

OCCURRENCE OF PATHOGENIC *Vibrio cholerae* SEROGROUPS O1 AND O139 IN SOME ESTUARIES OF TANZANIA

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ABSTRACT

In this study, the occurrence of culturable *Vibrio cholerae* serogroup O1 and O139 in three selected Tanzanian Estuaries, Mzingu, Pangani and Ruvu were explored. A total of 480 water and plankton samples were collected along salinity gradients (fresh, brackish and marine waters). Samples were enriched in Alkaline Peptone Water (APW) and subsequently cultured on Thiosulfate Citrate Bile-Salt Sucrose (TCBS) agar. Resulting isolates were serially identified by oxidase test, API-20E test and monoclonal antibodies for *V. cholerae* serogroup O1 and O139. A total of 670 presumptive *V. cholerae* isolates were obtained from 416 samples (86.7%), where 137 (20.4%) were oxidase test positive. Out of the oxidase test positive, 96 (70.8%) were further confirmed by API 20E to be *V. cholerae* while only 8 isolates were ascertained by monoclonal antibodies to be *V. cholerae* O1 and 1 isolate to be *V. cholerae* O139. This indicated that, apart from the few *V. cholerae* O1 and O139 confirmed serogroups, there is still a large percentage of *V. cholerae* non-O1/O139. Similarly, both *V. Cholerae* serogroups O1 and non-O1/O139 were observed in clinical samples suggesting a close link between the existence of the bacterium in aquatic environments and cholera outbreak.

Key words: Tanzanian Estuaries, *V. cholerae*, serogroup O1/O139

INTRODUCTION

Cholera is a waterborne infectious disease caused by the bacterium *Vibrio cholerae*, which colonizes the small intestine in humans (Emchet al. 2008). The disease is characterized by devastating watery diarrhoea, caused by an enterotoxin known as cholera toxin (CT) produced by the bacterium. The infection leads to rapid dehydration, which may cause death within a few hours to untreated patients (Emchet al. 2008). *V. cholerae*, a comma-shaped

bacterium, gram-negative rods, is found abundantly in marine environment. It is an oxidase positive and when grown on Thiosulfate Citrate Bile-Salt Sucrose (TCBS) agar it produces flat, golden-yellow colonies in the first 18 hours of incubation (Uchiyama 2000). *V. cholerae* displays a wide variation in colony morphology ranging from round to irregular and a wide range of salinity tolerances (Peterson 2002, Karl 2007).

Since the discovery that *V. cholerae* is not only a pathogen but also a marine bacterium it has been reported in coastal environments around the world, both in areas where cholera is endemic and in cholera disease-free areas (Islam and Sack, 1994, Huq et al. 2001, Karunasagar et al. 2003). Today, it is well known that cholera occurs in regions with natural aquatic reservoirs where the bacteria can persist in a free-living state, or in association with phytoplankton, zooplankton and detritus (Nelson et al. 2009). These natural aquatic reservoirs play a significant role in cholera epidemiology by favouring persistence of the pathogen in periods between epidemics (Vezzulli et al. 2010). Thus, the dynamics of cholera has been linked to climate and oceanographic conditions such as temperature, salinity, rainfall, wind direction, nutrients, chitin and plankton (de Magny et al. 2008, Fernandez et al. 2009, Vezzulli et al. 2010, Filho et al. 2011, Traerup et al. 2011).

Water temperature has been shown to be one of the most important factors that govern the seasonal and geographical variation of *V. cholerae* (Lippert et al. 2002, Alamet et al. 2007, Igbinosa and Okoh 2008). Also, Paz (2009) showed that air temperature and sea surface temperature at local scale, as well as anomalies at hemispheric scales, had significant impact on cholera incidence in the South and Eastern Africa. It has also been suggested that aero plankton and adult midges carry *V. cholerae* between bodies of water (Broza et al. 2005). Similarly, Paz and Broza (2007) suggested that the monsoon wind direction was associated with cholera dissemination patterns. They showed a relationship between the intercontinental distribution of cholera epidemics and dominant wind direction over land. In addition, surveillance reports suggest that cholera usually strikes villages in coastal regions before cases occur inland (Nair et al. 1994, Kierec 2003).

Cholera can only be transmitted to humans through eating food or drinks contaminated with enterotoxin producing *V. cholerae*. In developed world, seafood is the most common cause of cholera while in developing world water is often the source of infection (Sackett et al. 2004, Griffith et al. 2006). There are more than 200 serogroups of *V. cholerae* based on the O-antigens. However, only serogroup O1 and O139 have been found to cause cholera epidemic and pandemic in humans (Igbinosa and Okoh 2008). The O1 serogroup of *V. cholerae* is divided into two biotypes, classical and El Tor with the latter being responsible for the most recent cholera pandemic (Butterton and Calderwood 1995, Faruque et al. 2004). The El Tor type is further classified into Inaba, Ogawa, and Hikojima serotypes (Ojha 2013). However, more recent studies have shown the existence of *ctxA* in certain strains of non-O1/O139 serogroups (*V. cholerae* O10, O11, O12, O35, O37 and O75) of both clinical and environmental origin (Chakraborty et al. 2000, Nandi et al. 2000). These strains have been implicated as the causative agents of cholera-like diseases in India and United States of America (Tobin-D'Angelo et al. 2008, Chatterjee et al. 2009, Dutta et al. 2013).

Cholera cases in Tanzania escalated from 1,671 cases in 1977 to 40,226 in 1997 (Ministry of Health 1997, WHO 1998) with some of the highest fatality rates (5.5%) in Africa. Since then outbreaks of various magnitudes continued to re-occur, where by Lugomela et al. (2014) reported number of cholera cases amounting to 43,560 on mainland Tanzania between year 2004 to 2010. More recently high number of cases amounting to 23,258 was reported by Narra et al. (2017) to have happened between 15th August 2015 and 26th November 2016. The study by Lugomela et al. (2014) showed that five coastal regions of mainland Tanzania, Tanga, Pwani, Dar es Salaam,

Lindi, and Mtwara with approximately 20% of the total population, accounted for almost 50% of the cholera cases and 40% of the total mortality. The authors linked this to proximity of the coastal regions to the marine environment, which as stated before is the natural reservoir for *V. cholerae*. The seasonal cholera outbreaks in Tanzania have been linked to seasonal rainfall and or floods (Mhalu et al. 1987, Acosta et al. 2001) while hospital outbreak transmissions were associated with close person-to-person contact (Mhalu et al. 1984). However, it is worth noting that outbreaks may also occur during the dry season, especially when there is a shortage of clean potable water (Mpazi and Mnyika 2005, Lugomela et al. 2014, Narra et al. 2017).

Studies on the association between cholera outbreaks and environmental factors in East Africa are limited to the Lake Victoria Basin (Shapiro et al. 1999, Olago et al. 2007). More recently however, Dalusi et al. (2015) identified toxigenic *V. cholerae* in estuaries of Tanzania using conventional PCR techniques. Similarly du Preez et al. (2010) working in an estuarine of Beira, Mozambique, detected the O139 strain using direct fluorescent antibody methods. However, du Preez and co-workers were unable to culture and obtain an isolate for

this serogroup, nor could they confirm their results using molecular techniques. Thus the objective of this study was to elucidate the occurrence of the pathogenic *V. cholerae* strains, O1 and O139 based on culture methods in relation to some environmental parameters. In addition, environmental isolates of *V. cholerae* serogroups O1 and O139 were compared with clinical isolates from cholera patients of Tanga and Dar es Salaam Regions, Tanzania.

MATERIAL AND METHODS

Study sites and station

Sampling was done in three coastal regions; Tanga, Pwani and Dar es Salaam, of Tanzania. These regions most often face cholera outbreaks (Lugomela et al. 2014). In each region, one estuarine system (Study Site) was selected; Pangani Estuary in Tanga, Ruvu Estuary in Pwani, and Mzinga Creek in Dar es Salaam. In each Study Site, three sampling Stations (ST) were established depending on the level of salinity; Station 1 was in the riverine (fresh water), Station 2 in brackish waters and the Station 3 in the marine waters. The locations of the sampling stations are shown in Table 1 and they are the same as those of Dalusi et al. (2015) as the two studies were done simultaneously.

Table 1: Geographical positions of the Sampling Stations (ST) for *V. cholerae* and environmental parameters

SAMPLING STATIONS	MZINGA CREEK	RUVU ESTUARY	PANGANI ESTUARY
ST 1	-	06°25.454'S 038°50.517'E	05°24.413'S 038°55.546'E
ST 2	06°53.329'S 039°18.910'E	06°23.338'S 038°51.405'E	05°23.157'S 038°56.919'E
ST 3	06°51.964'S 039°17.556'E	06°22.203'S 038°53.772'E	05°25.981'S 038°58.948'E

Samplings and measurements of environmental parameters

Sampling for environmental parameters was conducted on a monthly basis from July 2009 to June 2010 during high tide water periods between 08.00 and 11.00am. Triplicate water samples were collected at each station (on the shores and at the middle of the creek) by submerging the 2 litre bottle to a depth of about 20 cm. Plankton samples were obtained by towing a 20 µm and 80 µm mesh sized plankton net using a motorized boat for about 5 minutes. Water samples for determination of inorganic nutrients (phosphate and nitrate and ammonium) were then filtered through 0.45 µm pore sized Millex-GS Millipore filters (France). The filtered water samples were kept in 100 mL acid cleaned plastic vials and immediately kept cool on ice for transport to a deep-freezing facility. In the laboratory, analyses for NO₃⁻, NH₄⁺ and PO₄³⁻ were done using a Shimadzu spectrophotometer (Japan) as described by Parsons et al. (1984). Other environmental parameters including salinity, water temperature, pH and Dissolved Oxygen (DO) were recorded *in-situ* using a multi-parameter probe (Horriba U-10, Japan). Water and plankton samples were collected in separate sterile bottles of 500 ml and kept in a cool box. The samples were then transported to laboratory for analysis.

Isolation and quantification of *V. cholerae*

Isolation of *V. cholerae* strains from water and plankton samples was done on a monthly basis for nine months (October 2009 to June 2010) by filtering 250 ml of water onto a 0.45 µm filter membrane (Millex-GS Millipore filters, France). For plankton samples, 25 ml were filtered onto 0.45 µm filter membrane. The filters were then inoculated into 15 ml Alkaline Peptone Water (APW), and incubated at 37°C for 8 hours, as described by Alam et al. (2006). The culture was further sub-cultured on Thiosulfate Citrate Bile-Salt Sucrose (TCBS) agar medium at 37°C for 24 hours. Bacterial colonies, which were sucrose-positive and yellow, were selected as putative *V. cholerae*

and inoculated into fresh sterile TCBS medium. The isolates were analyzed biochemically and serologically at Muhimbili University of Health and Allied Sciences, Tanzania. For quantitative analysis, 0.5 ml of water samples were inoculated on TCBS agar media using plate spread technique and incubated at 37°C for 24 hrs (Choopun et al. 2002, Alarm et al. 2006). The resulting 1 – 3 mm yellow colonies were counted as presumptive colony forming units (cfu) of *V. cholerae* (Choopun et al. 2002).

Biochemical and serological identification of *V. cholerae* isolates

Sucrose-positive yellow colonies of 1–3 mm diameter on TCBS agar from both water and plankton samples were sub-cultured on nutrient agar (NA) medium at 37°C for 24 hours and subjected to oxidase test for preliminary identification of *V. cholerae*, as described by Isenberg (2004). Oxidase positive (dark purple color within 10 seconds) isolates were subjected to Analytical Profile Index (API) tests to confirm the presence of *V. cholerae*. The tests were done using API 20 E kit (BioMérieux, USA), which consists of twenty biochemical tests noted especially by the color reactions for amino acid decarboxylations (Arginine Dihydrolase through Ornithine Decarboxylase) and carbohydrate fermentations (Glucose through Arabinose). Confirmed *V. cholerae* isolates were subjected to polyvalent *V. cholerae* serogroup O1 and polyvalent *V. cholerae* serogroup O139 agglutinating antisera, respectively, to identify the presence of *V. cholerae* serogroups O1 and O139. *V. cholerae* serogroup O1, were further analyzed for serotype Ogawa and Inaba using specific agglutinating antisera tests as described by Furniss and Donovan (1974).

Screening of Clinical Isolates of *V. cholerae*

The source of clinical isolates of *V. cholerae* O1 used in this study were from Microbiology Laboratory of Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania. These were collected from 12 cholera patients (admitted between August 2009 and December 2011) in Korogwe District Hospital in Tanga (8) as well as Mburahati and Buguruni Health Centers in Dar es Salaam Region (4). The clinical isolates were sub-cultured in TCBS and treated in the same way as the environmental isolates.

Statistical analyses

Data is presented as mean values \pm Standard Deviations and compared among sites and stations by using Kruskal Wallis (KW) followed by the post hoc Dunn's Multiple Comparison Test due to the fact that data was not normally distributed. Correlations were performed using the Spearman's Rank Correlation Test. All analyses were done using GraphPad Instant tm 1990–1993 software (GraphPad Software Inc. San Diego, USA). A P-value of ≤ 0.05 was considered statistically significant.

Ethical consideration

Ethical clearance (NIMR/HQ/R.8a/Vol.IX/1155) for the current study was obtained from the National Institute for Medical Research (NIMR) of the Ministry of Health and Social Welfare.

RESULTS

Environmental parameters

Mean values for environmental parameters

are presented in Table 2. As expected, salinity and conductivity values tended to increase from the fresh water to marine water Stations. Salinity ranged from an annual average value of 1.58 ± 2.73 recorded at Station 1 in Ruvu Estuary to the highest mean value of 33.6 ± 2.42 at Station 3 in Mzinga Creek. Conductivity was lowest with an annual average value of 3.67 ± 4.08 mS/cm at Station 1 in Ruvu Estuary to a highest value of 44.9 ± 2.03 mS/cm at Station 3. Water temperature on the other hand did not show a clear trend among the sampling stations with an average value of 28.3 ± 2.15 °C at Station 1 in Mzinga Creek and the highest mean value of 30.2 ± 3.10 °C at Station 2 in Mzinga Creek. pH values generally increased as one moves from Freshwater Station to the Marine waters ranging from an annual average value of 7.76 ± 0.46 at Station 2 in Mzinga Creek to 8.14 ± 0.27 at Station 3 in Pangani Estuary. The amount of Dissolved Oxygen in the water column did not show a clear trend among the sampling stations and ranged from the lowest annual mean value of 5.20 ± 1.19 mg/l at Station 3 in Mzinga Creek to 6.83 ± 2.37 mg/l at Station 2 at the same Mzinga Creek. Phosphate levels were lowest (0.91 ± 0.88 $\mu\text{mol/l}$ at Station 2 in Mzinga Creek to the highest value of 3.50 ± 1.21 $\mu\text{mol/l}$ at Station 1 in Pangani Estuary. Nitrate concentration was lowest (2.31 ± 2.07 $\mu\text{mol/l}$) at Station 3 in Ruvu Estuary and highest (9.74 ± 5.02 $\mu\text{mol/l}$) at Station 2 in Mzinga Creek. The amount of ammonia in the water column ranged between lowest annual mean value of 0.56 ± 0.34 $\mu\text{mol/l}$ at Station 2 in Mzinga Creek to a highest value of 1.46 ± 1.27 $\mu\text{mol/l}$ at Station 1 in Pangani Estuary.

Table 2: Annual mean values (\pm Standard Deviations) of the environmental parameters and nutrients (Phosphate, Nitrates and Ammonium), in the Study Sites and Study Stations

Parameter	Mzinga Creek		Ruvu Estuary			Pangani Estuary		
	ST2	ST 3	ST1	ST2	ST3	ST1	ST2	ST3
Salinity	24.0 \pm 6.94	33.6 \pm 2.42	1.58 \pm 2.73	13.2 \pm 9.75	32.9 \pm 2.66	4.44 \pm 4.84	14.7 \pm 8.04	31.3 \pm 4.27
Conductivity	33.3 \pm 13.5	43.2 \pm 4.22	3.67 \pm 4.08	18.1 \pm 13.2	44.9 \pm 2.03	8.31 \pm 6.62	24.5 \pm 8.26	43.5 \pm 1.11
Temperature	30.2 \pm 3.10	29.3 \pm 2.08	28.3 \pm 2.15	28.5 \pm 1.85	28.7 \pm 1.69	29.0 \pm 1.96	29.2 \pm 1.99	28.7 \pm 1.82
pH	7.76 \pm 0.46	8.07 \pm 0.29	7.48 \pm 0.43	7.81 \pm 0.47	8.21 \pm 0.23	8.01 \pm 0.37	7.99 \pm 0.34	8.14 \pm 0.27
DO	6.83 \pm 2.37	5.20 \pm 1.19	6.56 \pm 1.19	6.32 \pm 1.21	5.78 \pm 1.42	5.97 \pm 0.84	6.65 \pm 1.41	6.29 \pm 1.49
Phosphate	0.91 \pm 0.88	1.00 \pm 0.85	2.72 \pm 1.93	1.45 \pm 0.05	1.00 \pm 1.06	3.50 \pm 1.21	2.03 \pm 1.13	1.18 \pm 1.40
Nitrate	9.74 \pm 5.02	4.03 \pm 2.42	8.75 \pm 1.88	6.79 \pm 4.44	2.31 \pm 2.07	5.81 \pm 3.18	5.05 \pm 3.60	1.37 \pm 0.87
Ammonium	0.56 \pm 0.34	1.17 \pm 1.01	1.38 \pm 1.17	0.67 \pm 0.38	0.55 \pm 0.31	1.46 \pm 1.27	0.62 \pm 0.39	0.54 \pm 0.37

Number of presumptive *V. cholerae* colonies

The total number of presumptive *V. cholerae* colonies in water samples is presented in Figure 1. In Pangani Estuary, the number of presumptive *V. cholerae* counts ranged from 14 cfu/ml as recorded at Station 1 and Station 3 in October 2009 to 532 cfu/ml at Station 1 in March 2010 (Figure 1A). Ruvu Estuary showed the lowest number (4 cfu/ml) at Station 2 in March 2010 to the highest value (206 cfu/ml) recorded in April 2010 at Station 2 (Fig. 1B). Mzinga Creek had the lowest and the highest values (14 cfu/ml and 262 cfu/ml) at Station 3 in December 2009 and January 2010 respectively (Figure 1C). Pangani site showed the highest mean value of 123.9 \pm 152.1 cfu/ml followed by Mzinga Creek (112.0 \pm 77.94) and Ruvu Estuary (44.96 \pm 47.89). There was a significant difference in the number of presumptive *V. cholerae* counts among the sampling sites (KW = 13.605, p = 0.0011) with Dunn's multiple comparison test indicating the differences to be between Pangani and Ruvu Estuaries (p < 0.01) as well as between

Mzinga Creek and Ruvu Estuary (p < 0.01) but not between Pangani and Mzinga Creek (p > 0.05). The mean values for number of presumptive *V. cholerae* was highest at Station 3 (97.41 \pm 134.2) followed by Station 2 (88.52 \pm 73.77) and Station 1 (86.33 \pm 120.5). However, there was no significant difference in number of presumptive *V. cholerae* colony counts among the sampling stations (KW = 0.4523, P = 0.7976). There was a significant positive correlation between the numbers of presumptive *V. cholerae* colonies with the amount of dissolved oxygen in the water column (r = 0.2378, p = 0.044). However, there was no significant correlation between the numbers of presumptive *V. cholerae* colonies with all other environmental variables tested in this study (r = 0.2179, p = 0.6041; r = 0.255, p = 0.5578; r = 0.4901, p = 0.2176; r = 0.2227, p = 0.5961; r = -0.0434, p = 0.9187; r = -0.1229, p = 0.7719; r = 0.1429, p = 0.7520 for salinity, conductivity, temperature, pH, Phosphate, Nitrate and Ammonia, respectively).

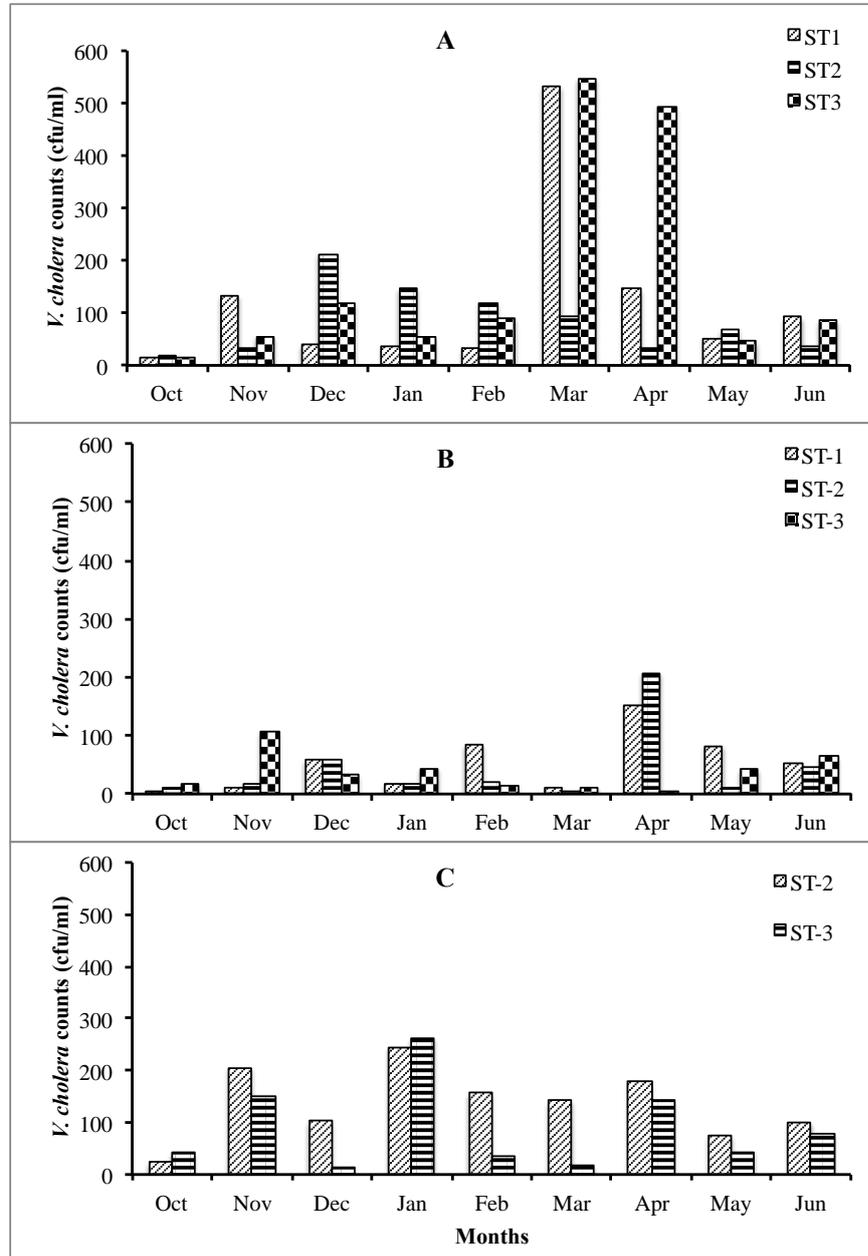


Figure 1: Total number of *V. cholerae* like colony counts in water samples collected from the various sampling stations at Pangani Estuary (A), Ruvu Estuary (B) and Mzingia Creek (C)

Pure culture of environmental and Clinical Isolates *V. cholerae*

A total of four hundred and sixteen (416) of

presumptive *V. cholerae* colonies were isolated on TCBS agar to pure culture from water and composite plankton samples. Out

of these, only 137 (33%) isolates were oxidase test positive of which 36 isolates were from fresh water, 47 from brackish and 54 from marine water. Of the 137 oxidase positive isolates, 96 isolates (about 23% of all *V. cholerae* like colonies on TCBS) were biochemically confirmed using API 20E kit as *V. cholerae* (Table 3). Out of the 96 API 20E positive, 26 were from fresh water Stations, 31 from brackish and 39 from the marine water.

Results from antisera agglutinations showed that, out of the 96 API 20E positive *V. cholerae* isolates, only 8 (8.3%) isolates were identified to be *V. cholerae* serogroup O1 whereby 2 of these isolates agglutinated to mono-valent antiserum as serotype Ogawa. One of these isolate originated from marine water samples in Ruvu Estuary while the other was from brackish plankton sample

from Mzinga Creek. An additional isolate of the *V. cholerae* serogroup O1 from marine plankton sample from Ruvu Estuary agglutinated to Inaba antiserum while the rest did not agglutinate to either of these two mono-valent antisera. One isolate, from marine water environment in Ruvu Estuary agglutinated to poly-valent *V. Cholerae* serogroup O139 antiserum.

Twelve clinical isolates on TCBS agar showed characteristic colonies suggestive of *V. cholerae* and were all oxidase and API 20E positive. Seven of these (58.33%) were serologically confirmed to be *V. cholerae* O1 serotype Ogawa. The remaining 5 (41.67%) isolates did not agglutinate with either O1 or O139 serogroups and therefore considered to be none-O1/O139 (Table 3).

Table 3: Number of environmental and clinical presumptive *V. cholerae* isolates that showed positive results against oxidase, API 20E and serological tests.

Sample identity	Station	Sample type	Oxidase Test	API 20	Type of serological test			Non-O1/O139
					Serogroup O1	Serotype (Ogawa/Inaba)	Serogroup O139	
Environmental samples								
Pangani	ST 1	Water	9	7	0	-	0	7
		Plankton	18	13	0	-	0	13
	ST 2	Water	2	1	0	-	0	1
		Plankton	14	11	0	-	0	11
	ST 3	Water	5	4	1	0	0	3
		Plankton	10	7	1	0	0	6
Ruvu	ST 1	Water	2	2	0	-	0	2
		Plankton	7	4	0	-	0	4
	ST 2	Water	2	2	0	-	0	2
		Plankton	9	4	0	-	0	4
	ST 3	Water	5	4	1	1 Ogawa	1	2
		Plankton	10	5	1	1 Inaba	0	4
Mzinga	ST 2	Water	7	4	0	0	0	4
		Plankton	13	9	2	1 Ogawa	0	7
	S 3	Water	5	4	0	-	0	4
		Plankton	19	15	2	0	0	13
Total			137	96	8	3	1	87
Clinical Samples								

DSM	-	-	4	4	3	3 Ogawa	0	1
Tanga	-	-	8	8	4	4 Ogawa	0	4
Total			12	12	7	7	0	5

- = not tested; DSM = Dar es Salaam

DISCUSSION

Our culture-based analysis indicated that the numbers of presumptive *V. cholerae* colonies in the water along the coast of Tanzania are comparable to those reported for coastal areas of Bangladesh (Haque 2011). Significantly higher number of presumptive *V. cholerae* was found at Pangani Estuary and Mzinga Creek compared to Ruvu Estuary. This may be attributed to the fact that Pangani Estuary and Mzinga Creek are much close to human settlements compared to Ruvu Estuary where the closest town of Bagamoyo is some 5 km away. Although there was no significant difference in the number of presumptive *V. cholerae* among the sampling stations, a general increase towards Station 3 (marine waters) was noted. According to Tamplin et al. (1990), the bacterium, *V. cholerae* is a halophilic organism, which requires elevated pH and NaCl for optimal growth. However, growth requirements for the bacterium based on culture-based characterization should be cautiously interpreted, as requirements in nature may be quite different.

The fact that only 23% of the TCBS isolates were confirmed API 20E test to be *V. cholerae* strains could be due to failure of the TCBS technique to select only for *V. cholerae*. Due to failure of TCBS isolation technique to select *V. cholerae*, the obtained number may be considered a fraction of existing total *V. cholerae*. Several literatures have indicated that over 99% of the environmental bacteria are non-culturable or difficult to culture even though are viable (Rappe and Giovannoni 2003, Binsztein et al. 2004, Imamura et al. 2015). Moreover, *V. cholerae* persists in aquatic environments predominantly in a non-culturable state, a

situation known as the viable-but-non-culturable state (Alam et al. 2007). Furthermore, during the dormant stage when the reproduction condition is not suitable, the populations of *V. cholerae* sink and survive in the silt at the bottom of the estuary, appearing completely absent from plankton populations and waters until conditions are suitable for reproductive success (Colwell et al. 1996).

To the best of our knowledge this is the first quantitative study on *V. cholerae* in Tanzanian coastal waters confirming the occurrence of similar strain in environmental and clinical samples using culture techniques. The occurrence of *V. cholerae* in water samples and clinical isolates in Tanzania corresponds to a study by Rivera et al. (2001) of which clinical and environmental isolates were obtained from Brazil, Peru, Chile, Mexico, India and United States of America as well as Lipp et al. (2003) who collected environmental samples in Peruvian waters and Alam et al. (2006) in Bangladesh. Since *V. cholerae* O1 and O139 serogroups have major epidemiologic roles in cholera, this necessitated the importance of this study in investigating whether *V. cholerae* present in the Tanzanian coastal environment belong to either of these pathogenic groups. The results of this study revealed the occurrence of culturable strains of pathogenic *V. cholerae* O1 and O139 in the coastal environments. This suggests that the source of the cholera outbreak from these patients could be from the natural water bodies. The results mirror other studies that have recovered serogroup O1 in environmental samples and patients (Acosta et al. 2001, Naha et al. 2013) as compared to serogroup

O139. Serogroup O1 is known to be responsible for the 7th (1961 to 1975) pandemic (Nandi et al. 2000, Fenget al. 2008, Ramamurthy and Nair 2010) and is commonly reported in clinical studies of cholera endemic outbreaks in East Africa (Feikin et al. 2010).

The current study confirms the occurrence of toxigenic *V. cholerae* O139, which was reported for the first time in the marine environments of Tanzania by Dalusi et al. 2015. This serogroup has rarely been reported for environmental samples and in Africa. Another report on environmental *V. cholerae* O139 is that by du-Preezet al. (2010) who conducted their study in an estuarine of Beira, Mozambique. According to the authors, the possibility of a *V. cholerae* O139 outbreaks are seldom considered in Africa. This however suggests that if the *V. Cholerae* O139 would cause infections, a subsequent consequence could be difficulties in preparedness due to inexperience in detecting the causative agent, which would lead to difficulties in management and disease control. We therefore recommend that the Ministry of Health of Tanzania be aware of the existence of this serogroup in estuarine environment and to take precautions for possible cholera outbreak caused by *V. cholerae* O139.

Serological analysis indicated that two out of the eight environmentally isolated strains of serogroup O1 belonged to serotype *Ogawa* and one to serotype *Inaba*, while all seven clinical isolates belonged to serotype *Ogawa*. This observation suggests that the remaining majority of the serotypes in environmental *V. cholerae* serogroup O1 in the coastal waters are neither *Ogawa* nor *Inaba* but may belong to alternative serotypes such as *Hikojima* (Ojha 2013), which was not analyzed in the current study. Similar result were obtained by Acosta et al. (2001), where the authors isolated *V. cholerae* strains from stool specimens and

river water samples that were all *Ogawa*, indicating the importance of this serotype in Tanzania. Other studies elsewhere have shown the two serotypes to occur sporadically at different cholera outbreaks (Pal et al. 2006). *V. cholerae* O1 strains are known to be interconvert and to switch between the two known serotypes *Ogawa* and *Inaba* (Garget al. 2000). Serotype switching may be related to immune pressure on the prevailing serotype, as it has been shown that these serotypes are not uniformly sensitive to antibiotics (Pal et al. 2006).

The majority of *V. cholerae* in the current study were found to be from non-O1/O139 serogroups. Such results have also been reported elsewhere (Chakraborty et al. 2000, Nandi et al. 2000). Importantly, many reports suggest that *V. cholerae* non-O1/O139 serogroups with cholera toxin gene (*ctxA*) may be the main causative agents of cholera-like diseases (e.g. Bagchi et al. 1993, Dalsgaard et al. 1995, Chakraborty et al. 2000, Nandi et al. 2000). However, some studies e.g. Dutta et al. (2013) have shown that *V. cholerae* non O1/O139 serogroups without the cholera toxin (*ctxA*) genes can also cause cholera-like diarrhoea. These results suggest that the presence of all serotypes of *V. cholerae* in aquatic environments should be given careful attention, as they may potentially become virulent. Further epidemiologic studies are recommended to determine the ecology, virulence factors, and public health role of the recovered *V. cholera* non O1 and O139 serogroups in the area.

In conclusion, results of this study showed that, pathogenic *V. cholerae* colonizes a wide range of natural aquatic environments as they were isolated from water and plankton samples in the marine, brackish and riverine waters. The presence of the pathogenic *V. cholerae* serogroup O1 and O139 as well as non-O1/O139 from the natural environment

and in cholera patients suggest a close link between the coastal waters and cholera epidemics in the area. The current findings call for more investigation over period to establish the persistence of the different serotypes in the Western Indian Ocean aquatic environments and in patients during cholera outbreaks. Furthermore, studies on antibiotic sensitivity of different serotypes are also very important so as to get prepared in case of outbreak of uncommon *V. cholerae* strains.

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REFERENCES

- Acosta CJ, Galindo CM, Kimario J, Senkro K, Urassa H, Casals C, Corachán M, Eseko N, Tanner M, Mshinda H, Lwilla F, Vila Jand 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. and Colwell RR 2006 Toxigenic *Vibrio cholerae* in the aquatic environment of Mathbaria. Bangladesh. *Appl. Environ. Microb.* **72**: 2849–2855.
- Alam M, Sultana M, Nair GB, Siddique AK, Hassan NA, Sack, RB, Sack DA, Ahmed KU, Sadique A, Watanabe H, Grim CJ, Huq A and Colwell RR 2007 Viable but non culturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. *PNAS*, U.S.A. **104**: 17801–7806.
- Bagchi K, Echeverria P, Arthur AD, Sethabutr O, Serichantalergs O and Hoge CW 1993 Epidemic diarrhea caused by *Vibrio cholerae* non-O1 that produced heat-stable toxin among Khmers in a camp in Thailand. *J. Clin. Microbiol.* **31**: 1315–1317.
- Binsztein N, Costagliola MC, Pichel M, et al. 2004 Viable but non culturable *Vibrio cholerae* O1 in the Aquatic Environment of Argentina. *Appl. Environ. Microb.* **70**:7481–7486.
- Broza M, Gancz H, Halpern M and Kashi Y 2005 Adult non-biting midges: possible windborne carriers of *Vibrio cholerae*. *Environ. Microb.* **7**: 576–585.
- Butterton JR and Calderwood SB 1995 *Vibrio cholerae* O1. In: Blaser MJ, Smith PD, Ravdin JI, Greenberg HB and Guerrant RL (Eds.) *Infections of the gastrointestinal tract*. Raven Press, pp. 649-670.
- Chakraborty S, Mukhopadhyay AK, Bhadra RK, Ghosh AN, Mitra R, Shimada T, Yamasaki S, Faruque SM, Takeda Y, Colwell RR and Nair GN 2000 Virulence genes in environmental strains of *Vibrio cholerae*. *Appl. Environ. Microb.* **66**: 4022–4028.
- Chatterjee S, Gosh K, Raychoudhuri A, Chowdhury G, Battacharya MK, Mukhopadhyay AK, Ramamurthy T, Battacharya SK, Klose KE and Nandy RK 2009 Incidence of 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.
- Choopun N, Louis V, Huq A and Colwell R 2002 Simple procedure for Rapid Identification of *Vibrio cholerae* from the Aquatic Environment. *Appl. Environ. Microb.* **68**: 995–998.
- Colwell R, Brayton P and Herrington D 1996 Viable but non-culturable *Vibrio*

- cholerae* O1 revert to a cultivable state in the human intestine. *World J. Microbiol. Biotech.* **12**: 28–31.
- Dalusi L, Lyimo TJ, Lugomela C, Hosea KMM and Sjolting S 2015 Toxigenic *Vibrio cholerae* identified in estuaries of Tanzania using PCR techniques. *FEMS Microbiol. Lett.* **362**(5): 1–8.
- Dalsgaard A, Albert MJ, Taylor DN, Shimada T, Meza R, Serichantalergs O and Echeverria P 1995 Characterization of *Vibrio cholerae* non-O1 serogroups obtained from an outbreak of diarrhea in Lima, Peru. *J. Clin. Microbiol.* **33**: 2715–2722.
- De Magny G, Murtugudde R and Sapiano MR 2008 Environmental signatures associated with cholera epidemics. *PNAS USA*, **105**: 17676–17681.
- du Preez M, Van Der Merwe MR and Cumbana A 2010A survey of *Vibrio cholerae* O1 and O139 in estuarine waters and sedimentsof Beira, Mozambique. *Water SA* **36**: 615–20.
- Dutta D, Chowdhury G, Pazhani GP, Guins S, Dutta S, Gosh S, Rajendran K, Nandy RK, Mukhopadhyay AK, Battacharya MK, Mitra U, Takeda Y, Nair GB and Ramamurthy T 2013 *Vibrio cholerae* Non – O1, Non – O139, serogroups and cholera-like diarrhea, Kolkata, India. *Emerg. Infect. Dis.* **19**(3): 464–467.
- Emch M, Feldacker C, Islam MS and Ali M 2008 Seasonality of cholera from 1974 to 2005: a review of global patterns. *Int. J. Health Geogr.* **7**(31): 1–13.
- Faruque SM, Chowdhury N, Kamruzzaman M, Dziekman M, Rahman MH, Sack DA, Nair GB and Mekalanos JJ 2004 Genetic diversity and virulence potential of environmental *Vibrio cholerae* population in a cholera-endemic area. *PNAS, U.S.A.* **101**: 2123–2128.
- Feikin DR, Tabu CW and Gichuki J 2010 Short report: Does water hyacinth on East African lakes promote cholera outbreaks? *Am. J. Trop. Med. Hyg.* **83**: 370–3.
- Feng L, Reeves PR, Lan R, Ren Y, Gao C, Zhou Z, Ren Y, Cheng J, Wang W, Wang J, Qian W, Li D and Wang L 2008A recalibrated molecular clock and independent origins for the cholera pandemic clones. *PLoS One* **3**: 1–11.
- Fernandez M, Bauernfeind A, Jimenez JD, Gil CL, El Omeiri N and Guibert DH 2009 Influence of temperature and rainfall on the evolution of cholera epidemics in Lusaka, Zambia, 2003–2006: analysis of a time series. *Trans. RoyalSoc. Trop.Med. Hyg.* **103**: 137–143.
- Filho JEM, Lopes RM, Rivera ING and Colwell RR 2011 *Vibrio cholerae* O1 detection in estuarine and coastal zooplankton. *J. Plankt. Res.* **33**: 51–62.
- Furniss AL and Donovan TJ 1974 Technical Method: The Isolation and Identification of *Vibrio cholerae*. *J. Clin. Pathol.* **27**: 764–766.
- Garg P, Chakraborty S, Basu I, Datta S, Rajendran K, Bhattacharya T, Yamasaki S, Bhattacharya SK, Takeda Y, Nair GB and Ramamurthy T 2000 Expanding multiple antibiotic resistance among clinical strains of *Vibrio cholerae* isolated from 1992–7 in Calcutta, India. *Epidemiol. Infect.* **124**: 393–399.
- Griffith DC, Kelly-Hope LA and Miller MA 2006 Review of reported cholera Outbreaks Worldwide, 1995–1998. *Am. J. Trop. Med. Hyg.* **75**(5): 973–977.
- Haque M 2011 Seasonal prevalence of cholera pathogen (*Vibrio cholerae*) in water and plankton samples in the southern coastal part of Bangladesh. *Ind. J. Sci. Techn.* **4**(9): 1101–1104.
- Huq A, Sack RB, Colwell R 2001 Cholera and global ecosystems. In: *Ecosystem Change and Public Health - A Global Perspective* 2001. The John Hopkins University Press, Baltimore: pp 327–352.
- Igbinosa EO and Okoh AI 2008 Emerging *Vibrio* species: an unending threat to public health in developing countries. *Res. Microbiol.* **159**: 495–506.
- Imamura D, Mizuno T, Miyoshi S and

- Shinoda S 2015 Stepwise changes in viable but non culturable *Vibrio cholerae* cells. *Microbiol. Immunol.* **59**: 305–310.
- Isenberg HD 2004 *Clinical Microbiology Procedures Handbook*. 2nd Edition. America Society for Microbiology Press. Washington DC. ISBN: 1-55581-243-0, 2298 pp.
- Islam, M.J. and Sack, R.B. 1994. Probable role of blue-green algae in maintaining endemicity and seasonality of cholera in Bangladesh: a hypothesis. *J. Diarrhoeal. Dis. Res.* **12**: 245–256.
- Karl DM 2007 Microbial oceanography: paradigm, processes and promise. *Nat. Rev. Microbiol.* **5**: 759-769.
- Karunasagar IRI, Joseph B and Kennedy B 2003 OmpU genes in non toxigenic *Vibrio cholerae* associated with aquaculture. *J. Appl. Microbiol.* **95**: 338–43.
- Kieriec KWP 2003 The *Vibrio cholerae* O139 O-antigen polysaccharide is essential for Ca²-dependent biofilm development in sea water. *PNAS USA*, **100**: 14357–14362.
- Lipp EK and Huq A, Colwell RR 2002 Effects of global climate on infectious disease: The cholera model. *Clin. Microbiol. Rev.* **15**: 757–770.
- Lipp EK, Rivera ING, Gil AI, Espeland EM, Choopun N, Louis VR, Cohen ER, Huq A and Colwell RR 2003 Direct detection of *Vibrio cholerae* and ctxA in Peruvian coastal water and plankton by PCR. *Appl. Environ. Microb.* **69**(6): 3676–3680.
- Lugomela C, Lyimo TJ, Namkinga LA, Moyo S, Goericke R and Sjöling S 2014 Co-variation of Cholera with Climatic and Environmental Parameters in Coastal Regions of Tanzania. *Western Indian Ocean J. Mar. Sci.* **13**(1): 93 – 105.
- Mhalu FS, Mtango FD and Msengi AE 1984 Hospital outbreaks of cholera transmitted through close person-to-person contact. *Lancet* **2**: 82-84.
- Mhalu FS, Mtenga WM and Mtango FD 1987. Seasonality of cholera in Tanzania: possible role of rainfall in disease transmission. *East Afr. Med. J.* **64**: 378-87.
- Ministry of Health; Health Information and Research Section 1997. Health statistics abstracts. Tanzania: Ministry of Health.
- Mpazi VM and Mnyika KS 2005 Knowledge, attitude and practices regarding Cholera outbreaks in Ilala municipality of Dar Es Salaam region, Tanzania. *East Afr. J. Pub. Health* **2**: 6–12.
- Naha A, Chowdhury G, Ghosh-Banerjee J, Senoh M, Takahashi T, Ley B, Thriemer K, Deen J, Seidlein LV, Ali SM, Khatib A, Ramamurthy T, Nandy RK, Nair GB, Takeda Y and Mukhopadhyay AK 2013. Molecular characterization of high-level-cholera-toxin-producing El Tor variant *Vibrio cholerae* strains in the Zanzibar Archipelago of Tanzania. *J. Clin. Pathol.* **51**: 1040–5.
- Nair GB, Shimada T, Kurazono H, Okuda J, Pal A, Karasawa T, Mihara T, Yoshihiko Uesaka Y, Shirai H, Garg S, Kumar P, Mukhopadhyay SAK, Ohashi T, Tada J, Nakayama T, Fukushima S, Takeda T and Takeda Y 1994 Characterization of phenotypic, serological, and toxigenic traits of *Vibrio cholerae* O139 Bengal. *J. Clin. Microbiol.* **32**: 2775–2779.
- Nandi B, Nandi RK, Mukhopadhyay GB, Nair GB, Shimada T and Ghose AC 2000 Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the outer membrane protein OmpW. *J. Clin. Pathol.* **38**: 4145–4154.
- Narra R, Maeda JM, Temba H, et al. 2017 Notes from the Field. Ongoing Cholera Epidemic — Tanzania, 2015–2016. *MMWR Morb. Mortal. Wkly. Rep.* **66**:177–178.
- Nelson EJ, Morris JG, Calderwood SB and Camili A 2009 Cholera transmission: the host, pathogen and bacteriophage dynamic. *Nat. Rev. Microbiol.* **7**: 693–702.

- Ojha AK 2013 Study of *Vibrio cholerae*, its types Ogawa, Inaba serotypes & E. coli antimicrobial susceptibility in Delhi Region, M.V.I.D. Hospital. *Biolife* **1(4)**: 246–250.
- Olago D, Marshall M, Wandiga SO, et al. 2007 Climatic, Socio-economic and Health Factors Affecting Human Vulnerability to Cholera in the Lake Victoria Basin, East Africa. *Ambio*, **36**: 350–358.
- Pal BB, Khumtia HK, Samal SK, Das SS and Chotray GP 2006 Emergence of *Vibrio cholerae* O1 Biotype El Tor Serotype Inaba Causing outbreaks of cholera in Orissa, India. *Jap. J. Infect. Dis.* **59**: 266-269.
- Parsons TR, Maita Y and Lalli M 1984 *A manual of chemical and biological methods for seawater analysis*. Pergamon Press, Oxford. 173pp.
- Paz S 2009 Impact of temperature variability on cholera incidence in Southeastern Africa, 1971–2006. *Eco. Health*, **6**: 340–345.
- Paz S and Broza M 2007 Wind direction and its linkage with *Vibrio cholerae* dissemination. *Environ. Health Perspect.* **115(2)**: 195–200.
- Peterson K 2002 Expression of *Vibrio cholerae* virulence genes in response to environmental signals. *Curr. Iss. Intest. Microbiol.* **3**: 29–38.
- Rappe Mand Giovannoni S 2003 The uncultured microbial majority. *Ann. Rev. Microbiol.* **57**: 369–39.
- Rivera IG, Chun J, Huq A, Sack RB and Colwell RR 2001 Genotypes associated with virulence in environmental isolates of *Vibrio cholerae*. *Appl. Environ. Microb.* **67(6)**: 2421–2429.
- Ramamurthy T and Nair GB 2010 Evolving identity of epidemic *Vibrio cholerae*: past and the present. *Sci Cult.* **76**: 5–6.
- Sack DA, Sack BR, Nair BG and Siddique AK 2004 Cholera. *The Lancet* **363**: 223–233.
- Shapiro RL, Otieno MR, Adcock PM, Phillips-Howard PA, Hawley WA, Kumar L, Waiyaki P, Nahlen BL and Slutsker L 1999 Transmission of epidemic *Vibrio cholerae* in rural western Kenya associated with drinking water from Lake Victoria: an environmental reservoir for cholera. *Am. J. Trop. Med. Hyg.* **60**: 271–276.
- Tamplin ML, Gauzens AL, Huq A, Sack DA and Colwell RR 1990 Attachment of *Vibrio cholerae* O1 to Zooplankton and Phytoplankton of Bangladesh water. *Appl. Environ. Microb.* **56**: 1977–1980.
- Tobin-D'angelo M, Smith AR, Bulens SN, Thomas, S, Hodel M, Izumiya E, Morita M, Watanabe H, Marin C, Parsons MB, Greene K, Cooper K, Haydel D, Bopp C, Yu P and 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**: 1035–40.
- Traerup SLM, Ortiz RA and Markandya A 2011 The costs of climate change: A case study of cholera in Tanzania. *Int. J. Environ. Res. Public Health.* **8**: 4386–4405.
- Uchiyama H 2000 Distribution of *Vibrio* species isolated from Aquatic Environments with TCBS. *Environ. Health Prev. Med.* **4**: 199-204.
- Vezzulli LP, Huq CA and Colwell RR 2010 Environmental reservoirs of *Vibrio cholerae* and their role in cholera. *Environ. Microbiol. Rep.* **2**: 27–33.
- World Health Organisation (WHO) 1998 Cholera in 1997. *Weekly Epid. Rec.* **73**: 201–208.