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Genetic relationship between clinical and environmental *Vibrio cholerae* isolates in Tanzania: A comparison using repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) fingerprinting approach

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The bacterium causing cholera, *Vibrio cholerae*, is a marine organism and coastal waters are important reservoirs of the organism. There are more than 200 serogroups of *V. cholerae*, of which serogroups O1 and O139 are known to be the causative agent of the cholera. The main virulent factor in *V. cholerae* is cholera toxin gene (ctx) that is found from the epidemic O1 and O139 strains, but may also be found in some strains other than O1 and O139 (non-O1 and non-O139). In this study, 48 *V. cholerae* strains isolated from three estuaries of Tanzania and 20 stool isolates were characterized in terms of their serogroups and possession of ctx gene and then compared using two PCR based fingerprinting methods: Enterobacterial repetitive intergenic consensus (ERIC) sequences and repetitive extragenic palindromic (REP) sequences. All the stool isolates and twelve of the environmental isolates belonged to serogroup O1 while the remaining 36 environmental isolates were defined as non-O1/O139. The entire stool isolates and 21 of the environmental isolates had the cholera toxin gene (*ctxA*). Both ERIC and REP methods gave almost unique fingerprints for each strain and confirmed high genetic heterogeneity among the different cholera strains. Higher similarity was observed in REP-PCR (70-100%) than in ERIC-PCR (62-100%), indicating different discriminative power of these methods. Environmental isolates clustered together with clinical isolates at ≥90% similarity level suggesting their great potential of producing pathogenic strains that may be the causative agents for the frequent observed cholera outbreaks particularly along the coast.

Key words: *Vibrio cholerae,* enterobacterial repetitive intergenic consensus (ERIC)-PCR, repetitive extragenic palindromic (REP)-PCR, estuaries of Tanzania.

INTRODUCTION

Cholera epidemics and pandemics are a major health problem causing death, particularly in low-income countries. It is a waterborne infectious disease caused by the Gram-negative bacterium *Vibrio cholerae*, when it colonizes the

small intestine in humans. The disease is characterized by devastating watery diarrhea, caused by an enterotoxin known as cholera toxin (CT), which is produced by the bacteria. The reemergence of cholera is presenting unprecedented challenges and scientists have for long time questioned how *V. cholerae* could seemingly disappear-undetected in water samples; with no new cases reported, and then almost spontaneously arise again. Exactly what awakens the bacterium from their dormant state is unknown, but it is thought to involve a combination of different factors, such as water temperature, water salinity, nutrients and phytoplankton biomass that provide the "right" conditions (Islam et al., 2007). Climatic factors have also been shown to be significant in cholera epidemics (Lobitz et al., 2000; Lipp et al., 2002; Fernández et al., 2009) due to the fact that the historical context of the disease has a close link to specific seasons and biogeographical zones.

There are more than 200 serogroups of *V. cholerae*, of which serogroups O1 or O139 are known to cause the cholera disease in humans. The main virulent factor in *V. cholerae* is cholera toxin gene (ctx) that is found from the epidemic O1 and O139 strains, but it is also found from other *V. cholerae* strains (non-O1 and non-O139) that have been hypothesized to be the source of new epidemics (Lipp et al., 2002; Peterson, 2002). Earlier studies have shown that *V. cholerae* is capable for horizontal gene transfer, where for example the O139 strain has arose from the *V. cholerae* O1 strain (Bik et al., 1995; Waldor and Mekalanos 1994). In addition, *V. cholerae* non-O1 and non-O139 strains can acquire genes for toxin production by horizontal gene transfer (Faruque et al., 1998). In Tanzania, a recent study has also shown the existence of both serogroup O1 and O139 in estuary ecosystems (Lugomela et al., 2011). Interestingly, the authors also found a number of *V. cholerae* non-O1/non-O139 to carry the cholera toxic gene *ctxA*, raising the question whether these strains pose a health risk in the region.

A wide variety of techniques are used to study the pathogenicity and identification of *V. cholerae* (Nandi et al., 2000; Binsztein et al., 2004; Rao and Surendran, 2010; Teh et al., 2011a; Kumar et al. 2014). Repetitive element sequence-based PCR fingerprinting studies have repeatedly revealed a high degree of diversity among nontoxigenic and toxigenic *V. cholerae* isolates from various sources (Teh et al., 2011a). Such methods include enterobacterial repetitive intergenic consensus (ERIC-PCR) sequences and repetitive extragenic palindromic (REP-PCR) sequences (Rao and Surendran, 2010). The ERIC sequences are 126 bp and highly conserved at nucleotide sequence level but their chromosomal locations differ between species (Hulton et al., 1991). The ERIC sequence in *V. cholerae* is located near the haemolysin gene (Wong and Lin, 2001). The REP-PCR method is based on the presence of 38-bp REPs in *Enterobacteriaceae* and other bacteria and has been applied to many species (Wong and Lin, 2001).

Tanzania, like many other countries in sub-Saharan Africa, has consistently been affected by cholera cases. Outbreaks of various magnitudes continue to re-occur across the country and especially along the coastal regions. Studies on the association between cholera outbreaks and environment in Tanzania are limited. The seasonal cholera outbreaks have been linked to rainfall, dry season and/or floods (Acosta et al., 2001). The major risk factors have been linked to rivers and bathing water contaminated by sanitary effluents or eating dried fish from contaminated water. However, there is no study that has assessed the genetic relationships of the *V. cholerae* isolated from patients and those occurring in the natural environment in Tanzania. This may have led to limitation on proper implementation of preventive interventions to contain the outbreak of epidemic cholera. In this study, we used multiple PCR targeting *V. cholerae* specific regions (species-specific outer membrane protein *ompW,* cholera toxin gene (ctx), serogrouping of O1-rfb and O139-rfb) to assess occurrence of pathogenic strains of *V. cholerae* on three estuaries of Tanzania. The genetic heterogeneity between environmental isolates and clinical isolates were also determined using two PCR fingerprinting approaches: the ERIC-PCR and REP-PCR sequencing methods.

MATERIALS AND METHODS

Isolation of *V. cholerae* and DNA extraction

Samples for isolation of environmental *V. cholerae* strains were collected from Pangani Estuary (Tanga Region), Ruvu Estuary (Pwani Region) and Mzinga Creek (Dar es Salaam Region) on monthly bases from July 2009 to June 2010. At each site, three sampling stations were set, that is., in fresh water (Station 1), brackish water (Station 2) and marine water (Station 3). In each sampling station, water and plankton (collected by concentrating 100 L through 20 µm plankton net) samples were taken and stored in pre-sterilized bottles. A total of 48 environmental (14 from water samples and 34 plankton samples) that were confirmed as *omp*W positive were used in this study.

Isolation *V. cholerae* was done at Muhimbili Hospital laboratory by following standard techniques. First, the samples were enriched using alkaline peptone water (APW) followed by sub culturing on thiosulfate citrate bile-salt sucrose (TCBS) agar media (both are selective media for *V. cholerae*). After incubations, colonies showing the characteristic *V. cholerae* were picked and purified. Clinical *V. cholera* strains, isolated from cholera patient stools were obtained from Muhimbili national culture collection. The clinical strains, referred as stool in the text, were re-cultured in TCBS

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medium. A total of 20 viable cultures for this study were derived from cholera patient's stool from Dar es Salaam and Tanga between 2009 and 2012. All strains were then preserved in 70%. ethanol before DNA extraction. The DNA extractions were done as described by Jia et al. (2004). The extracted DNA was suspended in 50 µl of TE buffer and stored at 4°C

PCR typing of *V. cholerae* for REP and ERIC PCR

PCR for the detection of *V. cholerae* was performed using species-specific ompW primers *ompW*F ('5-CACCAAGAAGGTGACTTTATTGTG-3') and *ompW*R ('5-GGTTTGTCGAATTAGCTTCACC-3') followed by detection of enterotoxigenic *V. cholerae* by using ctx primers *ctx*AF ('5-CTCAGACGGGATTTGTTAGGCACG-3') and *ctx*AR ('5-TCTATCTCTGTAGCCCCTATTACG-3') as described in Alam et al. (2006). To confirm the presence of *V. cholerae* O1 and O139, PCR was performed by using O1-rfb [01*rfb*F ('5-GTTTCACTGAACAGATGGG-3') and 01*rfb*R ('5-GGTCATCTGTAAGTACAAC-3')] and O139-rfb [0139*rfb*F (5-AGCCTCTTTATTACGGGTGG-3') and 0139*rfb*R ('5-GTCAAACCCGATCGTAAAGG-3') specific primers, respectively. Enterobacterial ERIC-PCR was performed using ERIC1R (5'-ATGTAAGCT CCTGGGGATTCA C-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGC G-3') while REP-PCR was performed using REP1D (5'-NNN RCGYCGNCA TCMGGC-3') and REP2D (5'-RCGYCTTATCMGGCCTAC-3') as described in Rao and Surendran (2010).

PCR amplification was performed as previously described by Rao and Surendran (2010), with minor modifications. Thus, PCR mix composed of 10x buffer [100 mM KCl, 20 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA], 2 µM of dATP, dCTP, dGTP and dTTP, 3 U of Taq polymerase, 5 µM of primers and 5 ng of template DNA in a final volume of 50 µl. The amplifications were carried out in a S1000TM Thermal cycler programmed as follows: PCR cycle conditions for REP-PCR and ERIC-PCR was done by an initial denaturation at 95°C for 7 min, then 30 amplification cycles of denaturation at 90°C for 30 s, annealing at 45°C for 1 min, extension at 65°C for 3 min, final extension at 70°C for 10 min. After each PCR reaction, the correct PCR product was confirmed by running 10 µl of PCR product on 1% agarose gel electrophoresis in 1× TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.0]) containing 1 µg/ml SYBR safe (Invitrogen, Carlsbad, CA, USA).

The electrophoretic patterns of ERIC-PCR and REP-PCR were analyzed using a computer software package on Quantity One ver. 4.1.0 (Bio-Rad). Dendograms were analyzed using PC-ORD program and produced with MEGA6 using the unweighted average pair Group method (UPGMA) (Sneath and Sokal, 1973).

RESULTS

A total of 68 *V. cholerae* strains (48 environmental and 20 stool isolates) that were confirmed as *omp*W positive were used in this study (Figures 1 and 2). Twelve (12) of the environmental isolates, both from water and plankton samples, as well as the entire stool isolates belonged to serogroup O1. Thus, the remaining 36 isolates were defined as non-O1/O139 (Figures 1 and 2). There was no strain that belonged to serogroup O139. Furthermore, the results revealed that 21 of the environmental isolates and all the stool isolates had the cholera toxin gene (*ctxA*) (Figures 1 and 2).

ERIC-PCR fingerprinting

The fingerprints of *V. cholerae* isolates consisted of one to 12 amplification bands, ranging in size from 263 to 3633 bp (Figure 3). Each strain gave almost a different ERIC fingerprint although common bands were observed on most of the isolates at 565 and 425 bp (48 and 40 isolates, respectively). Comparison of different strains showed extensive diversity among the *V. cholerae* isolates and the similarity ranged between 62-100%. Analyzing the similarity among the different strains with the diversity database software MEGA6 employing the UPGMA allowed the identification of four different clusters (ERIC groups) at 80% similarity level (Figure 1). The stool isolates as well as environmental isolates were found from all ERIC groups and the samples were divided without any clear division based on the source, cholera toxin gene or serogroup. Observing the results at 90% similarity level (Figure 1), 16 different clusters were obtained, where most of the clusters contained only stool isolates or environmental isolates. The different sampling stations or environmental samples (water and plankton) were not separately clustered. However, six clusters contained a mixture of clinical and environmental isolates (B83, T198, D588, D228, T181, B19, B144, B134, B118, B160, T124, B33, BI46 and B32) with at least 90% similarity level (Figure 1).

REP-PCR fingerprinting

Analysis using REP-PCR showed 3 to 14 bands for the 68 isolates. The size of these bands ranged from 220 to 3168 bp and the fingerprint patterns were showing high disparity between the samples (Figure 4). However, most isolates demonstrated a common band at 1020 bp (55 isolates) and the similarity between the isolates varied between 70-100%. A total of seven genomic REP groups were clustered among the 68 isolates of *V. cholerae* using the MEGA6 program and UPGMA method at 80% similarity level (Figure 2). As was the case with ERIC-PCR analysis, all REP groups contained isolates both from stool and environmental samples (Figure 2). Likewise, no environment, serogroup or cholera toxin gene based division was seen. Clustering at 90% similarity level revealed 15 clusters (Figure 2). However, only two clusters contained both stool and environmental (B211 and T181) isolates at 90% similarity level. Isolate T181 also clustered with stool isolate in ERIC-PCR analysis.

DISCUSSION

This study confirmed earlier observations on the presence of the toxigenic *V. cholerae* O1 and non-O1/O139 in the estuaries of Tanzania (Lugomela et al., 2011). In particular, *V. cholerae* serogroup O1 that was

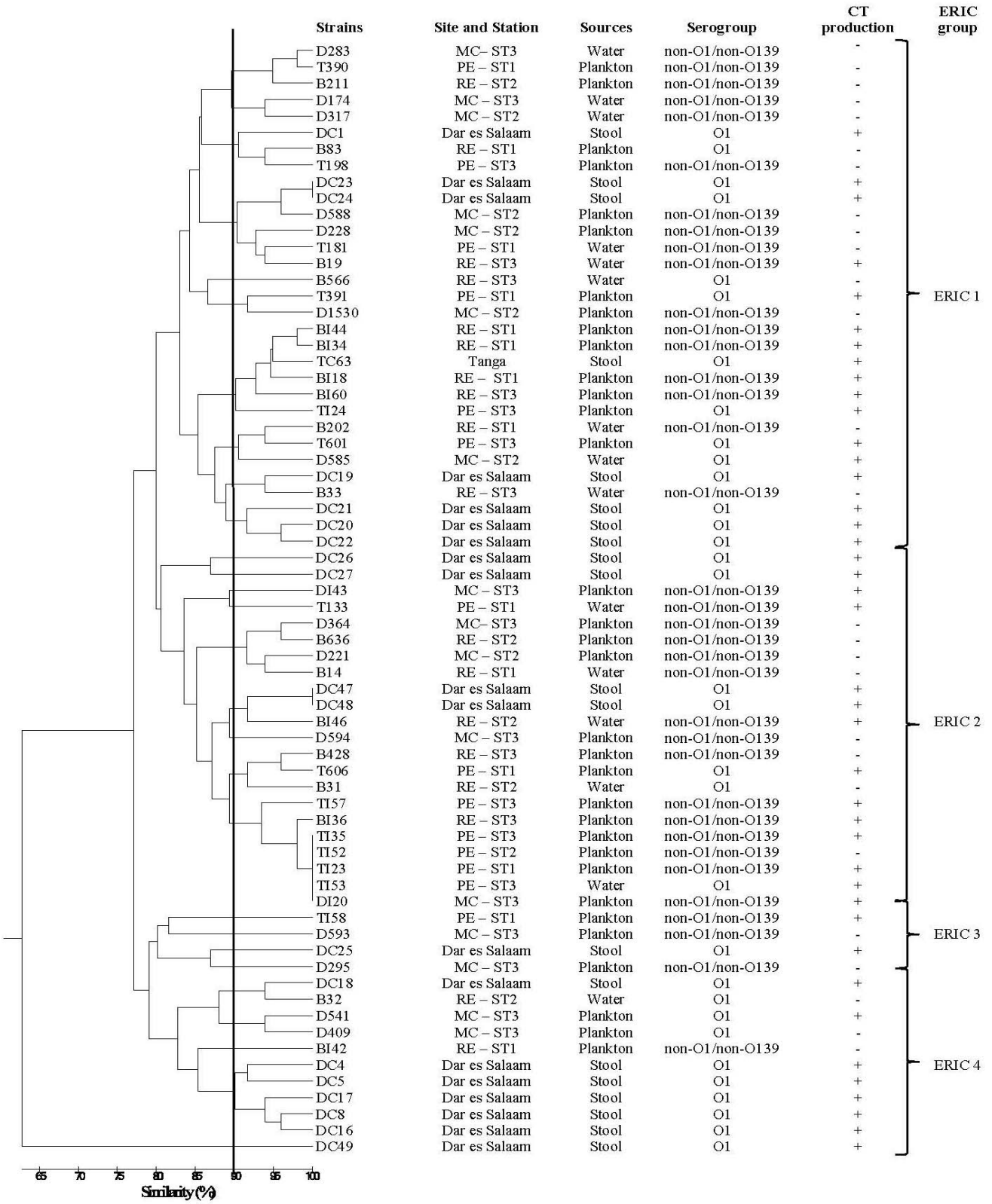

Figure 1. Dendrogram established by the diversity database software package (MEGA6) and UPGMA on the basis of the ERIC-PCR profiles of *V. cholerae* stains. Clustering at 80% similarity level is presented with ERIC groups and similarity at 90% level with black vertical line. The sample description and genetic characterization are presented for each sample (PE = Pangani Estuary, RE = Ruvu Estuary, MC = Mzinga Creek, DSM = Dar es Salaam, STI = Station 1, ST2 = Station 2, ST3 = Station 3, Zoo = zooplankton and Phyto = phytoplankton).

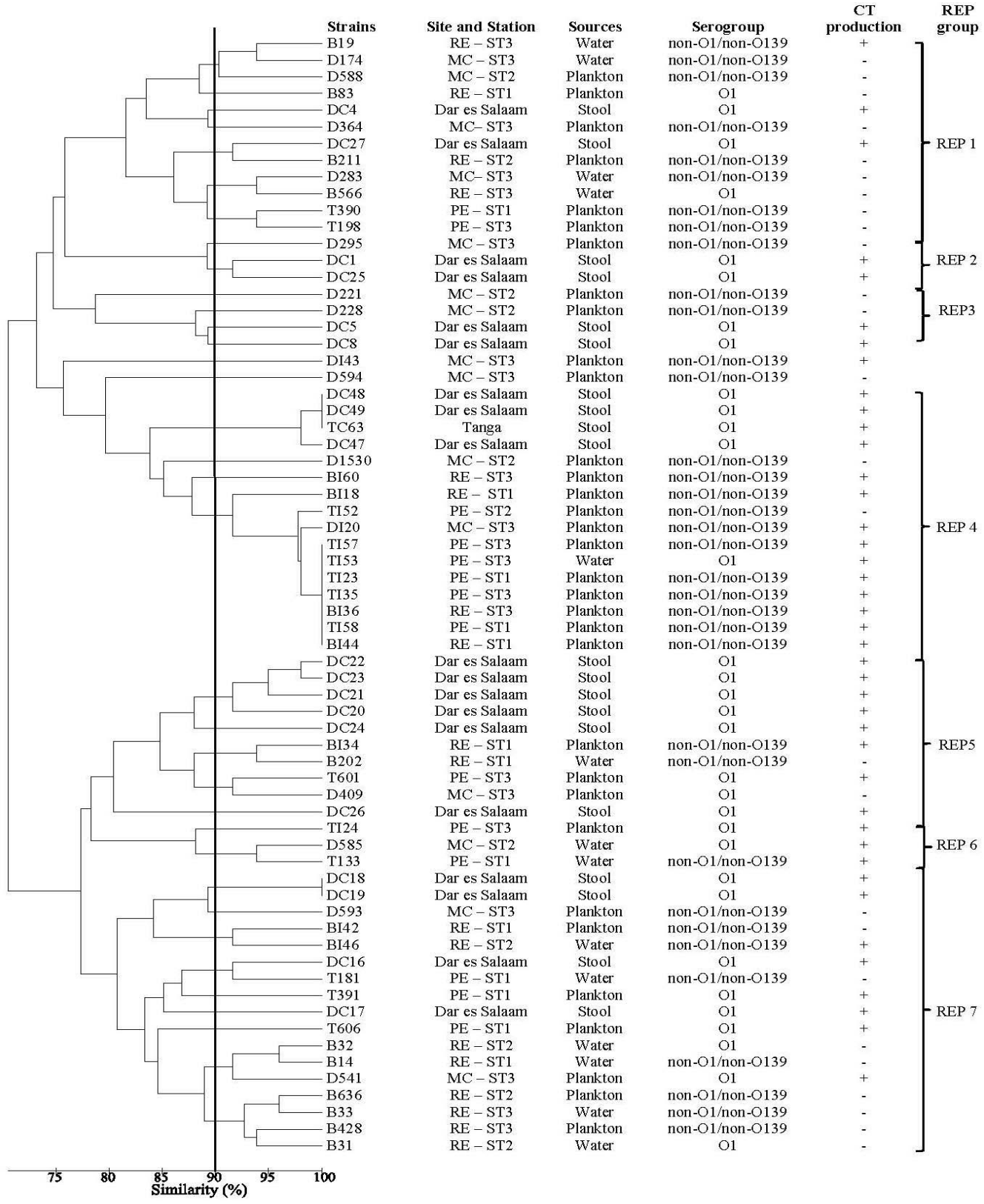

Figure 2. Dendrogram established by the diversity database software package (MEGA6) and UPGMA on the basis of the REP-PCR profiles of *V. cholerae* stains. Clustering at 80% similarity level is presented with REP groups and similarity at 90% level with black vertical line. The sample description and genetic characterization are presented for each sample (PE = Pangani Estuary, RE = Ruvu Estuary, MC = Mzinga Creek, DSM = Dar es Salaam, STI = Station 1, ST2 = Station 2, ST3 = Station 3, Zoo = zooplankton and Phyto = phytoplankton).

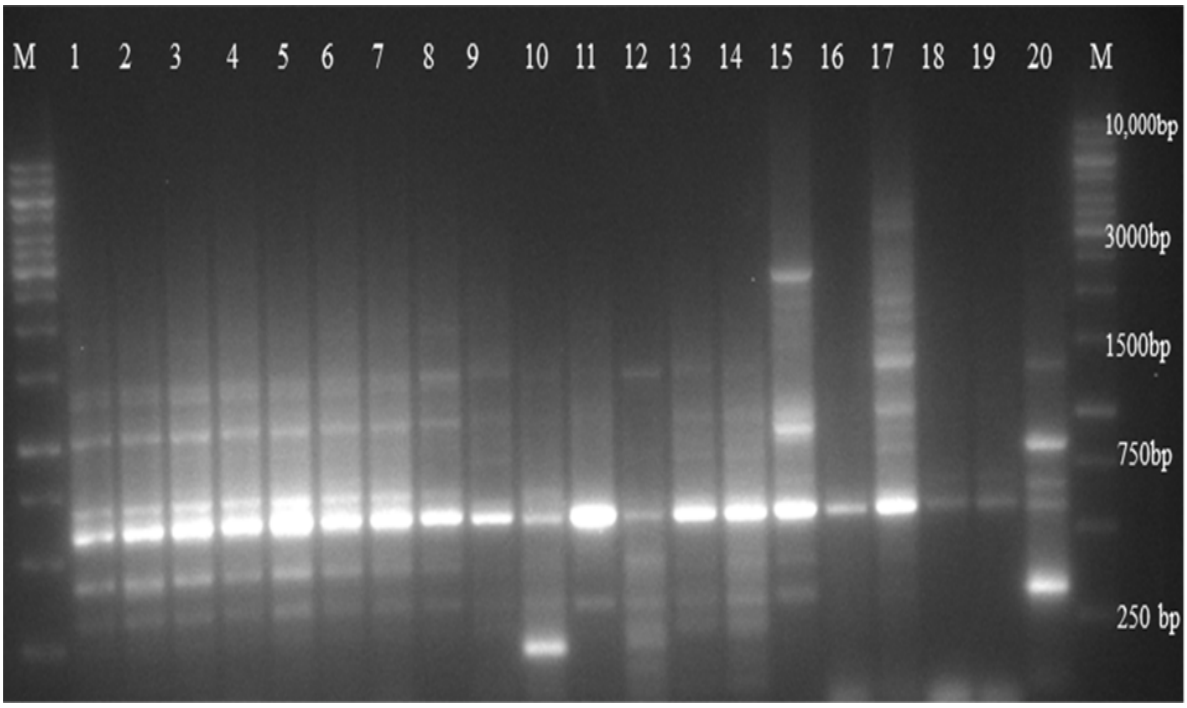

Figure 3. An Example of DNA fingerprint pattern of *V. cholerae* isolates using the ERIC-PCR. Lane 1-20 is TI58, TI35, TI52, TI23, TI53, BI36, DI20, TI57, BI60, BI42, BI18, BI46, DC47, DC48, DI43, TC63, DC49, BI44, BI34 and TI24, respectively. Lane M: 1 kb DNA Molecular weight maker.

commonly encountered is known to be responsible for the 7th (1961 to present) cholera pandemic (Feng et al., 2008). Indeed, all isolate from cholera patient's stool samples were *V. cholerae* Serogroup O1, which gives the implication that they are responsible for the observed frequent cholera outbreaks in Tanzania. Nevertheless, majority of *V. cholerae* strain that were isolated from the environment belonged to non-O1/O139 serotypes which is in agreement with what has been reported elsewhere (Teh et al., 2011a; Waturangi et al., 2012). Interestingly, over one-third of the non-O1/O139 *V. cholerae* strains were also found to possess the cholera toxic gene (ctxA). This is however not peculiar as other reports have also shown the existence of ctxA in certain strains of non-O1/O139 *V. cholerae* of both clinical and environmental origin (Chakraborty et al., 2000; Nandi et al., 2000). Indeed, strains belonging to serogroups non-O1/O139 have from time to time been implicated as the causative agents of cholera-like diseases in various parts of the world. These include serogroups such as O10, O11, O12, O35, O37 and O75 (Tobin-D'Angelo et al., 2008; Chatterjee et al., 2009; Dutta et al., 2013). This implies that the occurrence of non-O1/O139 strains in the aquatic environment of Tanzania can also be a source of cholera-like diarrhea as have been reported elsewhere. Since typically, the *V. cholerae* O1 and O139 are known as the causative agent of cholera (Nandi et al., 2000) a better understanding of the potential pathogenicity of *V. cholerae* non-O1/O139 strains is important and calls for further studies.

The results of ERIC-PCR fingerprinting showed that the 68 *V. cholerae* isolates clustered into 4 and 17 different genotypes at 80 and 90% similarity level, respectively. This is in agreement with other studies, which showed extensive genetic diversity of *V. cholerae* (Rao and Surendran, 2010; Teh et al., 2011a; Waturangi et al., 2012) as well as in *Vibrio parahaemolyticus* (Wong and Lin, 2001). Interestingly, 14 environmental isolates clustered with eight clinical isolates in six clusters at 90% similarity level. This suggests that the coastal waters of Tanzania harbor great potential for pathogenic *V. cholerae* strains that may also initiate cholera outbreaks. This could be accelerated through e.g. gene transfer process, which has been reported in various studies (Faruque et al., 1998; Blokesch, 2014).

Similarly, when applying REP- PCR on the 68 isolates, a total of 7 and 15 clusters were revealed at 80 and 90% similarity level, respectively. However, at a similarity level of 90% and above, ERIC-PCR showed more genetic variation among the *V. cholerae* isolates as compared to REP-PCR suggesting that ERIC approach is more powerful as it is less complex but more discriminative. This observation is similarly to that of Maluping et al. (2005) and Waturangi et al. (2012) who showed that REP PCR is less reproducible and yields relatively small number of products. On the contrary, in a study by Wong

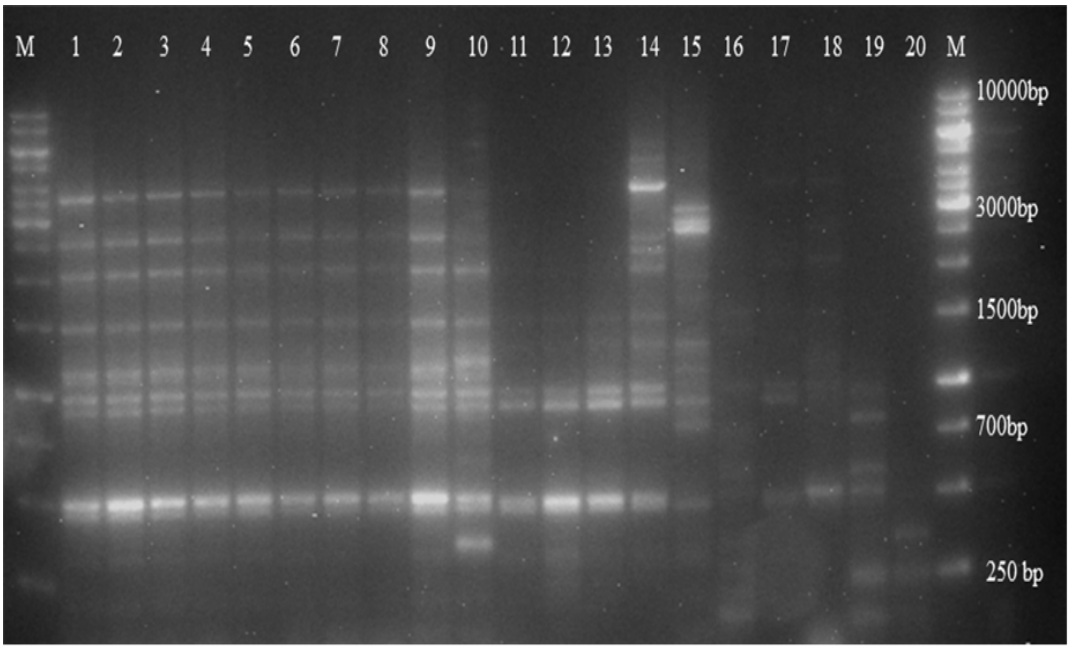

Figure 4. An example of DNA fingerprint pattern of *V. cholerae* isolates using the REP-PCR. Lane 1-20 is isolates TI58, TI35, TI57, TI23, TI53, BI36, BI34, DI20, TI52, BI18, DC47, DC48, TC63, DC49, DI43, BI60, BI44, BI42, BI46 and TI24. Lane M: 1kb DNA Molecular maker.

and Lin (2001) on *V. parahaemolyticus*, the authors suggested that REP-PCR was preferable to ERIC-PCR because of the greater reproducibility of its fingerprints. Nevertheless, the two methods did not give clear division among the groups based on neither source nor ctxA gene nor serogroup. This is similar to previous reports that show no clear distinction between the sources, ctxA gene as well as serogroups (Teh et al., 2011a; Waturangi et al., 2012). Thus, other finger typing methods such us Random Amplified Polymorphic DNA (RAPD), MultiLocus Sequence Typing (MLST) and Multi-virulence Locus Sequencing Typing (MVLST) methods (e.g. Teh et al., 2011b; Kumar et al., 2014) are recommended for further analysis of the genetic variations and relatedness of the isolated strains.

Conclusion

In conclusion, both ERIC and REP methods gave almost unique fingerprints for each strain. Comparison of similarities among the different profiles confirmed the genetic heterogeneity among *V. cholerae* strains. The two methods show no common cluster that contained similar strains composition possibly due to their different discriminatory powers. The high similarity between some of the environmental and stool isolates suggests that environmental strains are the causative agents of the frequent observed cholera outbreaks in coastal regions of Tanzania. The role of *V. cholerae* non-O1/O139 that contains the cholera toxin gene as agents for diarrheal diseases in coastal areas of Tanzania calls for further investigation.

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Conflict of interest

The authors did not declare any conflict of interest.

REFERENCES

Acosta CJ, Galindo CM, Kimario J, Senkro K, Urassa H, Casals C, Corachán M, Eseko N, Tanner M, Mshinda H, Lwilla F, Vila J, Alonso PL (2001). Cholera outbreak in Southern Tanzania: risk factors and patterns of transmission. Emerg. Infect. Dis. 7:583-587.

Alam M, Sultana M, Nair GB, Sack RB, Sack DA, Siddique K, Ali A, Huq A, Colwell RR (2006). Toxigenic *Vibrio cholerae* in the aquatic

environment of Mathbaria, Bangladesh. Appl. Environ. Microbiol. 72(4):2849-2855.
Bik EM, Bunschoten AE, Grow RD, Mooi FR (1995). Genesis of the novel epidemic *Vibrio cholerae* O139 specific strain: evidence of horizontal gene transfer of genes involved in polysaccharide synthesis. EMBO J. 14:209-216.
Binsztein N, Costagliola MC, Pichel M, Jurquiza V, Ramizer FC, Akselman R, Vacchino M, Huq A, Colwell RR (2004). Viable but nonculturable *Vibrio cholerae* 01 in the aquatic environment of Argentina. Appl. Environ. Microbiol. 70:7481-7486.
Blokesch M (2014). The lifestyle of the *Vibrio cholerae* forster gene transfer. Microbe 9:64-70.
Chakraborty S, Mukhopadhyay AK, Bhadra RK, Ghosh AN, Mitra R, Shimada T, Yamasaki S, Faruque SM, Takeda Y, Colwell RR, Nair GN (2000). Virulence genes in environmental strains of *Vibrio cholerae*. Appl. Environ. Microbiol. 66:4022-4028.
Chatterjee S, Ghosh K, Raychoudhuri A, Chowdhury G, Bhattacharya MK, Mukhopadhyay AK, et al. (2009). Incidence, virulence factors, and clonality among clinical strains of non-O1, non-O139 *Vibrio cholerae* isolates from hospitalized diarrheal patients in Kolkata, India. J. Clin. Microbiol. 47:1087-95.
Dutta D, Chowdhury G, Pazhani GP, Guin S, Dutta S, Ghosh S, Rajendran K, Nandy RK, Mukhopadhyay AK, Bhattacharya MK, Mitra U, Takeda Y, Nair GB, Ramamurthy T (2013). *Vibrio cholerae* Non-O1, Non-O139 Serogroups and Cholera-like Diarrhea, Kolkata, India. Emerg. Infect. Dis. 19(3):464-467.
Faruque SM, Albert MJ, Mekalanos JJ (1998). Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae.* Microbiol. Mol. Biol. Rev. 62:1301-1314.
Feng L, Reeves PR, Lan R, Ren Y, Gao C (2008). A recalibrated molecular clock and independent origins for the cholera pandemic clones. PLoS One 3: e4053. doi: 10.1371/journal.pone.0004053.
Fernández MÁL, Bauernfeind A, Jiménez JD, Gil CL, El Omeiria N, Guiberte DH (2009). Influence of temperature and rainfall on the evolution of cholera epidemics in Lusaka, Zambia, 2003-2006: analysis of a time series. Trans. Royal Soc. Trop. Med. Hyg. 103: 137-143.
Hulton CSJ, Higgins CF, Sharp PM (1991). ERIC sequences: a novel family of repetitive elements in the genomes of Escherichia coli, *Salmonella typhimurium* and other enterobacteria. Mol. Microbiol. 5: 825-834.
Islam MS, Jahid MIK, Rahman MM, Rahman MZ, Islam MS, Kabir MS, Sack DA, Schoolnik GK (2007). Plankton associated *Vibrio cholerae* in the aquatic environment of Bangladesh. Microbiol. Immunol. 51: 369-379.
Jia N, Penga B, Wangb G, Wanga S, Penga X (2004). Universal primer PCR with DGGE for rapid detection of bacterial pathogens. J. Microbiol. Meth. 57:409-413.
Kumar P, Mishra DK, Deshmukh DG, Jain M, Zade AM, Ingole KV, Goel AK, Yadava PK (2014). *Vibrio cholerae* O1 Ogawa El Tor strains with the ctxB7 allele driving cholera outbreaks in south-western India in 2012. Infect, Genet. Evol. 25:93-96.
Lipp EK, Huq A, Cowell RR (2002). Effect of global climate on infectious disease: The cholera model. Clin. Microbiol. Rev. 15:757-770.
Lugomela C, Shayo R, Hosea K, Namkinga L, Lyimo T, Moyo S, Ochiewo J, Mwangi S, Goericke R, Sjöling S (2011). Distribution of pathogenic *Vibrio cholerae* strains in aquatic environments in coastal areas of East Africa: Implication to cholera outbreaks and control. A report for MASMA Project MASMA/OR/2009/01, WIOMSA. 59 pp.
Maluping RP, Ravela C, Lavilla-Pitogo CR, Krovacek K, Romalde JL (2005). Molecular typing of the *Vibrio parahaemolyticus* strains isolated from Phillippines by PCR based methods. J. Appl. Microbiol. 99:383-391.
Nandi B, Nandi RK, Mukhopadhyay GB, Nair GB, Shimada T, Ghose AC (2000). Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the outer membrane protein OmpW. J. Clin. Microbiol. 38:4145-4154.
Peterson KM (2002). Expression of *Vibrio cholerae* virulence genes in response to environmental signlas. Curr. Issues Intest. Microbiol. 3: 29-38.
Rao BM, Surendran PK (2010). Genetic heterogeneity of non-O1 and non-O139 *Vibrio cholerae* isolates from shrimp aquaculture system: a comparison of RS-, REP- and ERIC-PCR fingerprinting approaches. Appl. Microbiol. 51:65-74.
Sneath PHA, Sokal RR (1973). *Numerical Taxonomy.* The Principles and Practice of Numerical Classification. San Francisco, CA W.H. Freeman.
Teh CSJ, Thong KL, Osawa R, Chua KH (2011a). Comparative PCR-based fingerprinting of *Vibrio cholerae* isolated in Malaysia. J. Gen. Appl. Microbiol. 57:19-26.
Teh CSJ, Chua KH, Thong KL, (2011b). Genetic variation analysis of *Vibrio cholerae* using multilocus sequencing typing and multi-virulence locus sequencing typing. Infect. Genet. Evol. 11:1121-1128.
Tobin-D'Angelo M, Smith AR, Bulens SN, Thomas S, Hodel M, Izumiya H, Arakawa E, Morita M, Watanabe H, Marin C, Parsons MB, Greene K, Cooper K, Haydel D, Bopp C, Yu P, Mintz E (2008). Severe Diarrhea Caused by Cholera Toxin–Producing *Vibrio cholerae* Serogroup O75 Infections Acquired in the Southeastern United States. Clin. Infect. Dis. 47:1035-1040.
Waldor MK, Mekalanos JJ (1994). ToxR regulates virulence gene expression in non-O1 strains of *Vibrio cholerae* that cause. Infect. Immun. 62:72-78.
Waturangi DE, Joanto I, Yogi Y, Thomas S (2012). The use of REP- and ERIC-PCR to reveal genetic heterogeneity of *Vibrio cholerae* from edible ice in Jakarta, Indonesia. Gut Pathogens. 10:1186-1757.
Wong H, Lin C (2001). Evaluation of typing of *Vibrio parahaemolyticus* by three PCR methods using specific primers. J. Clin. Microbiol. 39: 4233-4240.