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Atypical and dual biotypes variant of virulent SA-NAG-*Vibrio cholerae*: an evidence of emerging/evolving patho-significant strain in municipal domestic water sources

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Abstract

Introduction and purpose: The recent cholera spread, new cases, and fatality continue to arouse concern in public health systems; however, interventions on control is at its peak yet statistics show continuous report. This study characterized atypical and patho-significant environmental *Vibrio cholerae* retrieved from ground/surface/domestic water in rural-urban-sub-urban locations of Amathole District municipality and Chris Hani District municipality, Eastern Cape Province, South Africa.

Methods: Domestic/surface water was sampled and 759 presumptive *V. cholerae* isolates were retrieved using standard microbiological methods. Virulence phenotypic test: toxin co-regulated pili (tcp), cholera toxin, protease production, lecithinase production, and lipase test were conducted. Serotyping using polyvalent antisera (Bengal and Ogawa/Inaba/Hikojima) and molecular typing: 16SrRNA, OmpW, serogroup (Vc-O1/O139), biotype (*tcpAClas/El Tor*, *HlyAClas/El Tor*, *rstRClas/El Tor*, *RS1*, *rtxA*, *rtxC*), and virulence (*ctxA*, *ctxB*, *zot*, *ace*, *cep*, *prt*, *toxR*, *hlyA*) genes were targeted.

Result: Result of 16SrRNA typing confirmed 508 (66.9%) while OmpW detected/confirmed 61 (12.01%) *V. cholerae* strains. Phenotypic-biotyping scheme showed positive test to polymyxin B (68.9%), Voges proskauer (6.6%), and Bengal serology (11.5%). Whereas Vc-O1/O139 was negative, yet two of the isolates harbored the cholera toxin with a gene-type *ctxB* and *hlyAClas*: 2/61, revealing atypical/unusual/dual biotype phenotypic/genotypic features. Other potential atypical genotypes detected include *rstR*: 7/61, *Cep*: 15/61, *ace*: 20/61, *hlyAElTor*: 53/61, *rtxA*: 30/61, *rtxC*: 11/61, and *prtV*: 15/61 respectively.

Conclusion: Although additional patho-significant/virulent genotypes associated with epidemic/sporadic cholera cases were detected, an advanced, bioinformatics, and post-molecular evaluation is necessary. Such stride possesses potential to adequately minimize future cholera cases associated with dynamic/atypical environmental *V. cholerae* strains.

Keywords: Cholera, *Vibrio cholerae*, Phenotypes, Genotypes, Patho-significance, Virulence indices, Unusual/atypical biotype

Introduction

Cholera, a disease that is associated with dirt, gutter, unhealthy contact with contaminated material and other unhygienic practices has been affirmed to be associated with the pathogen *Vibrio cholerae*. This organism

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has been classified according to the Bergeys manual of identificative bacteriology among the family Vibrionaceae with more than twelve human and animal pathogenic strains identified. Over the years, there have been reports of sustained implication of the pathogen in acute watery diarrhea, wound infections, otitis media, neonatal meningitis, septicemia, sporadic, and cholera cases (Lu et al. 2014; Marin et al. 2013; Talkington et al. 2011). World Health Organization (WHO) and other investigators in vibriology have affirmed the annual burden of cholera cases to range from 1.3 million to 4.0 million with annual mortality rate ranging from 21,000 to 143,000 worldwide (Ali et al. 2015; WHO facts 2018). Due to their high clinical relevance, they are subdivided into serogroups, serotypes, phage types, and biotype (Momba and Azab El-Liethy 2017). Serogrouping are based on their extracellular polysaccharide into O1, O139, and somatic antigen non-agglutinating *V. cholerae* (SA-NAG-*Vibrio cholerae* or non-O1/non-O139) (Igbinosa et al. 2009). Biotyping is based on their first place and time of isolation which include Classical, El Tor, Bengal, Calcutta (Mandal et al. 2011). However, two biotypes have been globally distributed and implicated in outbreak reported cases (El Tor and Classical *V. cholerae*). Evident in the early studies was the observation of an El Tor biotype which was first isolated from camp quarantined pilgrims of the El Tor City, located at Sinai Peninsula, Egypt in 1905. According to Zaw et al. (2016) and Mandal et al. (2011), biotyping cholera *Vibrio* members possess a major epidemiological relevance in the classification scheme of the family Vibrionaceae especially as the pathogen has shown in history, a remarkable disappearance and re-establishment as well as dominance since 1992 (Mandal et al. 2011; Zaw et al. 2016).

Reports from various *Vibrio* studies have revealed that there exist some new genetic variants of dual biotype with virulent genes suggesting an ongoing evolution as reported by various investigators (Faruque et al. 2007; Goel et al. 2008; Grim et al. 2010; Nair et al. 2006; Zaw et al. 2016). Further results from the study of Ansaruzzaman et al. (2004) in Mozambique, after the cholera outbreak episode in Eastern African countries revealed the presence of an atypical variant of *V. cholerae* with dual biotype and resistant to phage type IV. These reports were affirmed in the studies of Faruque et al. (2007) and Das et al. (2007), who observed two tandem copies of prophage with similar sequence type as *CTX Φ ^{clas}* in the pathogen small chromosome. Other investigators of *Vibrio* have also reported the occurrence of dual phenotype and genotype among pathogenic *V. cholerae* (Ceccarelli et al. 2011; Lee et al. 2009; Nair et al. 2006; Nguyen et al. 2009; Safa et al. 2008; Zaw et al. 2018). Such features are positive and/or negative to chicken red cell

agglutination test, Voges Proskauer test, sensitive, and/or resistance to both polymyxin B and phage V but resistance to phage IV (Safa et al. 2010).

Such strains also anchor their genomic composition on two functional but distinct gene clusters, namely, the satellite phage (*RS1*) and *CTX-phi* (*CTX Φ*) phage (Castillo et al. 2018; Heidelberg et al. 2000). The classical biotype is shown to possess only the *CTX Φ* , whereas the El Tor biotype possesses both the satellite phage (*RS1*) and *CTX Φ* where *RS1* is located adjacent to *CTX Φ* as depicted in Fig. 1A, B below (Castillo et al. 2018; Heidelberg et al. 2000; Safa et al. 2010). Present as gene content of *CTX Φ* are other two distinct clusters referred to as *RS2* and core. The *RS2* is also a satellite phage-like particle as the *RS1*; however, it is similar in genetic content as the *RS1* except that it lacks the anti-repressor protein which promotes transcription of *CTX Φ* during the generation and gene expression of infectious particles (*rstC*) (Banerjee et al. 2014; Safa et al. 2010; Waldor et al. 1997). The core consists of accessory genes needed for secretion and assembling of infectious particles such as CT (*ctxA*, *ctxB*), *psh*, *cep*, *g^{II}ICTX*, *Ace*, and *Zot* (Fig. 1A). The satellite phage (*RS1*) consists of *rstA* gene which is responsible for replication of infectious particles, *rstB*, which is necessary for integration of viral particles while *rstR* and *rstC* are necessary for regulation of site specific recombination and gene expression of viral particle, although the *rstC* is an anti-repressing protein (Rezaie et al. 2017; Banerjee et al. 2014).

It is important to note that the *ctx B* gene is present in both the wild-type *CTX-phi* and the atypical genome. Although the *ctx B* gene differs in its amino acid sequences, the *CTX-phi* gene of the altered/atypical has a notable genome similarity with the Classical *CTX-phi* genes except for the difference which occurred at *rstR*. The difference in *ctxB* genotypes is associated with the global distribution and the wave of evolution over a period of time (Castillo et al. 2018; Banerjee et al. 2014; Safa et al. 2010; Heidelberg et al. 2000). The gene types found in atypical or altered strains of the pathogen are mainly of *RS1* and *CTX Φ* (*RS2* and core) origins which include *rstA*, *rstR*, *rstB*, *rstC*, and *CTX Φ* (*cep*, *ace*, *zot*, *rstA*, *rstB*, *rstR*, *psh*, *g^{II}ICTX*, *ctxA*, *ctxB*). Other patho-significant determinants of clinical relevance are *rstR^{cla}*, *rstR^{El Tor}*, *rtxA*, *rtxC*, and other virulent characteristics (Plaza et al. 2018; Kim et al. 2014; Lee et al. 2009; Nguyen et al. 2009; Safa et al. 2006, 2008; Faruque et al. 2007; Das et al. 2007). The inclusion of the RTX toxin is evident as earlier reported by Heidelberg et al. (2000) in a comparative genomics study which shows that located at the far end of the *CTX Φ* is a region encoding an RTX toxin (*rtxA*), and its activator (*rtxC*) with nucleotide

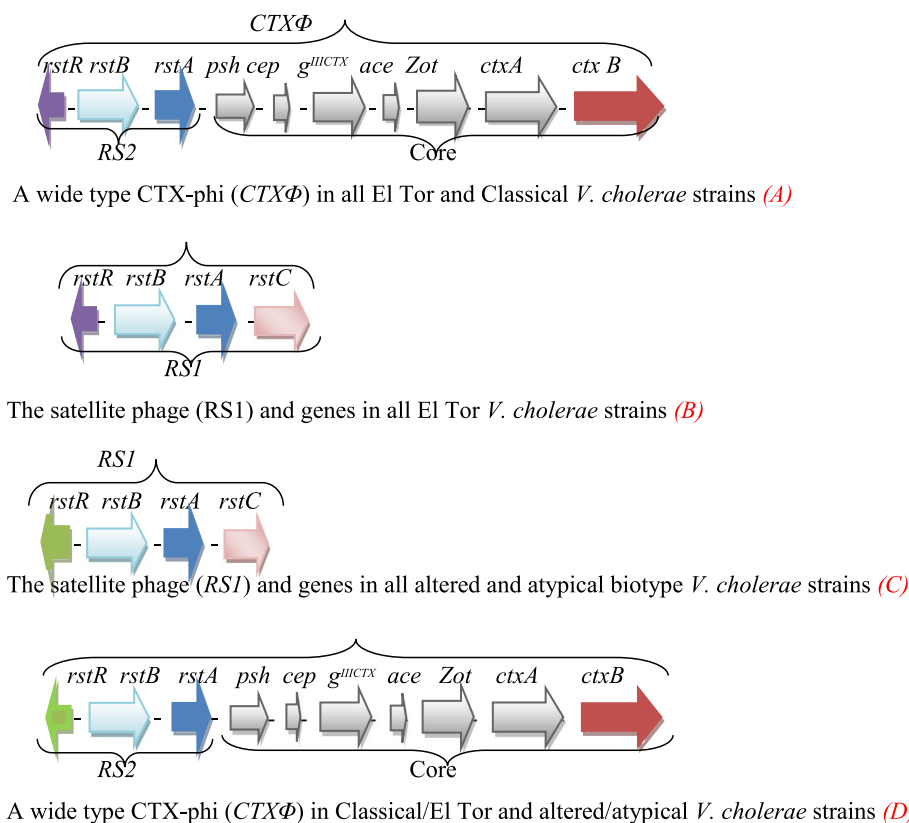


Fig. 1 The genomic composition of the CTX-phi and satellite phage (RS1) in *V. cholerae*. A wide type CTX-phi (CTXΦ) in all El Tor and Classical *V. cholerae* strains (A). The satellite phage (RS1) and genes in all El Tor *V. cholerae* strains (B). The satellite phage (RS1) and genes in all altered and atypical biotype *V. cholerae* strains (C). A wide type CTX-phi (CTXΦ) in Classical/El Tor and altered/atypical *V. cholerae* strains (D)

composition suggesting the RTX region was horizontally acquired along with other sensor histidine kinase/response regulator. It also suggests that the regulators affect the expression of some closely related RTX transcriptional units (Castillo et al. 2018; Plaza et al. 2018; Kim et al. 2014). Atypical strains are rarely reported among environmental SA-NAG-*V. cholerae* strains. In the current study, we confirm atypical/unusual trait/genes both phenotypically and genotypically among environmental SA-NAG-*V. cholerae* or non-O1/Non-O139 *V. cholerae* as evidence of emerging/evolving patho-significant strain in municipal domestic water sources.

Material and methods

Study area and sample collection

One liter of domestic water sources was collected onto sterile Nalgene bottles from each of the twenty sampling points which include the waste water treatment plants, receiving water shed, rivers, irrigation canals, dam, and tap water with geographical coordinates as follows: S 31° 54.548', E 026° 50.715', S 31° 54.548', E 026° 50.715', S

31° 54.548', E 026° 50.715', S 31° 54.548', E 026° 50.715'. These samples were collected from Amathole District municipality and Chris Hani District municipality of Eastern Cape Province, South Africa. The sampled treatment plant receives municipal wastewater from household/domestic usage and release effluents after treatment into receiving water shed, rivers, dams, and irrigation canal. Such released water is used by the rural population for swine husbandry, irrigation of farm lands, drinking, household/domestic activities, and recreational activities. Most irrigated farm lands with sampled water cultivate and harvest large variety of vegetables both local and commercial farm lands in sub-urban and peri-urban area of study municipalities (Queens Town, Alice). Water samples were collected from treated final effluent before discharge, point of discharge for receiving body of water, 500 m downstream, and 500 m upstream of the receiving water discharge point. Samples were collected monthly between February 2018 and August 2018. Specified water samples were collected onto 1 L screw capped glass Nalgene containers, pre-cleaned/washed using non-ionic detergents and rinsed/bathe in smooth running tap

water. Additional and further pre-cleaning includes treatment with 5% concentrate of hydrogen chloride acid and finally with deionized water. Prior to collection of water specimen at various sampling points, 1.7 mL volume of sterile 1% sodium thiosulphate solution was added aseptically to specimen bottles. At the collection site/location, midstream water sampling was done by dipping each sterile-secured sample bottle to approximately 20-30 cm below water surface, projecting the open-mouth of glass bottle or container against water flow direction. Collected samples were thereafter transported in cooler boxes containing ice-packs to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice, for respective physicochemical and microbial analyses within 6 to 8 h after collection.

Physicochemical analysis

The various sample site geographical coordinates were taken, the distance from analysis laboratory taken, the flow of water into municipality observed, and the wastewater treatment plant capacity design taken. The physicochemical parameters was measured using the following: HANNA HI 96711 free/residual and total chlorine meter for chlorine estimation after pre-preparation with the Merck lovibond DPD-1 tablets; HANNA is a multiple parameter waterproof meter made in Romania Europe (www.hannainst.com). Other machines including HACH HQ40D an optimum density meter; HACH Model CO150 conductivity meter; HACH DR 900 a multiple parameter meter HACH Company/Hach Lange GmbH, 2013 Model 210A, Orion Laboratories and Life Science membrane filtration system Model No: DOA – P730 – BN, Patr No: 13158 were used to analyze the various physiological properties of the collected water samples on/out site. The study encompasses sampling within three seasons of study area, (Winter, Summer, and Autumn).

Bacterial strains isolation

Following the previous methods as described by various investigators with few modifications, filtered water samples (vacuum pump/pressure pump: model No. DOA-P730-BN) (www.lasec.co.za) using 0.45- μ m nitrocellulose paper (Merck, Germany, www.merck-chemicals.com) were plated onto pre-prepared Thiosulphate Citrate Bile Salts-Sucrose agar (TCBSA) in triplicate and incubated at 37 °C for 24 h (Huq et al. 2012; Igbinosa et al. 2009; Pfeffer and Oliver 2003; Rivera et al. 2013; Uddin et al. 2018). Presumptive yellow and green *V. cholerae* colonies were sub-cultured onto nutrient agar to ascertain the purity subsequently. Three to five colonies per-plate were stored in aliquot on glycerol stock.

Further cultural morphology, colonial morphology, and biochemical characteristics of representative strains were observed/recorded as suspected or presumptive colonies which were subculture onto TCBSA and subsequently onto nutrient agar (NA) to ascertain/confirm purity. These characteristic tests include Gram reaction, oxidase test, motility test, Voges Proskauer test (VP test), and D-mannitol catabolism, while positive strains were selected for confirmation as *V. cholerae*/non-*Vibrio* strains using serotyping, molecular typing scheme, biotyping serogrouping, and pathotyping as specified by investigators (Beshiru and Igbinosa 2018).

Vibrio numerical density/count

The method as described by Huq et al. (2012) and Rivera et al. (2013) was employed for isolation with few modifications. Each of the samples was separated into plankton free and plankton associated. Plankton-free samples were filtered after a tenfold dilution treatment using 0.45- μ m nitrocellulose filter membrane (Merck filter membrane Germany, www.merck-chemicals.com), filtered membrane were plated in triplicate onto thiosulphate citrate bile salts-sucrose agar (TCBSA) and incubated at 37 °C for 24 h (Pfeffer and Oliver 2003; Baumann et al. 1984). Plankton-associated samples were pre-treated by centrifuging at 5000 rpm for 1 min and 1 ml of concentrated homogenates inoculated onto 9-ml sterile Alkaline peptone water (APW). The prepared plankton associated culture was incubated for 6-8 h and subcultured directly onto thiosulfate citrate bile salt-sucrose agar (TCBSA) and incubating at 37 °C for 24 h (Huq et al. 2012; Alam et al. 2006; WHO 2004; Oliver and Kapper 1997).

Presumptive identification, biochemical reaction, and in vitro virulence test

Pure colonies of presumptive *V. cholerae* isolates were tested using Gram reaction, oxidase reaction, motility and D-mannitol catabolism, Voges-Proskauer, and resistance to polymyxin B. An in vitro presumptive virulence test of isolates was determined using the string test, protease production (protein degrading enzyme test), lipase production (Ryan test or lipid degrading enzyme test), lecithinase production (lecithin degrading enzyme test), and cholera red production (Rivera et al. 2013; Huq et al. 2012; Alam et al. 2006; WHO 2004). Although these determinative tests may not be sufficient for virulence specific identity, they are used as preliminary/presumptive pathotyping procedure for both clinical/environmental *V. cholerae* members. Isolates that reveal positive/negative reactions to these aforementioned multiple tests were selected presumptively for gene-based confirmation as virulent non-agglutinating O1/O139 *V. cholerae* members.

Storage of *V. cholerae* strains

Yellow and green single colony from a fresh culture were purified and stored in aliquot {containing 50% glycerol in 1 ml of sterile Luria Bertani broth on sterile 2 ml graduated skirted-Cryo-preservation tubes (Starlabs, Milton Keynes, UK)}. The tubes were vortexed briefly to ensure thorough mixing of contents and immediately stored frozen at -80°C .

Optimum resuscitation broth (ORB)

The optimum resuscitation broth {nutrient broth (Oxoid CM0733)} was used for preparation of isolate dilutions during water samples collection and isolation of *V. cholerae*. It was prepared following appropriately the steps on manufacturer's instruction manuals. A 9.5 g quantity of nutrient broth powder was dissolved in 1 liter of distilled water and sterilized by autoclaving at 15 psi, 121°C for 15 min. Aliquot was stored aseptically at room temperature prior usage.

Phenotypic biotyping of strains

There are two basic biotypes of *V. cholerae somatic* serogroups, which are Classical and El Tor biotypes. Although a third biotype known as the Bengal biotype appeared as emerging member, it has remained in the Asian continent. The phenotypic biotyping test for isolates was applied using polymyxin B susceptibility, hemolysin test, and Voges-Proskauer test (Forbes et al. 2007).

Polymyxin B (PB) sensitivity

A modified technique as previously described by Han and Khie (1963) was applied. Mueller–Hinton agar plates were inoculated with standardized saline culture of test organism (4 h at 35°C) to obtain confluent growth and then 50 unit PB antibiotic disk was placed on the surface of medium, was inverted, and was incubated overnight at 37°C . Classical biotype *V. cholerae* shows a 12–15 mm zone of inhibition, whereas El Tor Biotype shows complete resistance.

Voges-Proskauer test

A prepared methyl red and VP reagent was added to pure 24 h culture of the presumptive isolates. A deep red coloration of medium as the reagent is added to the culture indicates a positive result.

Bengal biotype using serology

The MAS-AGGL-M15001 Bengal antisera were purchased from the Davis diagnostics {(Pty) Ltd., 141 Oak Avenue, Ferndale, Randburg, 2194, Gauteng, South Africa (www.daviesdiagnostics.co.za)}. A single colony from a pure 18–24 h growth culture plate was smeared on a grease-free slide containing two drops of normal saline.

Two drops of Bengal antisera was added and homogenized following manufacturer instructions. Positive report was recorded on observation of agglutination.

Cholerae red

Alkaline peptone water (APW) [VM 768100 704, Merck KGaA, 64271 Darmstadt, Germany (www.merck-chemicals.com)] was prepared according to manufacturer's instruction. Pure 24 h culture of isolates was inoculated onto prepared broth and incubated for 24 h. Few drops of concentrated H_2SO_4 were added to the 18–24 h broth culture growth of APW. Positive results were considered when the pale yellow color of APW is turned to pink or pale red.

Molecular typing methods for confirmation

DNA extraction

The boiling method as previously described by Maugeri et al. (2006) was applied. A single colony of the overnight pure culture of presumptive isolate was harvested and/or suspended in ice cold nuclease free 0.5 molar TE {Tris ethylene diamine tetracetate (EDTA)} in a 1.5-mL microfuge tube. The tube was heated to boiling using Lasec heating block (www.lasec.co.za) for 10 min and cooled centrifuged at 15,000 rpm for 8 min in Eppendorf centrifuge (Hermle Microrifuge; Labortechnik GmbH Germany). Supernatant of the above preparation was use as bacterial lysate or DNA template during the PCR amplification/detection experiments.

Detection

All primer sequences were synthesized by Inqaba Biotechnical industries (Pty) Ltd. (www.inqababiotech.co.za). PCR amplification and detection were performed using target specific 16SrRNA primer sequences VF169-(5'-GGA TAA CC/TA TTG GAA ACG ATG-3') and VR744-(5'-CAT CTG AGT GTC AGT G/ATC TG3') (617bp) (Yong et al. 2006). The isolates were further confirmed using the *OmpW* (304 bp) while other genetic-based elements as well as regions associated with *V. cholerae* virulence, serogroup, and biotype were detected as previously described (see Table S1 below for primer sequence and references). Virulence genes: *ctxA* (*ctxB*), *VSP-II* (*VC0511*, *VC0513*), *VPI-I* (*tcpA*), *heat stable enterotoxin* (*NAG-ST*), *Zot*, *ace*, *cep*, *prtV*, *enterotoxigenic hemolysin* (*hlyA*), *RTX toxin* (*rtxA*) *rtxC*, and *RS1* were detected. The transcriptional regulator of biotype-specific repeat sequence (*rstR*), *ctxB* alleles, and *rfb* gene specific for O1/O139 serogroup genes were also amplified.

PCR amplification

The amplification was carried out in a 200- μl microfuge tube. Fifty picomolar to 1 micromolar DNA extract was

used for the PCR in a final volume of 25 μ l, a GoTaq[®]G2 green master mix supplied in 2X Green GoTaq[®]G2 pH: 8.5 reaction solution containing, dNTPs {400 μ M each of dATP, dGTP, dCTP, and dTTP}, 3 mM MgCl₂, and GoTaq[®]G2 DNA polymerase at optimal concentration for efficient PCR amplification as specified by Promega Corporation USA (www.promega.com). Primer concentration of 0.5 μ M with the PCR machine (Bio-Rad T100[™] thermal cycler) was used. The thermocycling condition were 4 min at 94 °C followed by 35 cycles of 94 °C for 1 min, 53.0 °C for 1 min, and 72.0 °C for 1 min and a final extension step at 72.0 °C for 8 min. Other virulent genes cycling conditions are as shown in Table S1 below.

Tris acetate-EDTA (TAE) or Tris Borate-EDTA (TBE) buffer for gel electrophoresis

A TAE stock solution was prepared by adding 242 g of Trizma base, 5.7 ml glacial acetic acid and 100 ml 0.5M EDTA (pH: 8.0) into a 1 L of deionized water to make a 1 L 50 \times TAE buffer (pH 8.0). The working solution consists 20 ml of the stock solution to 980 ml of deionized water (DW) to prepare a 1 L of 1 \times TAE buffer.

Gel electrophoresis

A small-sized gel was prepared by adding 1.5 g of agarose (Sigma Aldrich, UK) powder and 100 ml 1 \times TAE buffer. The mixture was dissolved in a 250-ml conical flask, boiled on a microwave for approximately 3 min using high heat with intermittent mixing, allowed to cool to about 50 °C before adding 2 μ l per 50 ml of ethidium bromide. The gel was casted, allowed to polymerize, transferred onto an electrophoresis tank filled with 1 \times TAE buffer to mark. PCR products or amplicons from a thermal cycling process were mixed with loading buffer (bromophenol blue, 0.01% w/v, glycerol, 50% v/v, EDTA, 0.025 M pH 8.0) at the ratio of 4:1 (sample:buffer) before loading into the comb wells. The DNA ladder or molecular marker was then loaded without DNA-loading dye. Thereafter, samples were electrophoresed at an appropriate voltage of 100 V for 50 min until the dye front was 2 cm away from the end of the gel. The power was switched off and the electrophoresis tank was disconnected from the power pack. The gel was carefully removed from the casting tray and photograph using the Gel Doc imaging system (Bio Rad, USA).

Statistical analysis

Pearson correlation was employed to describe and determine level of significances between *Vibrio* colonial mean cell density/count and the recorded physicochemical parameters in relation to the study sites. This is done to determine the statistical significance of the physicochemical parameters to the proliferation tendency of

such emerging strains and/or the *Vibrio*-colonial density. Such generated data were transferred onto Excel spread sheets to develop tables while other generated data were described using figures.

Results

The environmental SA-NAG *V. cholerae* strains retrieved was phenotyped and genotyped using microbiological culture dependent methods and PCR. A total of 61 (12.01%) isolates were PCR confirmed after the detection of 508 (66.9%) genus specific isolates from 759 presumptive isolates during the study as shown in Fig. 2 and Table 2. The phenotypic character of isolates also further showed that some of them were producer of the cholera toxin as depicted by the cholera toxin red test (Fig. 3, Table 2). The serological test reveals some of them as Bengal (7/61) serotype, whereas 68.9% (42) of isolates showed susceptibility to polymyxin B test, while 6.6% (4) were Voges-Proskauer test positive, protease test [203; (26.8%)] (Fig. 4; Table 2).

The PCR detection of the *CTX-phi*, *RS 1*, *RTX cluster*, *HLY-A* cluster, and the *rstR* region of the Classical and El Tor biotype revealed the presence of various genes of emerging relevance. Table S1 shows the various sets of oligonucleotide-primer sequences ranging from genus specific *16SrRNA* primer (Yong et al. 2006) to specie specific *OmpW* gene (Nandi et al. 2000) which confirms the various isolates as *V. cholerae*. The *ctxA* gene was not detected during the study whereas *ctxB* and other toxigenicity associated genes such as *RTX*, *cep*, and *ace* were detected among isolates. All the detected gene markers are located within the *CTX-phi*, satellite phage, *RTX* cluster, and the hemolysin A cluster of the classical and El Tor strains of *V. cholerae* (Safa et al. 2010; Goel and Jiang 2010; Heidelberg et al. 2000; Waldor and Mekalanos 1996). The detection of the *RTX* toxin (repeat toxin) cluster reveals virulence determinants while *rtxC* gene detection reconfirm that these isolates where of the El Tor biotype as previously reported by Chow et al. (2001). The PCR detection of El Tor and the Classical hemolysin gene in 3.27% (2/61) of the isolates analyzed also confirms the occurrence of emerging and unusual genotype among the strains. Further studies are currently ongoing on these atypical *V. cholerae* members in our laboratory and environment which include whole genome sequence analysis, bioinformatics, and phylogenetic study of these members.

From Table 1 above, it was observed that at count 10¹, there was a significant positive correlation between free chlorine (0.304, $p < 0.05$); conductivity (0.418, $p < 0.05$); specific conductivity (0.453, $p < 0.05$); TDS (0.432, $p < 0.05$); salinity (0.357, $p < 0.05$); temperature (0.265, $p < 0.05$); TSS (0.413, $p < 0.05$); and TBS (0.402, $p < 0.05$) and

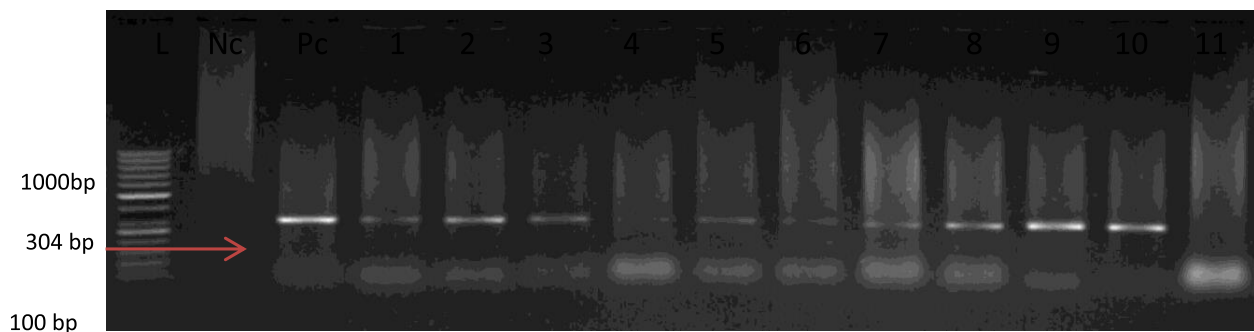


Fig. 2 Electrophoresis photomicrogram *OmpW* gene detected at 304 bp L represents a molecular marker of 1.2 kb, Pc is a positive control DSM 19130 *V. cholerae*, Nc is a negative control DSM 8224 *Plesiomonas shigelloides*, while numbers 1-10 are positive isolates while isolate 11 is negative

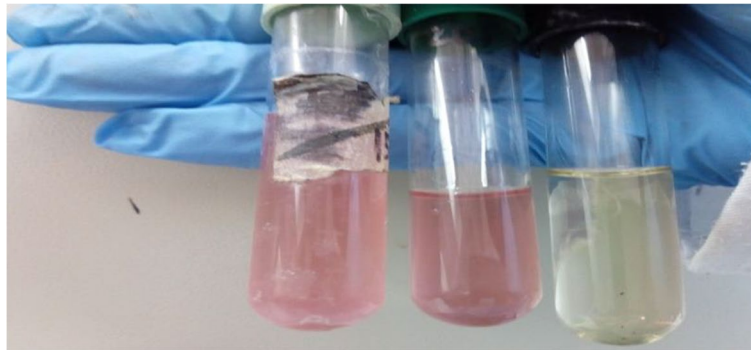


Fig. 3 Cholera red test for *V. cholerae* and a negative control DSM 8224 *Plesiomonas shigelloides*

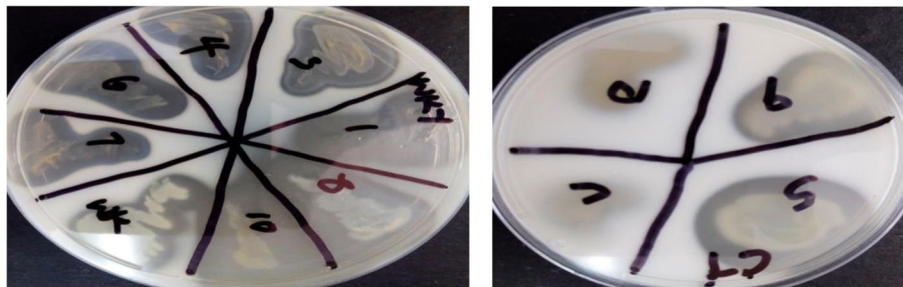


Fig. 4 Protease expression test for *V. cholerae* and a negative control DSM 8224 *Plesiomonas shigelloides*

the *Vibrio*-colonial count. There was also a significant negative correlation between redox potential (-0.453 , $\rho < 0.05$); resistivity (-0.362 , $\rho < 0.05$); DO (-0.562 , $\rho < 0.05$); and DO5 (-0.557 , $\rho < 0.05$) with *Vibrio*-colonial count. The positive significant correlations indicate that as the parameters increases the count at 10^1 increases also significantly. While for the negative significant correlation, as the parameters increases significantly, the count reduces due to anaerobiosis.

It also shows that there was no significant correlation between mVpH (-0.054 , $\rho > 0.05$); pH (-0.056 , $\rho > 0.05$);

Atm pressure (-0.022 , $\rho > 0.05$); and BOD (-0.052 , $\rho > 0.05$) with *Vibrio*-colonial count at 10^1 . Although there is no significant correlation, the result shows that they were all negatively correlated. This is inferred as follows: as the parameters increases in value, the *Vibrio* count reduces.

At count 10^3 , there was a significant correlation between free chlorine (0.305 , $\rho < 0.05$); conductivity (0.268 , $\rho < 0.05$); specific count (0.268 , $\rho < 0.05$); TDS (0.337 , $\rho < 0.05$); salinity (0.257 , $\rho < 0.05$); temperature (0.258 , $\rho < 0.05$); TSS (0.409 , $\rho < 0.05$); and TBS (0.397 , $\rho < 0.05$) and *Vibrio*-colonial counts. There was also negative

Table 1 Pearson correlation of physio-chemical parameters and Vibrio-colonial counts

Parameters (units)	Descriptive statistics		Correlation with counts		
	N	Mean \pm standard error	10 ¹	10 ²	10 ³
Free chlorine (mg/L)	216	0.38 \pm 0.49	.304**	.249**	.305**
mVpH	216	-29.45 \pm 63.09	-.054	-.021	.005
pH	216	7.60 \pm 0.99	-.056	.061	-.016
Redox potential (mV)	216	49.06 \pm 94.84	-.453**	-.026	.028
Conductivity (μ S/cm)	216	441.45 \pm 161.22	.418**	.170*	.268**
Specific count (μ S/cm)	216	364.84 \pm 220.90	.453**	.208**	.361**
Resistivity (m Ω .cm)	216	0.00 \pm 0.00	-.362**	-.177**	-.249**
TDS (mg/LTDS)	216	223.52 \pm 130.84	.432**	.200**	.337**
Salinity (psu)	216	0.23 \pm 0.15	.357**	.144*	.257**
Temperature ($^{\circ}$ C)	216	14.03 \pm 4.17	.265**	.136*	.258**
Atm pressure (psi)	216	13.18 \pm 1.61	-.022	.001	.007
DO (mg/L)	216	6.16 \pm 3.49	-.562**	-.307**	-.508**
DO5 (mg/L)	216	4.32 \pm 3.34	-.557**	-.317**	-.442**
BOD (mg/L)	216	1.80 \pm 1.93	-.052	-.005	-.153*
TSS (mg/L)	216	67.88 \pm 98.52	.413**	.294**	.409**
TBS (NTU/FAU)	216	75.13 \pm 105.65	.402**	.292**	.397**

**Sig at 0.01

*Sig at 0.05

Table 2 Density and phenotypic characters of the atypical *V. cholerae* and other members

Sites	Presumptive isolates	Numbers confirmed	Cholerae red test	Invitro TCP test	String test	Phenotypes detected		
						Bengal serotype	Polymyxin B test	Voges-Proskauer test
WWTP	327	29	91	27	92	4	27	2
RWS	186	15	53	14	51	2	9	1
Rs	149	13	27	11	39	1	4	1
Ca	97	4	8	7	15	0	2	0
Total	759	61	179	59	197	7	42	4

correlation between resistivity (-0.249 , $\rho < 0.05$); DO (-0.508 , $\rho < 0.05$); DO5 ($0.442-0.317$, $\rho < 0.05$); and BOD (-0.153 , $\rho < 0.05$) with Vibrio-colonial count, while there was no significant correlation between mVpH (0.005 , $\rho > 0.05$); pH (-0.016 , $\rho > 0.05$); redox potential (-0.028 , $\rho > 0.05$), Atm pressure (-0.007 , $\rho > 0.05$); and with Vibrio-colonial count.

Discussion

New reports of *V. cholerae* strains continue to surface from diverse region of the world as some reports are associated with the environmental strains. Other reports queried emergence of new strains with high virulent nature necessitating public health interest. The sampled domestic water physico-chemical indices

show statistical significance with Vibrio-colonial density/counts (Table 1). This reveals the impact of physicochemical parameters on the proliferating emerging strains. The strains show positive reaction to diverse phenotypic virulent determinants including protease, oxidase, cholerae red test (Figs. 3, 4), and other atypical phenotypes of pathogenic *V. cholerae* as depicted in Table 2 above. The study revealed that 68.9% (42/61) of isolates were susceptible to polymyxin B test, whereas 6.6% (4/61) of isolates were Voges proskauer test positive, while 11.48% (7/61) were positive to the Bengal serological test (Table 2). This implies that the SA-NAG members are emerging with such dual/atypical biotype. This result is similar to the reports of numerous investigators who have observed the dual phenotype

among *V. cholerae* (Zaw et al. 2018; Safa et al. 2005, 2006, 2010; Nguyen et al. 2009; Chun et al. 2009; Das et al. 2007; Faruque et al. 2007). Observing variability in susceptibility to polymyxin B by the SA-NAG strains reveals the atypical biotype. It is important to note that the polymyxin sensitivity or resistance has been applied for several decades as an epidemiological bio-marker in the biotyping scheme for *V. cholerae*. This occurrence and other phenotypic virulent indices (Figs. 5, 6, 7, and Table 2) has aroused the need to source track the spread/distribution of the patho-biotype exhibiting emergent trait.

The study also reveals a remarkable occurrence of atypical/unusual genes which are found both in the Classical biotype and the El Tor biotype as previously reviewed by Safa and his colleagues (Safa et al. 2010). The observed genotype/phenotypes were located around the *RSI* and *CTX ϕ* regions of either the small and/or large chromosomes (Heidelberg et al. 2000; Safa et al. 2010). According to Faruque and his group, it was reported that these strains with atypical or altered and mixed biotype may have risen from primordial El Tor strains that lost their *CTX ϕ ^{El}* from their large chromosome with a subsequent tandem location of *CTX ϕ ^{Clas}* in the small chromosome (Faruque et al. 2007). Nguyen et al. (2009) also reported that these

variant biotype strains may possess their *CTX ϕ ^{Clas}* and *CTX ϕ ^{El Tor}* either in tandem array or located within two different chromosomes.

It is important to note that previous vibriology investigators studied the O1 and O139 pathogenic strains of *V. cholerae*, it appears that the environmental strains (SA-NAG-O1/O139) are currently evolving with related dynamics which calls for prompt survey and/or control measure to avert future difficult to control strain.

Other atypical markers were also observed among our isolates which were similar to those reported recently in the study of Jena et al. (2018) and Samanta et al. (2018), revealing that the occurrence of two biotypes in *V. cholerae* O1 strain, exhibits diverse pattern of susceptibility to polymyxin B. The result of the PCR detection (Figs. 5, 6, 7) as represented in Table 3 showed that fifteen of the confirmed SA-NAG *V. cholerae* isolates harbored *ace* (24.6%) gene, while fourteen harbored *cep* (23%) gene. These genes were harbored in the *CTX ϕ* region of the pathogen chromosomes which reveals the presence of the nucleic acid based genotype. In an earlier study of Chun et al. (2009), he referred to strains with the above genotype with the term “a shift or drift between two distinct phyletic lineages” which is seen to be occurring in the environmental SA-NAG strains, and indicates potential evolution/emergence of new

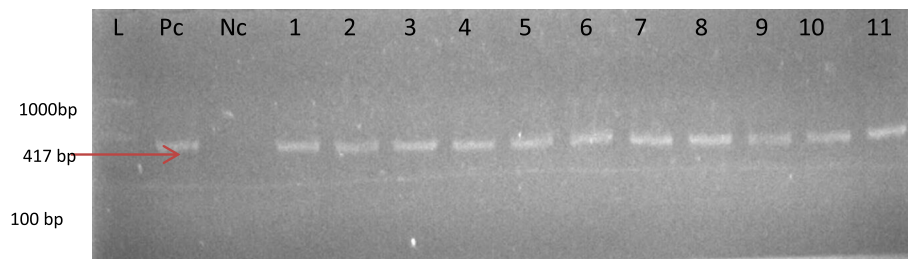


Fig. 5 Electrophoresis photomicrogram *rtxA* gene detected at 417 bp. L represents a molecular marker of 1.2 kb, Pc is a positive control *DSM 19130 V. cholerae*, Nc is a negative control *DSM 8224 Plesiomonas shigelloides*, while numbers 1-11 are positive isolates

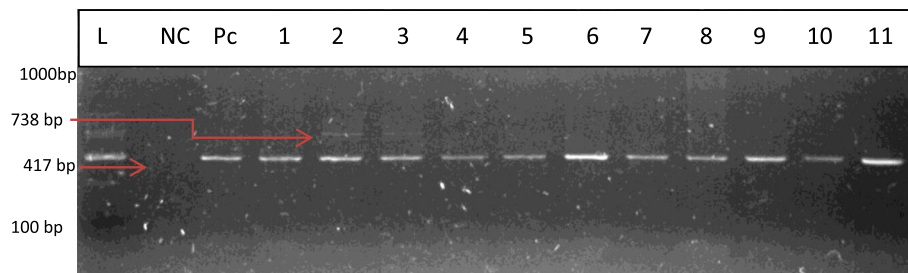


Fig. 6 Electrophoresis photomicrogram *hlyA* class (738 bp)/El Tor (481 bp) gene detected in atypical strains as depicted in isolate number 2 and 3. L represents a molecular marker of 1.2 kb, Pc is a positive control *DSM 19130 V. cholerae*, Nc is a negative control *DSM 8224 Plesiomonas shigelloides*, while numbers 1-11 are positive isolates for El Tor strains

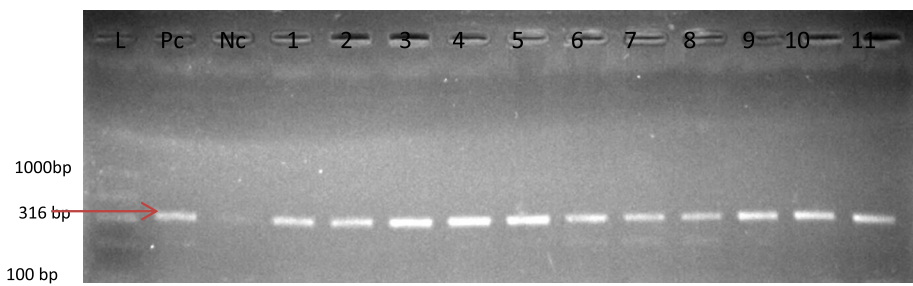


Fig. 7 Electrophoresis photomicrogram *ace* gene detected at 316 bp. L represents a molecular marker of 1.2 kb, Pc is a positive control *DSM 19130 V. cholerae*, Nc is a negative control *DSM 8224 Plesiomonas shigelloides*, while numbers 1-11 are positive isolates

strains with related pathogenic determinants. Estuarine environment has been shown as *V. cholerae* autochthonous habitat; however, the observation of such atypical or dual biotype strains among environmental *V. cholerae* shows evolving tendency which pose high risk to human and environmental health. Such risk and public health concerns are specifically directed to the urban and sub-urban individuals who use the studied water as their domestic water source. The need for adequate application of hygienic practice and supply of safe water cannot be over emphasized. This observation is also a pointer to the potential occurrence of a transitional representative of genetically identical strains, which possess high virulent potential as the pathogenic strain. It also implies that there is a possibility of an ongoing shift/drift of the environmental strains due to acquisition of new genes over time. Very indicative in the study was the observation of pathogenic emerging indices in somatic antigen non-agglutinating *V. cholerae* strains (*SA-NAG-Vc-1/139*) which were previously known non-pathogenic *V. cholerae* members.

Among other observed genotypes is an *RTX* toxigenic gene detected in the *RTX* cluster which is reported to encode cytotoxic reaction. Tables 3 and 4 show the numbers of isolates with related reports. It is reported to be physically linked to the *CTX-phi* in the *V. cholerae* genome (Heidelberg et al. 2000; Faruque and Mekalanos

2003) and associated with other residual features in attenuated cholera vaccines. About 49.2% of the isolates possess *rtxA* genotype whereas 18% possess the *rtxC* genotypes. These observations were also previously reported in the comparative genomics study of Heidelberg et al. (2000) and the characterization of *RTX* by Chow et al. (2001). It is said that the detection of *RTX* cluster is associated with actin depolymerisation when cross-linked with Hep-2. Such depolymerisation results villi effacement, hemorrhagic colitis, and bloody diarrhea in infection cases (Chow et al. 2001). This indicates that in any infection case associated with the atypical environmental strains of *SA-NAG V. cholerae*, bloody diarrhea and other symptoms cannot be removed from such cases. The study of Goel and Jiang (2010) also reported a similar occurrence of *RTX* (*rtxA* and *rtxC*) virulence and cytotoxin genes with such potential clinical symptoms.

Also revealed from this study was the detection of *cep* gene (23%) which is associated with the expression of *CTX-phi* coat protein among strains. This report is similar to the previous annual report of NICED (2004) which indicated that the detection of *cep* gene is of high and paramount clinical/epidemiological relevance. *Cep* gene detection may be used instead of *ctxA* gene detection while tracking the *CTX-phi* genes in *V. cholerae* isolates in both outbreak associated location and the environment.

Table 3 Number of confirmed/genotypes detected for atypical *V. cholerae* and other members

Sites	Confirmed isolates	Genotypes detected											
		RS 1							CTX-phi				
		<i>rstR</i>	<i>rstA</i>	<i>rstB</i>	<i>rstC</i>	<i>psh</i>	<i>g^{III}CTX</i>	<i>RS2</i>	<i>cep</i>	<i>ace</i>	<i>Zot</i>	<i>ctxA</i>	<i>ctxB</i>
WWTP	29	4	ND	ND	2	ND	ND	ND	8	11	6	0	2
RWS	15	2	ND	ND	0	ND	ND	ND	4	5	5	0	0
Rs	13	1	ND	ND	0	ND	ND	ND	2	3	3	0	0
Ca	4	0	ND	ND	0	ND	ND	ND	1	1	0	0	0
Total	61	7			2				15	20	14		2

Table 4 Numbers of other genotypes detected for atypical *V. cholerae* and other members

Sites	Confirmed isolates	Other genotypes							
		RS		HLYA		Altered/atypical strains	RTX		
		<i>rstRcl</i>	<i>rstREI Tor</i>	<i>hlyAcl</i>	<i>hlyAEI Tor</i>		<i>rtxA</i>	<i>rtxC</i>	<i>prtV</i>
WWTP	29	0	4	2	21	2	13	5	7
RWS	15	0	2	0	16	0	8	2	4
Rs	13	0	1	0	11	0	7	3	3
Ca	4	0	0	0	5	0	2	1	1
Total	61	Nil	7	2	53	2	30	11	15

The genotyping analysis of *hlyA* gene revealed that all isolated *SA-NAG-Vc-1/139* strains possessed the El-Tor hemolysin gene which is affirmed to be frequently observed among non-O1/non-O139 *V. cholerae* (*SA-NAG-Vc 1/139*) with the exception of two which possess both the Classical and El Tor gene type (Table 4 and Fig. 6). These genotype is said to be unusual among members of the *V. cholerae* family and rare among the non-O1/non-O139 *V. cholerae* strains. The detection of *hlyA* atypical genotype among the non-O1/non-O139 cholera *Vibrio* members indicates that strains are changing its nature within the environment over time. A previous study by Chatterjee et al. (2009) and Rivera et al. (2001) had reported the presence of the El Tor hemolysin gene (*hlyA*-El Tor) among non-O1 and non-O139 *V. cholerae* members; however, the dual phenotype was not observed in their study. Our position remains that the detection of the atypical *V. cholerae* has opened a new area of interest about the potential pathogen in our environmental and domestic water sources.

Conclusion

A notable report from this study is the observation of biotype gene content as well as other associated genes which are located within the small and large chromosome of the cholera *Vibrio*. It shows that present in the environment are emerging and evolving strains of *SA-NAG V. cholerae* which pose threat to the public in the urban and sub-urban area from which these isolates were retrieved. This is used to describe the potential evidence of virulence among environment associated *V. cholerae* (*SA-NAG-Vc-1/139*) isolated during the study. This study is also necessary as it provide reports of new area of research among *V. cholerae* with specific interest in relevant autochthonous diagnostic scheme, autochthony dependent vaccine design/application and need for other therapeutics. In avoidance of any doubt, more

biotype variants with other virulent indices and phenotypic characters may arise in the future which pose futuristic threat to public health. These emerging trend, change, or occurrence necessitate an urgent routine survey with a view to avoiding any looming outbreak. The need for research based and clinical management preparedness for such evolving and emerging strains remains or offers prime hope for possible future management of outbreaks associated with variant and/or altered biotype non-agglutinating *V. cholerae* O1/O139 in various environmental nexus.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13213-021-01661-5>.

Additional file 1: Table S1. The various primer sets, annealing temperature and expected amplicon size

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Authors' contributions

IBE: conceptualized, investigated, validate, analysis, data curation, writing original draft, project administration, and editing. AIO: editing, and review the submitted copy. UUN: conceptualization, validation, software, formal analysis, resources, data curation, review/editing, supervision, visualization, and funding acquisition. All authors have read and agreed on the current version.

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Availability of data and materials

The datasets as well as results presented in this study are available on request from the corresponding author.

Declarations**Ethics approval and consent to participate**

There is no identified violation of ethics in this study. The study was conducted to source track and characterizes the emerging indices of environmental and non-O1/non-O139 *V. cholerae* strain which are arising with unusual phenotypic/genotypic biotype features in water nexus. There was no clinically associated specimen hence ethical concerns, consent, and approval were not provided as specimen were environmental basis which was used in executing the study.

Consent for publication

The authors of this manuscript have read, corrected, and agreed on the final copy reports contained in this study.

Competing interests

The authors declare that they have no competing interests.

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