African Journal of Biology and Medical Research
Volume 2, Issue 2, 2019 (pp. 12-24)

www.abjournals.org

GENETIC RELATEDNESS AND CLONAL SIMILARITIES OF *VIBRIO CHOLERAE* FROM CLINICAL AND ENVIRONMENTAL SOURCES IN BAYELSA AND RIVERS STATES USING *Mbo* I AND *Alu* I Digestion of 16S rDNAs

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ABSTRACT: *This study entails the use of DNA fingerprinting procedure to determine the genetic relatedness and clonal relationship of clinical and environmental strains of Vibrio cholerae from clinical and environmental sources in Bayelsa and Rivers States. A total of forty (40) clinical and environmental (fresh water, brackish and seafood) were selected for DNA fingerprint. Chromosomal DNA was isolated using phenol/chloroform/isoamyl alcohol protocol, this was followed by the polymerase chain reaction (PCR) probe for 16S rDNA. The PCR products of 16S rDNA sequence was digestion with two restriction endonucleases Mbo I and Alu I. The electrophoresis gel image produced was used to construct similarity matrix index showing clonal relatedness of strains and the genetic tree was constructed to determine the clustering patterns among strains. The electrophoretic gel image of Mbo I restriction endonuclease digestion of 16S rDNA revealed 50-100% genetic homology with three (3) distinct DNA fingerprinting patterns and the genetic tree analysis revealed four (4) clusters of strains while Alu I digestion showed ten (10) distinct clustering patterns, the similarity matrix index of clonal relatedness ranged from 33.33-100% and ten (10) distinct clustering patterns from the genetic tree analysis. Many of the clinical strains from the same localities clustered 100% in genetic homology to fresh water strains which implies the source of infection.*

KEYWORDS: V.cholerae, Mbo I, Alu I, Chromosomal DNA, 16S rDNA, Genetic Relatedness, DNA Fingerprinting Procedure

INTRODUCTION

The cholera vibrio in the family *Vibrionaceae* remains a threat to public health especially in tropical climes and most especially under developed settlements of sub-Saharan Africa (Hutin *et al*., 2003; Usman *et al*., 2005; WHO 2012; Ayeni, 2014). *Vibrio cholerae* the bacterium associated with cholera is a Gram negative, facultatively anaerobic, oxidase positive curved or straight rod-shaped microorganism (Prescott *et al*., 2005;). The source of *Vibrio cholerae* transmission is predominantly water (Idika *et al*., 2000) and host is infected through ingestion of water and seafoods contaminated with this causative agent of cholera (Aladese and Enabulele, 2014).

The pathological effect is excessive release of fluids characterized by diarrhea and vomiting, which results into dehydration, elevated blood proteins and death through cardiac arrest (Faruque *et al*., 1998b). Historically, there have been seven recorded global epidemics of cholera since the onset of the first pandemic in 1817 (Faruque *et al*., 1998b). All pandemics were caused by classical biotype with exception of the seventh pandemic which was caused

by *El Tor* biotype (Faruque *et al*., 1998a). The seventh pandemic is ongoing, affecting many developing countries. In 1970, out of the 36 countries that had incidence of cholera 28 were newly affected countries and 16 were in Africa (Kaper *et al*., 1995). One of the worst cholera epidemics during the seventh pandemic occurred in Goma, Eastern Zaire (now Democratic Republic of Congo), in July 1994 (Siddique *et al*., 1995).

Cholera cases in Nigeria had been given significant attention over the years. In 1999, the outbreak in Kano State in Northern Nigeria recorded 815 cases and 28 deaths (Hutin *et al*., 2003). In 2010, 41,787 cases were reported with 4.1% case fatality ratio (CFR). Incidence was mostly in the Northern states including Borno, Bauchi, Adamawa and Katsina (World Health Organisation, 2012; Ukaji *et al*., 2015). Similarly, in 2015 an explosive cholera epidemic affected many creek settlements of Rivers and Bayelsa states South-south Nigeria. The World Health Organisation (WHO) in collaboration with the two state governments instituted treatment centres. In spite of these measures the epidemic continued to for several weeks spreading to other localities in both states. The logical reason given by epidemiologists is possible reinfection of the inhabitants from a contaminated source.

The use of molecular biology procedure especially Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) of 16S rDNA helps to understand the phylogenetic relationship among strains of microorganism (Yoon *et al*., 2003). This DNA fingerprinting approach can provide clonal similarities among closely related strains which can give insightful information on the cholera bacterium source of infection. This research is aimed to ascertain genetic similarities and variations among strains using Polymerase chain reaction (PCR)- Restriction Length Fragment Poymorphism (RFLP). This molecular procedure will provide information on the clonal similarities, genetic relatedness and effectively finger the source of the epidemic.

MATERIALS AND METHODS

Enrichment and Isolation of *V.cholerae* from Samples

Forty (40) clinical and environmental strains (fresh, brackish waters and sea-foods) were isolated from different localities in Rivers and Bayelsa. Isolation and identification of strains of *Vibrio cholerae* were done according to the methods described by Adebayo-Tayo *et al*. (2011) and Aladese *et al*. (2015).

Isolation of *V. cholerae* Chromosomal DNA

Stock cultures of *V. cholerae* were inoculated into Mueller-Hinton agar and incubated at 37^0C for 24hrs. Chromosomal DNA isolation of *Vibrio cholerae* strains were carried out using the phenol/chloroform/isoamyl alcohol procedure as described by Ausubel *et al*. (1987). The chromosomal DNA were kept below freezing temperature for the purpose of preservation for PCR and DNA fingerprinting analysis.

Polymerase Chain Reaction Probe for 16s RNA

Polymerase chain reaction probe for 16s rRNA for *Vibrio cholerae* was carried out based on the method described by Whitehouse *et al*. (2010) using the following primers

Vibrio cholerae (F): 5' AGA GTT TGA TYM TGG CTC AG 3'

Vibrio cholerae (R): 5' GAA ATT CTA CCC CCC TCT ACA G 3'

Polymerase chain reaction of 16s RNA was amplified in a thermocycler with 30 cycles at 59^0C.

Restricted Fragment Length Polymorphism using *Mbo*I

Restriction fragment analysis 16S rDNA fragments amplified by PCR was digested *Mbo*I with restriction endonuclease. *Mbo*I restriction enzyme digestions was carried out at 37^0C for 1-2 hrs. This was followed by electrophoresis on 2 % agarose gel medium. The resulting restriction patterns from 2.0 (w/v) agarose gel electrophoresis were the gel bands of DNA fragments spliced at specific sites. These bands were observed on U-V trans-illuminator at 312nm for presence and absence of traits. These restriction bands patterns on electrophoretic gel bands were digitally photographed on gel documentation machine.

Construction of Genetic Tree

The DNA fingerprints patterns produced by *Mbo*I restriction enzyme was evaluated using the Image master software (Pharmacia Biotechnology, Uppsala, Sweden). Informative DNA fragments (>100 bp) were scored for their presence or absence; similarity and divergence calculated. The similarity matrix indices and genetic distance trees were constructed using DICE and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering methods and the percentage of clonal relatedness was determined.

RESULT

The information on the source, location and date of isolation of forty (40) strains selected for DNA fingerprinting is shown on Table 1. The biochemical characteristics of strains (Table 2) showed negative reactions to Gram's reaction and positive reaction to lysine and ornithine decarboxylase tests. The strains were also motile with production of acid without gas from sugar fermentation (Table 2).

The electrophoretic gel image of *Mbo* I restriction endonuclease digestion of 16S rDNA (Figure 1) showed 50-100% genetic homology as shown in the similarity matrix index of clonal relatedness (Table 3). There are three (3) distinct DNA fingerprinting patterns and the genetic tree analysis revealed four (4) clusters of strains (Figure 2). Clinical and fresh water strains from Okoli'ile and Southern-Ijaw all clustered together with 100% clonal similarities (Figure 2).

The electrophoretic gel image of *Alu* I restriction endonuclease digestion of 16S rDNA showed five (5) distinct DNA fingerprinting patterns of over 100 base pair (Figure 3). The percentage of clonal relatedness and genetic identities of strains from the similarity matrix

index of clonal relatedness ranged from 33.33-100% (Table 4). The analysis genetic tree showed ten (10) distinct clustering patterns with many of the clinical and fresh water strains from same cholera epidemic locality clustering into 100% clonal similarities.

Strain 18 (fresh water) and strains 37 and 40 (clinical) from Ogbia all clustered 100% in genetic similarities to *Alu* I digestion. Clinical strains from Southern-Ijaw (strains 19 and 20) showed 100% genetic relatedness with fresh water strain 12. Similarly, strain 5 and strain 13 (fresh water) and clinical strain 21 all from Ebukuma also showed 100% clustering in clonal relatedness *Alu* I endonuclease digestion.

Table 1. List of Strains with Source, Location and Date of Isolation

Strains Designate	Source	Location	Date
Strains 1	Clinical	Ebukuma	February, 2015
Strains 2	Fresh water	Ikuru	March, 2015
Strains 3	Seafood	Sagbama	March, 2015
Strains 4	Clinical	Ikuru	March, 2015
Strains 5	Fresh water	Southern-Ijaw	February, 2015
Strains 6	Brackish water	Yenagoa	February, 2015
Strains 7	Clinical	Opobo	February, 2015
Strains 8	Seafood	Yenagoa	February, 2015
Strains 9	Brackish water	Yenagoa	March, 2015
Strains 10	Seafood	Opobo	February, 2015
Strains 11	Fresh water	Sagbama	March, 2015
Strains 12	Seafood	Ebukuma	February, 2015
Strains 13	Fresh water	Ebukuma	January, 2015
Strains 14	Fresh water	Okoli'ile	January, 2015
Strains 15	Clinical	Sagbama	March, 2015
Strains 16	Brackish water	Ogbia	March, 2015
Strains 17	Clinical	Ikuru	February, 2015
Strains 18	Seafood	Yenagoa	March, 2015
Strains 19	Clinical	Southern-Ijaw	January, 2015
Strains 20	Clinical	Southern-Ijaw	January, 2015
Strains 21	Clinical	Ebukuma	January, 2015
Strains 22	Clinical	Southern-Ijaw	January, 2015
Strains 23	Seafood	Ogbia	March, 2015
Strains 24	Clinical	Okoli'ile	February, 2015
Strains 25	Brackish water	Ikuru	March, 2015
Strains 26	Clinical	Okoli'ile	January, 2015
Strains 27	Fresh water	Ikuru	February, 2015
Strains 28	Brackish water	Southern-Ijaw	April, 2015
Strains 29	Clinical	Okoli'ile	January, 2015
Strains 30	Brackish water	Yenagoa	March, 2015
Strains 31	Seafood	Southern-Ijaw	April, 2015
Strains 32	Seafood	Southern-Ijaw	February, 2015
Strains 33	Brackish water	Ebukuma	January, 2015
Strains 34	Seafood	Opobo	January, 2015

Strains 35	Brackish water	Sagbama	March, 2015
Strains 36	Brackish water	Ogbia	March, 2015
Strains 37	Clinical	Ebukuma	January, 2015
Strains 38	Fresh water	Southern-Ijaw	March, 2015
Strains 39	Fresh water	Okoli'ile	February, 2015
Strains 40	Clinical	Ogbia	March, 2015

Table 2. Biochemical Characterisation of Isolated *Vibrio cholerae* from Rivers and Bayelsa State

Gram's reactions	–
Oxidase reactions	+
Motility	+
String test	+
Lysine decarboxylase	+
Arginine dihydrolase	–
Ornithine decarboxylase	+
5 % chicken erythrocytes	+
Glucose	Acid production (No gas)
Sucrose	Acid production (No gas)
Lactose	No acid production
Triple sugar iron	Acid/Acid reaction (No gas, No H_2S)
Growth in 0 % NaCl	+
Growth in 1 % NaCl	+
Growth in 3 % NaCl	+
Growth in 6 % NaCl	V

**(V) Some strains showed varying degrees positive and negative reactions*

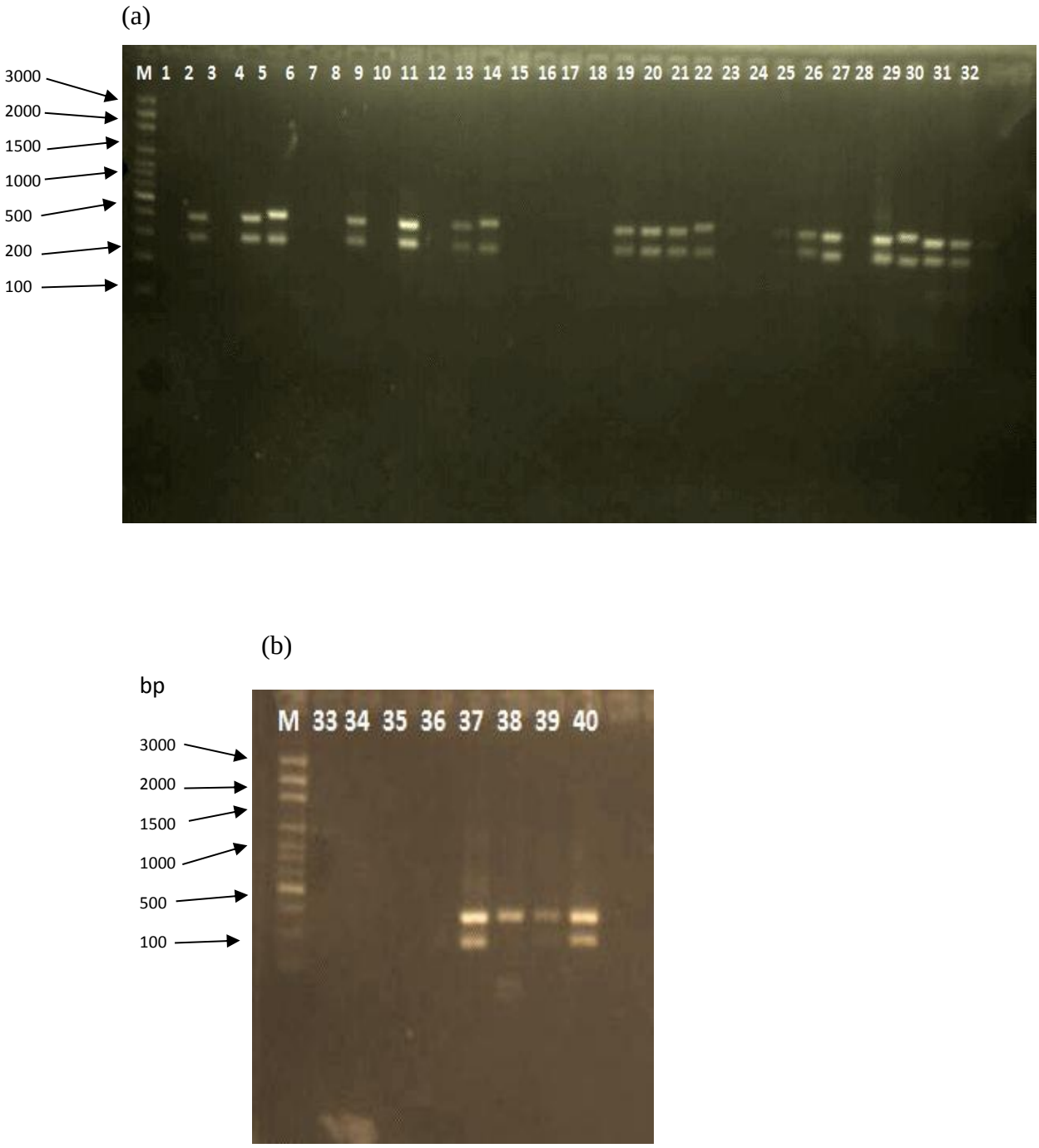


Figure 1. Agarose gel electrophoresis of PCR-RFLP products of *V. cholerae* strains from Bayelsa and Rivers State using *Mbo*1 restriction enzyme. Lanes: M, Molecular weight ladder; (a) Photographic gel images of *V. cholerae* strains 1-32; (b) Photographic gel images of *V. cholerae* strains 33-40

Table 3. The Matrix of Percentage of Clonal Relatedness Among Strains Using *Mbo* I Digestion

	Strain 2	**Strain 4**	**Strain 5**	**Strain 9**	**Strain 11**	**Strain 13**	**Strain 14**	**Strain 19**	**Strain 20**	**Strain 21**	**Strain 22**	**Strain 25**	**Strain 26**	**Strain 27**	**Strain 28**	**Strain 29**	**Strain 30**	**Strain 31**	**Strain 32**	**Strain 38**	**Strain 39**
Strain 2	100	50	100	100	100	50	100	100	100	50	100	100	100	100	50	100	100	100	75	100	100
Strain 4	50	100	100	100	50	100	100	100	50	100	100	100	100	50	100	100	100	75	100	100	
Strain 5	50	50	50	100	50	50	50	100	50	50	50	50	100	50	50	50	75	50	50		
Strain 9	100	100	50	100	100	100	50	100	100	100	100	50	100	100	100	75	100	100			
Strain 11	100	50	100	100	100	50	100	100	100	100	50	100	100	100	75	100	100				
Strain 13	50	100	100	100	50	100	100	100	100	50	100	100	100	75	100	100					
Strain 14	50	50	50	100	50	50	50	50	100	50	50	50	75	50	50						
Strain 19	100	100	50	100	100	100	100	50	100	100	100	75	100	100							
Strain 20	100	50	100	100	100	100	50	100	100	100	75	100	100								
Strain 21	50	100	100	100	100	50	100	100	100	75	100	100									
Strain 22	50	50	50	50	100	50	50	50	75	50	50										
Strain 25	100	100	100	50	100	100	100	75	100	100											
Strain 26	100	100	50	100	100	100	75	100	100												
Strain 27	100	50	100	100	100	75	100	100													
Strain 28	50	100	100	100	75	100	100														
Strain 29	50	50	50	75	50	50															
Strain 30	100	100	75	100	100																
Strain 31	100	75	100	100																	
Strain 32	75	100	100																		
Strain 38	75	75																			
Strain 39	100																				

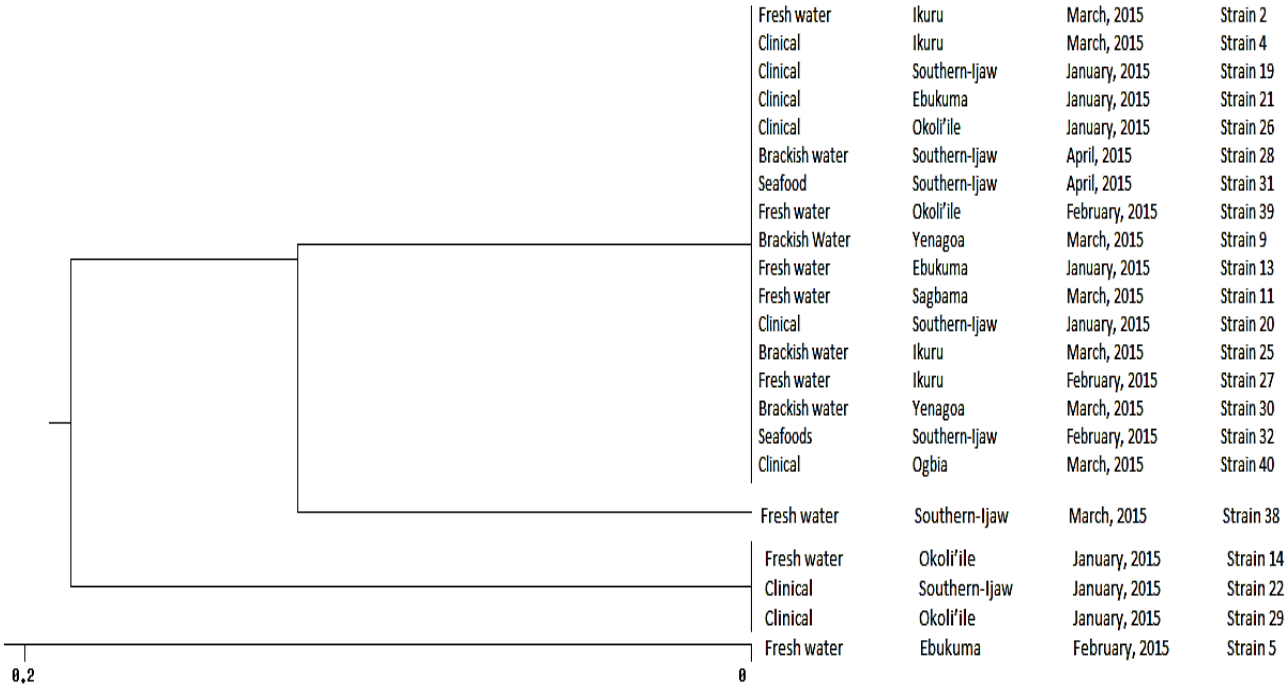
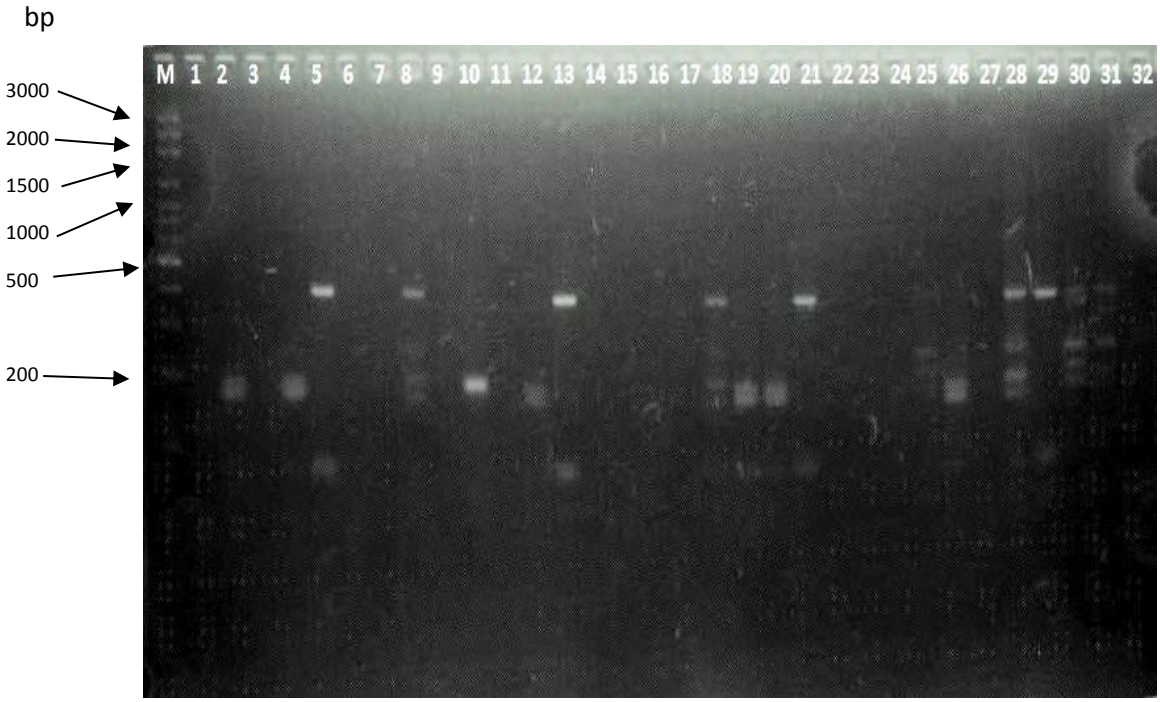


Figure 2. Genetic Distance Tree Showing Clonal Relatedness of Strains from *Mbo* I Digestion Fingerprints Patterns

(a)



(b)

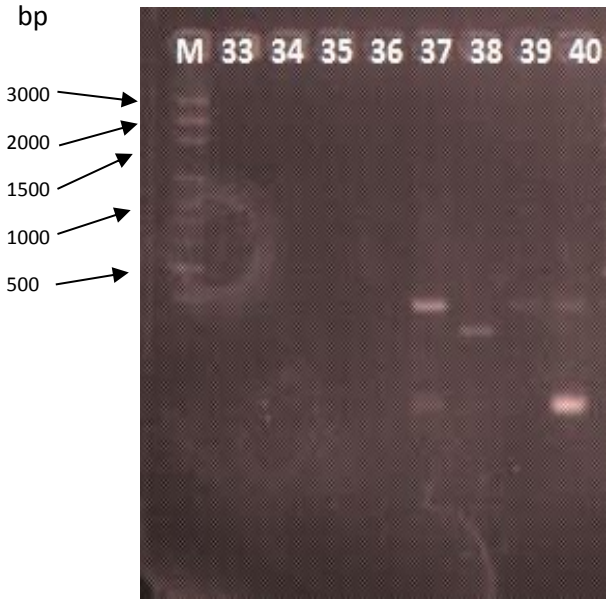


Figure 3. Agarose gel electrophoresis of PCR-RFLP products of *V. cholerae* strains from Bayelsa and Rivers State using *Alu*1 restriction enzyme. Lanes: M, Molecular weight ladder

Table 4. The matrix of percentage of clonal relatedness among strains using *Alu* I digestion

	Strain 2	**Strain 4**	**Strain 5**	**Strain 8**	**Strain 10**	**Strain 12**	**Strain 13**	**Strain 18**	**Strain 19**	**Strain 20**	**Strain 21**	**Strain 25**	**Strain 28**	**Strain 29**	**Strain 30**	**Strain 31**	**Strain 37**	**Strain 38**	**Strain 39**
Strain 2	100.00	66.67	83.33	50.00	66.67	66.67	66.67	66.67	66.67	66.67	83.33	66.67	50.00	50.00	66.67	66.67	50.00	50.00	66.67
Strain 4	66.67	83.33	50.00	66.67	66.67	66.67	66.67	66.67	66.67	83.33	66.67	50.00	50.00	66.67	66.67	50.00	50.00	66.67	
Strain 5	83.33	50.00	33.33	100.00	66.67	33.33	33.33	100.00	50.00	66.67	83.33	50.00	66.67	66.67	50.00	83.33	66.67		
Strain 8	33.33	50.00	83.33	83.33	50.00	50.00	83.33	66.67	83.33	66.67	66.67	83.33	83.33	33.33	66.67	83.33			
Strain 10	83.33	50.00	50.00	83.33	83.33	50.00	66.67	50.00	66.67	33.33	50.00	50.00	100.00	66.67	50.00				
Strain 12	33.33	66.67	100.00	100.00	33.33	83.33	66.67	50.00	50.00	66.67	66.67	83.33	50.00	66.67					
Strain 13	66.67	33.33	33.33	100.00	50.00	66.67	83.33	50.00	66.67	66.67	50.00	83.33	66.67						
Strain 18	66.67	66.67	66.67	50.00	66.67	83.33	50.00	66.67	100.00	50.00	83.33	100.00							
Strain 19	100.00	33.33	83.33	66.67	50.00	50.00	66.67	66.67	83.33	50.00	66.67								
Strain 20	33.33	83.33	66.67	50.00	50.00	66.67	66.67	83.33	50.00	66.67									
Strain 21	50.00	66.67	83.33	50.00	66.67	66.67	50.00	83.33	66.67										
Strain 25	83.33	33.33	66.67	83.33	50.00	66.67	33.33	50.00											
Strain 28	50.00	83.33	100.00	66.67	50.00	50.00	66.67												
Strain 29	33.33	50.00	83.33	66.67	100.00	83.33													
Strain 30	83.33	50.00	33.33	33.33	50.00														
Strain 31	66.67	50.00	50.00	66.67															
Strain 37	50.00	83.33	100.00																
Strain 38	66.67	50.00																	
Strain 39	83.33																		

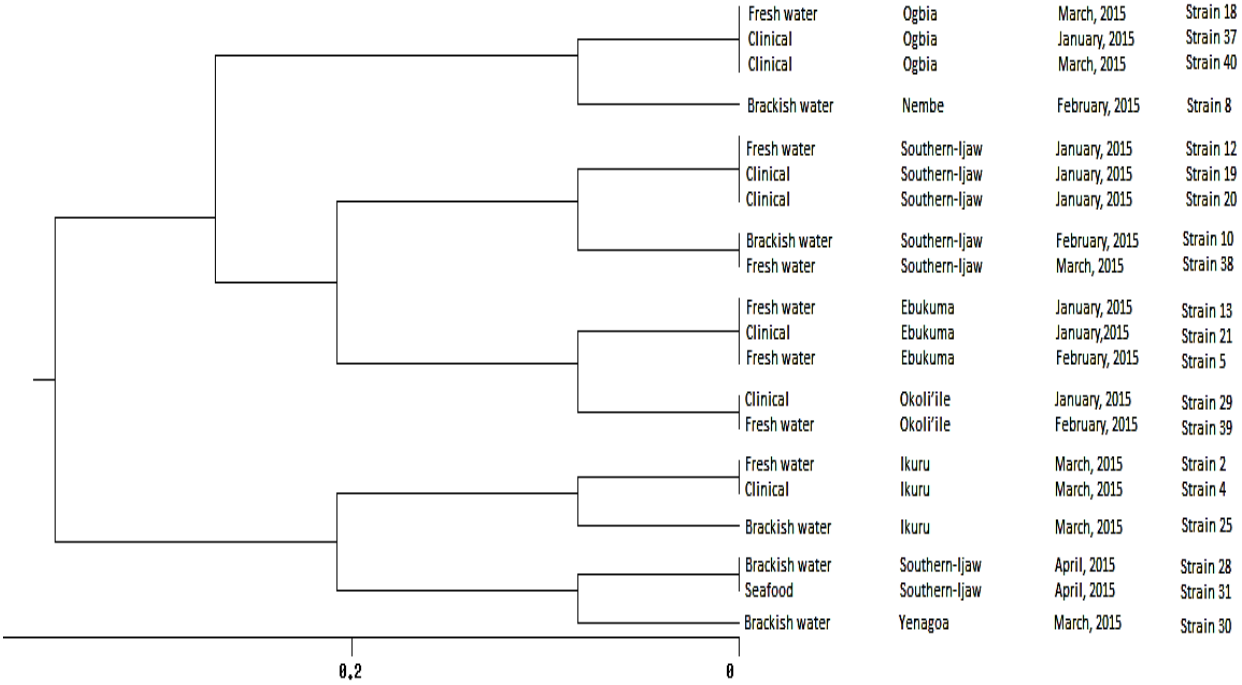


Figure 4. Genetic Distance tree Showing Clonal Relatedness of Strains from *Alu* I Digestion Fingerprints Patterns

DISCUSSION

The use of PCR-RFLP to trace the source of microbial infection and clonal relatedness employed in this study has been documented by earlier studies (Bialkowska-Hobrzanska *et al*., 1990a; Bialkowska-Hobrzanska *et al*., 1990b, Yoon *et al*., 2003; Zulkifli *et al.*, 2009; Hoffman *et al*., 2010). The *Mbo*I restriction endonuclease is usually derived from *Moraxella bovis* which spliced DNA fragments at 5'----- GATC----- 3' and 3'----CTAG---- 5' as recognition sites. The restriction fragment length polymorphic (RFLP) analysis of 16S rDNA of *V.cholerae* from different localities in Rivers State using the *Mbo*I restriction endonuclease showed 100% genetic homology of strain designate 14 (Fresh water, Okoli'ile) and strain 29 (Clinical, Okoli'ile). Similarly, strain 22 (Clinical, Southern-Ijaw) and strain 5 (Fresh water, Southern-Ijaw) also clustered with 100% clonal relatedness which could imply that the source of the epidemic of 2015 cholera outbreaks in the two localities is from the source of drinking waters in these communities.

The *Alu*I restriction endonuclease is derived from *Arthrobacter luteus* usually excise DNA fragments at 5'----- AG CT----- 3' and 3'----CT GA----- 5' as recognition sites. The restriction fragment length polymorphic (RFLP) DNA analysis using the *Alu*I on 16S RNA gene of *V.cholerae* strains from different localities in Rivers revealed most of the clinical strains clustered together with fresh water strains from the localities. The *Alu*I endonuclease DNA fingerprinting showed 100% clonal homology in clinical and fresh water strains from the same settlements. Strain 18 (fresh water) and strains 37 and 40 (clinical) from Ogbia all clustered 100% in genetic similarities. Clinical strains from Southern-Ijaw (strains 19 and 20) showed 100% genetic relatedness with fresh water strain 12. Similarly, strain 5 and strain 13 (fresh water) and clinical strain 21 all from Ebukuma also showed 100% clustering in clonal relatedness.

Past attempts to use epidemiological tools to trace the source of infection of an epidemic has been documented (Zulkifli *et al.*, 2009; Hoffman *et al*., 2010). Idika *et al*. (2000) and Hutin *et al.* (2003) used biochemical and serological typing to source for the possible cause of outbreaks of cholera in Lagos and Kano State. Both studies successfully fingered fresh waters from wells and ponds as possible sources of *V. cholerae* infections. The approach employed in this research uses the PCR-RFLP fingerprinting of 16S rDNA of strains to precisely trace the possible source of cholera outbreaks in these communities in Bayelsa and Rivers. This approach gives a better accuracy in epidemiological surveillance of the microbial sources of cholera epidemics. Conclusively, the use of molecular techniques to track sources of microbial infections will provide better information on the epidemiological surveillance of causative strains. This will undoubtedly lead to appropriate strategies and implementable frameworks that will target prevention and control of outbreaks in these localities.

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