respectively. Using the old phage type scheme of Basu and Mukerjee (1968), the observed disparity in percentages between T-2 and T4 phage types (55.8% vs. 44.2%; P > 0.05) was not significant (Table 2). The minor phage types seen included T-7 in 2010 only, T-12 with consecutive occurrence in 2009 to 2011 and T-13 detected only in 2009 cholera epidemic (Table 3). The relative frequency of occurrence of each phage type detected according to epidemic year between 2007 and 2013 using the old and new phage typing schemes are presented as shown in Figures 1 and 2. The 2010 cholera epidemic recorded the highest frequency of occurrence of the T-2 and T-4 phage types (Figure 1) and higher phage multiplicity involving T-27, T-24 and T-23 (Figure 2). Computation of case fatality rate per cholera epidemic years using the national epidemiological data revealed involvement of T-27 phage types in the previous cholera epidemics since 2008 and highest phage multiplicity in 2010 cholera epidemics (Table 4).

DISCUSSION

To further improve strains identification for the purpose of developing effective cholera mitigation measures in

Table 4. Case fatality rate in relation to phage multiplicity according to year of epidemic.

Outbreak year	CFR(%)[3, 4, 5]	Phage multiplicity (From this study)
2007	10.4	3 (T-10, T-17, T-23)
2008	4.8	3 (T-17, T-24, T-27)
2009	3.1	4 (T-12, T-13, T-23, T-27)
2010	4.1	8 (T-7, T-10, T -12, T-14, T-16, T-23, T-24, T-27)
2011	3.2	4 (T-12, T-16, T-24, T-27)
2013	3.4	2 (T-10, T-27)

CFR: Case fatality rate. [3,4, 5]Cited references in the reference list.

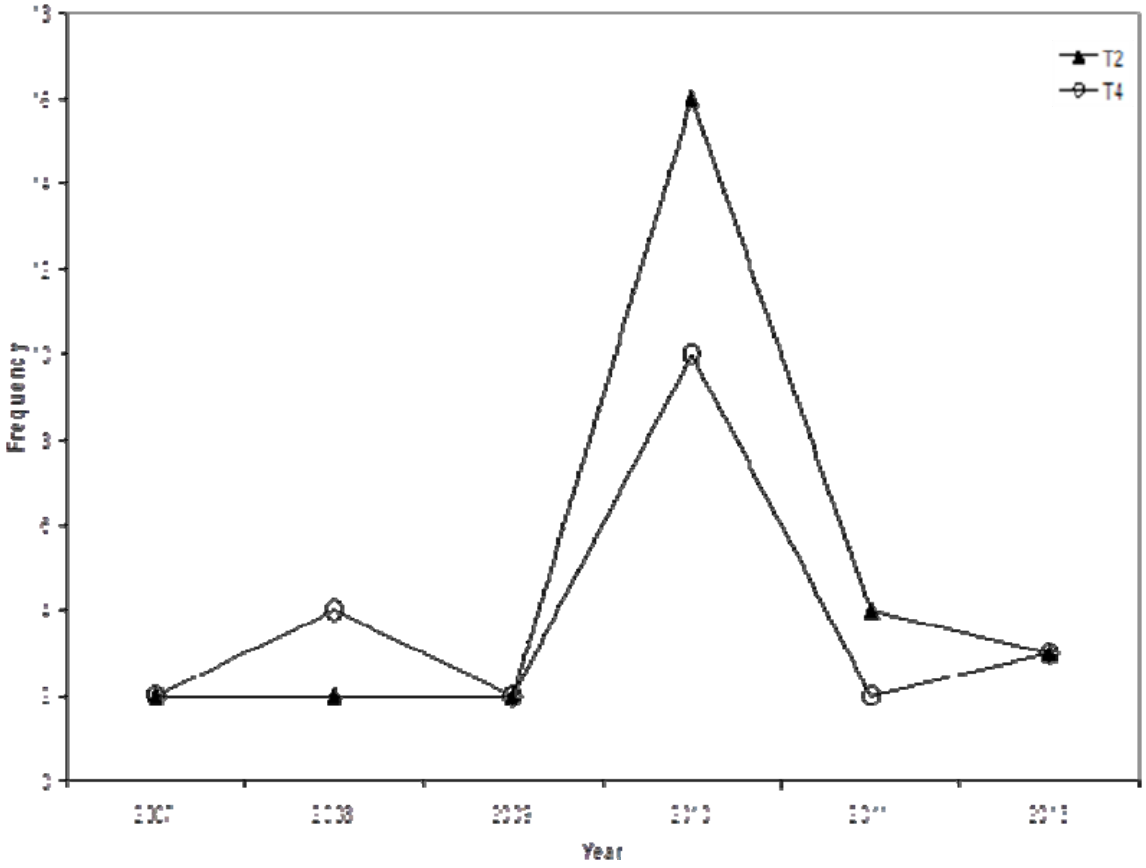

Figure 1. Variations in the incidence of *V. cholera* phage-type 2 and 4 during cholera epidemics in nine states in Nigeria (2007-2013)-Basu and Mukherjee typing scheme.

Nigeria, this study phage typed 52 representative strains of *V. cholera* 01 biotype El Tor recovered during epidemics from nine states from 2007 to 2013. In this study, all the strains were found typeable using the old scheme of Basu and Mukerjee (1968) in which 55.8% of the strains belonged to T-2 and 44.2% to T4 phage types. With regards to typeability, this old scheme was reported to fail in typing up 10% epidemic strains from India (Mukerjee, 1978). This disparity may be attributed to differences in sample size, geographical location and periods of epidemics from which strains were retrospectively analyzed in the two countries. However, the non-significant outnumbering of T-4 by T-2 phage types observed in this study further revealed the sample size limitation as agreed with previous reports, regarding the poor discriminatory power of the old typing scheme for epidemic strains identification (Mukerjee, 1978). This study also revealed the higher discriminatory power inherent in the new typing scheme as it enabled further discrimination of the T-2 and T-4 phage type strains into 10 distinct phage types. Despite limited sample size, our findings indicate that *V. cholerae* 01 biotype El Tor strains

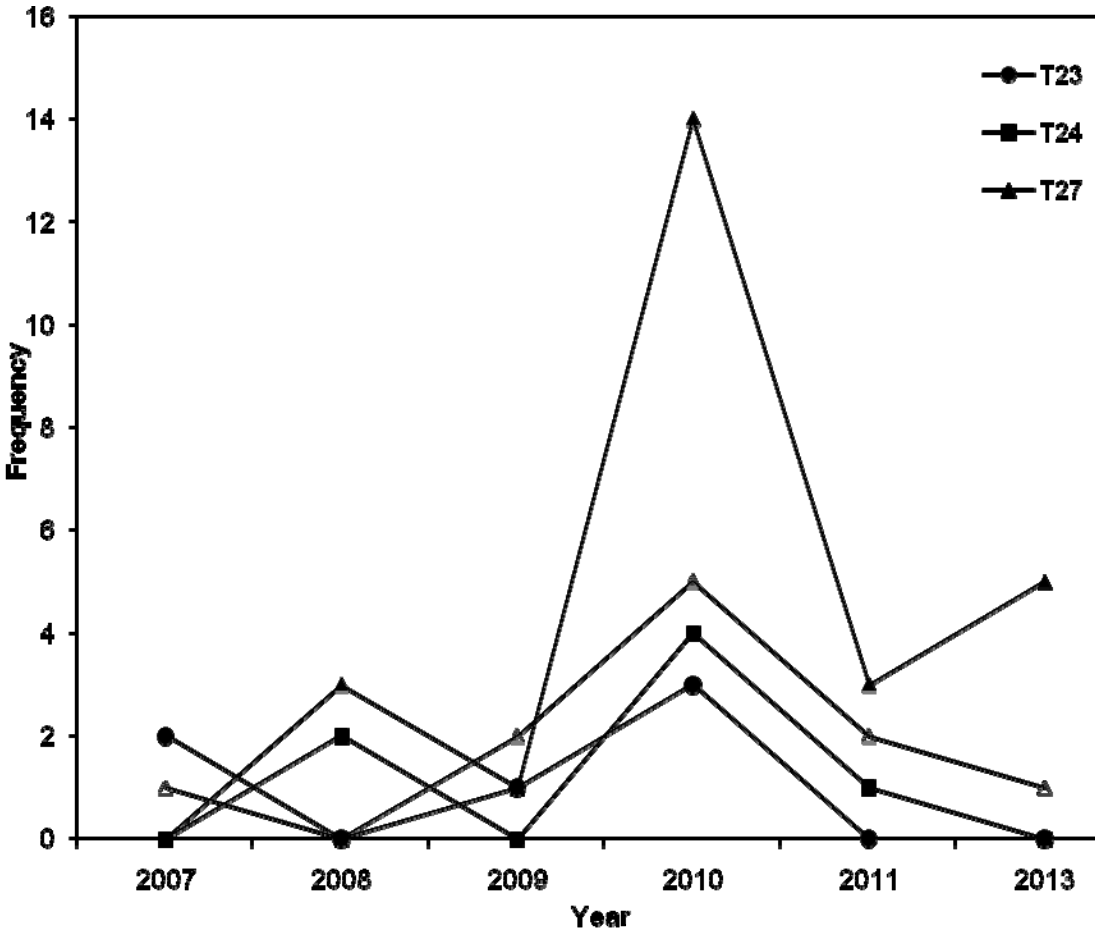

Figure 2. Variation in the incidence of *V. cholera* 01 phage types T23, T23, T27 and others during cholera epidemics in nine states in Nigeria (2007-2013)-New phage typing scheme.

belonging to multiple phage types have been driving cholera epidemics but at different levels of dominance in Nigeria since 2007. The most predominant phage type responsible for cholera epidemics since 2007 based on the nine studied states was found to be T-27, followed by equal dominance of T-23 and T-24 as well as T-10 and T-12. In India, the T-27 was also the predominant epidemic phage type, followed by T-26. The present study did not detect T-26 and found only once (that is, in 2009) the previously predominant phage types for the African region (Frost and Rowe, 1987) T-13. The latter has been shown to circulate majorly in Africa (Frost and Rowe, 1987). Therefore, to validate our present finding, regarding T-13 phage type absence, larger number of epidemic states from other epidemic states of the country will be needed. Of interest in this study is the higher frequency and multiplicity of phage types of epidemic strains responsible for cholera epidemics in 2010, the most devastating cholera epidemics in the country since 1971 (Wilson, 1971). Our results suggest that the extent of clonal diversity may also increase the risk of cholera severity and deaths during epidemics. This is because of the higher case fatality rate of 4.1% reported in the 2010 epidemic, involving 8 distinct phage types compared to 3.1 to 3.4% case fatality reported for 2009, 2011 and 2013 in which epidemic strains belonging to 3 to 5 phage types drove these successive cholera outbreaks in the studied states. The fact that higher case fatality rates of 4.8 and 10% were reported for 2008 and 2007 cholera epidemics suggest that other pathogen and host factors may also play a role in cholera severity and deaths. Recent studies in the country have identified these factors to include carriage of virulence genes for cholera toxin and haemolysin on the part of the pathogen and poor health seeking behavior on the part of the host (Oyedeji et al., 2013; Oladele et al., 2012).

Aside the limitation of small sample size, the observed predominance and clonal related of the T-27 phage type epidemic strains of *V. cholerae* 01 biotype El Tor need further investigation on a larger scale using other epidemiological markers. Another limitation of this study is lack of information on sites from which the phage typed isolates were recovered for 'black spot' identification to inform state-specific cholera control measures.

In conclusion, findings from this study indicate that multiple phage types of *V. cholerae* 01 biotype El Tor with the predominance of T-27 have been responsible for cholera epidemics in Nigeria since 2007. This study also revealed phage multiplicity to play a role in the documented higher case fatality of 2010 cholera epidemics compared to recent outbreaks in the country.

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Conflict of interests

The author(s) have not declared any conflict of interests.

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