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**Genetic characterization of non-O1/non-O139 *Vibrio cholerae* mobilome: a strategy for understanding and discriminating emerging environmental bacterial strains**

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**Conflict of interest**: the authors declare no potential conflict of interest, and all authors confirm accuracy.

**Availability of data and materials:** all datasets generated were analyzed during the study and included in the article for publication. Other additional datasets used for this study are available from the corresponding author upon request.

**Ethics approval:** the study was also conducted with a view to source-track and characterize the emerging and evolutionary dynamics of emerging environmental strains, with a redirected research interest on the relevance of these members of non-O1/non-O139 *V. cholerae* both in water and clinical nexus. There was no clinically associated specimen hence there was no ethical base concern as samples were of environmental origin.

**Informed consent for publication:** the authors have read and agreed to the final copy of the findings as contained in this manuscript. Additional written consent was not necessary as samples were of environmental origin.

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**Abstract**

Acute diarrhea and cholera (AWD/C) result in more than 21000 to 143000 global mortality annually and are associated with *Vibrio cholerae*. The pathogen has shown increasing evolutionary/emerging dynamics linked with mobilome or ubiquitous nature of mobile integrative genetic and conjugative elements (MIGCE), however, such dynamics are rarely reported amongst somatic-antigen non-agglutinating Type-1/-139 *V. cholerae* (*SA-NAG-T-1/139Vc*). The study reports the genetic detection of mobilome-associated indices in *SA-NAG-T-1/139Vc* as a potential strategy for differentiating/discriminating emerging environmental bacteria. Presumptive *V. cholerae* isolates were retrieved from five water sources, while strains were characterized/serogrouped and confirmed using simplex and comparative-genomic-multiplex Polymerase Chain Reaction (PCR). Genomic island (*GI-12det, GI-14det, GI-15det*); Phages (*TLC-phagedet, Kappa-phagedet*) and *ICEs* of the *SXT/R391* family genes (*SXT/R391-ICEs* integrase*, SXT-Hotspot-IV, ICEVchInd5Hotspot-IV, ICEVchMoz10Hotspot-IV*) were detected. Other rare *ICE* members such as *the ICEVcBan8att gene* and Vibrio Seventh Pandemic island detection (*VSP-II Integrase, Prototypical VSP-II*) were also detected. Results revealed that the 8.22% (61/742) *SA-NAG-T-1/139Vc* serogroup observed harbors the Vibrio Seventh Pandemic island integrase (34/61; 55.7%) and other rare genetic traits including; attB/attP (29/61; 47.5%, 14/61; 23%), integrative genetic elements (4/61; 6.56%), phage types (*TLC-phagedet:* 2/61; 3.28% and *Kappa-phagedet*: 7/61; 11.48%)

as well as the integrase genes (*INT1, Sul1, Sul2*) (29/61: 47.5%; 21/61: 34.4%; 25/61: 41%). Such genetic detection of mobilome determinants/MIGCE suggests potential discriminatory tendencies amongst *SA-NAG-T-1/139Vc*which may be applied in mobilome typing of evolving/emerging environmental bacteria. The need to encourage the application of such mobilome typing indices and continuous study of these strains is suggestive of interest in controlling future potential emerging environmental strains.

**Introduction**

Cholera remains the cause of death amongst poor sub-urban individuals who lack access to safe water as well as illegal immigrants/displaced population and refugee camp interns either due to environmental disaster or conflict.[1] Over the years, the causal pathogen (*Vibrio cholerae*) continues to emerge and re-emerge in pathogenic potential causing acute watery to bloody diarrhea amongst infected humans and animals.[2,3] Since their early detection in 1817 till date, the pathogens were the object of several waves of occurrence, including sixth members (which are biotyped as Classical), to the ongoing Seventh Pandemic El Tor O139 *V. cholerae*. It has spread resulting death of humans, especially in most Southern/Eastern African countries.[4,5] Various investigators have reported the autochthonous survival of the pathogen in aquatic environments where it thrives and acquires several pathogenic indices.[3] The pathogen is serogrouped into more than 203 somatic types but the O1 and the O139 members have been implicated in most epidemic and pandemic cases.[3] Other members are referred to as non-O1/non-O139 which include: O2, O4, O10, O14, O24, O70, etc.[3,6,7] These somatic antigen non-agglutinating type 1/139 (SA-NAG-T-1/139 *V. cholerae*) or non-O1/non-O139 *V. cholerae* were previously referred to as non-pathogenic, but in recent times reports have shown these members are implicated in sporadic pathogenic cases of watery diarrhea as well as outbreaks.[7-9] In some related studies from Africa, it was reported that there is a notable acquisition of genetic elements, resistance determinants to common antibiotics, and class 1 (*sul1*) or class 2 (*sul2*) integrase.[10-12] Such acquired genetic elements have been previously reported amongst non-agglutinating (NAG) *V. cholerae* strains which persist/thrive in the environment suggesting their environmental fitness, both in Asia (Bangladesh) and some parts of West Africa (Ghana).[13-15] Studies on the detection of integrase genes amongst environmental NAG *V. cholerae* members showed the mobilization

of integron and acquisition/loss of various gene cassettes[16,17] suggesting them as potential promoters of gene transfer and antibiotic resistance emergence.

Coincidentally, these strains also possess emerging virulent genotypes associated with mobile genetic elements (MGEs)[14-17] activities. MGEs are genomic features that can move within or between DNA molecules as well as cells. These elements provide mobility for genetic material which fuels adaptive potential for the existence or persistence of microbial life.[17] These elements are categorized as mobilome(transposons, plasmids, bacteriophages, and self-splicing molecular parasites)as they possess transfer capacities amongst cells[14,15-17] Mobilome genetic detection in any environmental bacteria is an indication of genomic plasticity, evolution, and/or the emergence of such strains.[14-20] Even so, genomic islands (*GI-12,-14, -15*) were used as distinguishing and associating factors with atypical/evolving bacterial strains.[19-21]

The *SXT/R391* families are other classes of integrative and conjugative genetic elements (*ICEs*) or mobile integrative genetic and conjugative elements (MIGCEs) whose presence is indicative of multiple antibiotic resistance amongst *V. cholerae.*[20] The mobilome genes have both been demonstrated in genomic islands (*GI*), integrative conjugative elements (ICEs), and prophages (*Kappa, TLC*) and they serve as distinguishing factor amongst the diverse phylogenetic lineage of *V. cholerae.*[17-20] In a bid to affirm such distinguishing factors in emerging/evolving potential pathogens thriving and proliferating within our environment, this study was conducted. This study genetically detects mobilome/MGEs genes amongst environmental SA-NAG*Vc*-T-1/139 strains as a strategy for discriminating emerging/evolving dynamics of substantial bacteria in the environment. Such a study would reveal the emerging tendency of major environmental potential pathogens. To address the aforementioned, we employed a rapid detection approach for mobilome typing, using simplex and comparative genomic two-way multiplex PCR techniques.

## Material and Methods

### *Design of study and area*

Aliquot samples from wastewater final effluents (WWFEs) or wastewater treatment plants(WWTPs), receiving water shed (RWS), rivers (R), earth canals (EC), dams (D) and tap water (T)were collected within six months in 2018 from three local municipalities in Eastern Cape, South Africa, for the isolation of non-O1 and non-O139 *V. cholerae* using standard microbiological techniques.[20,21] Forty-five samples each were processed in six months from four sampling sites/areas, namely H (Amathole Hill water-fall); C (Cathcart); Q (Queens

town); CF (Cofimvaba), as follows: WWFE/WWTP/RWS: 20, R: 10, D: 5, EC: 5, T: 5, to make a total of 1080 samples (45 × 4 × 6 samples). The specified water samples (1L each) were collected in triplicates from sites using pre-sterilized Nalgene 1L glass bottles,[20,21] stored in cooler boxes and shipped to the research laboratory within 5 hours for microbiological analysis on TCBSA(quadrant streaking onto pre-prepared Thiosulphate Citrate Bile Salts-Sucrose agar) plates and incubated at 37ºC for 24-48 h formicrobial isolation.[21,22] Five to ten yellow and green suspected colonies were sub-cultured from TCBSA onto nutrient agar to ascertain the purity of isolates; stored on glycerol stock in aliquots and subjected to an array of biochemical tests and serological tests. Some biochemical tests conducted after the Gram reaction include motility test, oxidase test, Voges Proskauer test (VP-test), and D-mannitol catabolism. The control strains (*MJ*1236, *CIRS*101, *MO*10, *B*33, *N*16961, *DSM* 19130 *V. cholerae,* and *DSM 8224Plesiomonas shigelloides*) were collected/sourced from Leibniz-Institut GmbH Germany and the Centre for Enteric Diseases (previously referred to as the Enteric Diseases Reference Unit) of the National Institute for Communicable Diseases (NICD), Division of Virology and Communicable Diseases Surveillance, School of Pathology, Faculty of Health Science, University of the Witwatersrand, Johannesburg, South Africa.

***Serological/serotypical identification of isolates***

Commercially sourced diagnostic antisera from Davis Diagnostics (Pty) Ltd, Gauteng, South Africa (www.daviesdiagnostics.co.za) such as MAS-AGGL-M11003 (411900) and MAS-AGGL-M11004 (411901) were employed during the study following manufacturer's instructions, on 24 h old single and pure culture colony.

***Microbial culture and molecular confirmation of isolates***

Briefly, all yellow morphologically distinct colonies from each TCBSA plate were picked and purified as presumptive strains after 24 – 48 h incubation at 37°C while isolated strains were stored in 20% saline/glycerol stock as presumptive environmental *V. cholerae* isolates at -80ºC refrigeration. Synthesized target-specific primer pairs for *V. cholerae* such as ***16SrRNA***:(VF169 5'-GGATAACC/TATTGGAAACGATG-3', VR7445'-CATCTGAGTGTCAGTG/ATCTG-3'), ***OmpW:*** *V.choleF*5'-CACCAAGAAGGTGACTTTATTGTG-3', *V.choleR*5'-GGTTTGTCGAATTAGCTTCACC-3') and other primer pairs for **Serogrouping:** (*Vc-O1F5'*-GTTTCACTGAACAGATGGG-3', *Vc-O1R5'*- GGTCATCTGTAAGTACAAC3',

*Vc-O139F5'*- AGCCTCTTTATTACGGGTGG-3', *Vc-O139R5'*- GTCAAACCCGATCGTA AAGG)[22,23]were applied on individual DNA extract from all presumptive isolates and positive amplicon bands size at 617bp, 304bp 192bp, 449bp were selected for the subsequent PCR (polymerase chain reaction) analysis. Confirmed isolates were serotyped and biotyped using further molecular typing methods (PCR and serology). The reference strains used with isolates were *V. cholerae* 7th pandemic reference strain O1 *V. cholerae* N16961 (acc. num. NC_002505), O1 *V. cholerae* B33 (acc. num. NZ_ACHZ00000000), O1 *V. cholerae* CIRS101 (acc. num. NZ_ACVW00000000), O139 *V. cholerae* MO10 (acc. num. NZ_AAKF00000000) and O1 *V. cholerae* MJ-1236 (acc. num. NC_012668), which were used to develop the multiplex PCR as designed by NICD, South Africa. Isolates belonging to our laboratory-typed culture collections were also analyzed to ascertain their mobilome genotype status and compared with reference strains.

***Mobilome individual loci Primer pairs used***

The mobilome gene target-specific PCR primer pairs were designed for both simplex and multiplex procedures to overcome dimer formation using the Java web bioinformatics tools.[24,25] Table 1 below shows the detailed primer sequence information, PCR amplicon expected band size, as well as the targeted gene regions. Primer pairs were synthesized by Inqaba Biotechnical Industries (Pty) Ltd. (Hatfield Pretoria, South Africa, an Africa's Genomics company; www.inqababiotec.co.za).

The first multiplex assay is a set of six primer pairs that detects the Vibrio 7th Pandemic Island II (*VSP-II*), integrase, and other *SXT/R391 ICEs hotspots*, while the second set of multiplex PCR technique detects five-gene pairs including the genomic islands (*GI-12, -14, -15*) and phages (*Kappa* and *TLC*).[23,25,26] The simplex PCR detected integron,$attB_{BAN8,}$ and *ICEVchBan8* integrase genes.[20,26] Negative gene detection showed no amplicon band size while positive gene detection showed a specific band size at the expected amplicon size for each specific gene (Table 1). Triplicate samples were processed by PCR techniques to avoid any experimental error as care was also taken to prevent isolate/strain duplication. The amplicons were sent for partial sequencing to confirm their identity (sequence reports were processed using bioEdit, MegaX, and Chromas and reported elsewhere).

***DNA extraction PCR amplification conditions and electrophoresis***

DNA extraction was achieved by boiling method as previously described by Maugeri *et al*.[27] Amplification was conducted using the Bio-Rad T100$^{TM}$Thermal cycler (T100$^{TM}$ thermal

cycler, Bio-Rad, Hercules, Califonia, USA; www.lasec.co.za), optimized for final reaction mix volume of 25µL, in a 200 µLmicrofuge tube. Fifty picomolar to one micromolar DNA extract was used for PCR, a GoTaq®G2 green master mix supplied in 2X Green GoTaq®G2 reaction buffer containing pH: 8.5, dNTPs (400 µM each of dATP, dGTP, dCTP and dTTP), 3 mM $MgCl_2$ and GoTaq®G2 DNA polymerase at optimal concentration for efficient PCR amplification as specified by Promega Corporation USA (Califonia USA; www.promega.com) was used. A primer concentration of 0.3 µM was used. The optimized PCR cycling conditions were as follows: initial denaturation at95ºC for 5 min in 35 cycles of denaturation at 95ºC for 45s, annealing at 59ºC for 35 s (Multiplex 1) and 64.5ºC for 60 s (Multiplex 2) and an extension of 72ºC for 60 s. It terminates with a final extension at 72ºC for 8 min and storage at 4ºC until post-PCR analysis. Amplified products (5µL) were electrophoresed in a 1.5% agarose gel electrophoresis at 100 V for 45 min. The gel was stained with 2 µL per 50 mLof 0.5 mg/Lethidium bromide and visualized using an alpha UV documentation system at 254 nm (Bio Rad Hercules, Califonia, USA).

***Data analysis***

The Paleontological Statistics Version 3.14 (the past3.zip software package 3.14)[28,29] was used for the divergent and cluster studies. The absence/presence of mobilome gene amongst isolates was used for strain discrimination/divergence/relationship. A dendrogram was generated by neighbor-joining (NJ) using the Euclidean similarity index of the past3.zip software package 3.14.

***Discrimination of various environmental isolates of SA-NAG-T-1/139***

Although isolates were recovered from the environment and water nexus, detected genes were used to differentiate isolates using a matrix plot and cluster analysis. A designed binary code of 0,1 (zero, one) was used to generate the matrix where zero represents an absence of a detected gene while one represents a detected or present gene. The detected mobilome profile was used to separate the isolates into groups as the presence and/or absence of gene was used to show strain relation and/or discrimination.[28,29]

**Results**

***16SrRNA and* V. cholerae *species specific gene detection***

The applied techniques retrieved 759 presumptive *Vibrio* strains from a total of 1080 samples. Retrieved isolates from water sources are as follows; H (Amathole Hill fall); (WWTP/RWS: 0, R: 57, D: 34, EC: 34) C (Cathcart); (WWTP/RWS: 17, R: 69, D: 58, EC: 44), Q (Queens town); (WWTP/RWS: 95, R: 73, D: 59, EC: 52), CF (Cofimvaba); (WWTP/RWS: 46, R: 49, D: 38, EC: 34). The 16SrRNA gene (617bp) detection confirmed 97.8% (742/759) as *Vibrio* species while the species-specific *OmpW* gene (304bp) detection confirmed 8.22% (61/742) as *V. cholerae* (Figure 1 and 2). The gene-based (PCR) serogrouping of sixty-one strains further confirmed them as somatic antigen non-agglutinating types 1/139*V. cholerae* or NAG *V. cholerae* strain or SA-NAG-T-1/139 *V. cholerae* strain. It is also important to note that there was no detected band for strains at 192 bp and 449 bp amongst tested strains.

***Mobilome typing***

Mobilome typing conducted using two different sets of multiplex PCR, two simplex PCR, and thirteen sets of primer pairs as described in section 1.2.2 yielded the following gel photos below. Amongst the confirmed (61/742) non-O1/non-O139*V. cholerae* isolates (Figure 1 and 2), 34/61 (55.7%) were positive for the *VSP II* integrase gene*,* 4/61 (6.56%) were positive to *SXT/R391 ICEs* integrase*,* 2/61 (3.28%) were positive to *SXTHotspot IV* in the multiplex 1 cascade. Whereas multiplex 2 detections showed 2/61 (3.28%) in *TLC Phage* and 7/61 (11.48%) in *Kappa Phage* (Figure 3, 4, 5; Table 2). There was no detected band size at the expected amplicon size for Prototypical *VSP-II, ICEVchInd5Hotspot IV, ICEVchMoz10, GI-12, GI-14,* and *GI-15* which may point to the absence of such genes. However, these detected genes are mobilome dynamics including *ICEs* genes families which indicates that our isolates possess mobilome-associated genes and potential evolving/emerging genetic dynamics with genotypes that may be applied as indicators for discriminating other emerging environmental organisms.

***Typing other rare mobile genetic elements associated genes***

In addition, other rare recombinatory machinery amongst environmental non-O1/non-O139*cholerae* strains which are atypical ICE genes (*ICEVchBan8attB/Ban8attP genes*) were detected. Such detection at 613 bp and 283 bp (47.5%, 29/61; 23%, 14/61; Figure 6) depicts

the integration of genomic island which is inserted in a t-RNA-Ser suggesting the formation of a hybrid element. Other additional observation from the study shows detection of class 1 and 2 integron genes (*INT1, Sul1, Sul2*) [47.5% (29/61), 34.4% (21/61), 41% (25/61)] amongst confirmed strains (Figure 7). This indicates mobility of gene cassettes or mobilization of integron and loss/acquisition of multiple gene cassettes. It also infers that the isolates were promoters of mobile genetic elements activities and multiple antibiotic resistance emergences.

**Discussion**

One of the suggestions from our previous review on the changing nature of environmental non-agglutinating *V. cholerae* strains, and cholera/acute watery diarrhea (C/AWD) control in a changing environment was the application of mobilome typing.[9,30] Employing such an approach in cholera environmental surveillance schemes would enhance futuristic discriminatory and improved control strategy which may encourage vaccine development and phage therapy. This study employs mobilome molecular typing using PCR detection technique on non-agglutinating *V. cholerae* strains and mobilome genes profile (genetic fingerprint) as shown in Table 1 above.

Amongst the sixty-one confirmed (61/742) non-O1/non-O139*V. cholerae* isolates (Figure 1 and 2), 55.7% possess the *VSP II* integrase gene*,* 6.56% for *SXT/R391 ICEs* integrase*,* 3.28% were positive to *SXTHotspot IV* in the multiplex 1 cascade. Whereas the multiplex 2 detection experiment showed *Phage* (3.28%) and *Kappa Phage* (7/61; 11.48%) (Figure 3, 4, 5; Table 2). Such detected mobilome genotype and *ICEs* genes families are potential evolutionary machinery amongst non-O1/non-O139*V. cholerae* strains. Their detection shows that such isolates possess evolving and emerging genetic dynamics with genotypes that may be applied as indicators for discriminating evolving bacterial strains. Following the reports from numerous investigators,[20,31-36] the detection of *SXT/R391 ICE* families amongst *V. cholerae* and other bacterial strains is associated with a tendency for incorporation/recombination of new genes into conserved regions either from the aquatic and/or clinical environment.[20,34,35] Bacterial strains that harbor such incorporated genes are also adjudged as recombinant strains and/or evolving/emerging strains. Based on evolutionary fitness, it is inferred from the study that the environmental strains of non-agglutinating O1/O139 *V. cholerae* with such positive mobilome genes/dynamics contribute to evolution and emergence hence their detection may be adjudged as emerging strains. This was the position of previous investigators, who reported that the detection of several MIGEs such as phages, integron, plasmids, transposons,

and integrative conjugative elements (*ICEs*) amongst *V. cholerae* reveal evolving tendencies and contribute to genome instability especially as such genes are integrated into either of the two *Vibrio* chromosomes.[10,16,17]

It is noteworthy that these mobilome genotypes act collectively to facilitate horizontal genetic exchange, change in gene-based characters, and transfer/promote the acquisition of numerous genes. This was the presentation/submission in the reports of Chun *et al*.,[34] Tang *et al.*,[37] and Mikalsen *et al*.[38] in earlier studies which show that the genome of various prokaryotes possesses dynamic genes or traits that evoke in them the tendency to acquire and/or share genetic materials. Such genetic and/or genome-borne materials [MGEs/Genomic islands (*GIs*) and integrative conjugative elements (*ICEs*)] serve as major driving forces of evolution amongst bacteria and possess relevant medical and environmental implications.[20] The findings from Siefert[39] and Deshpande *et al.*[40] studies also affirmed that the detection of mobilome genotypes indicates that such strains possess genomic agents of change that control gene-based characters (genome controlling elements).

During the study, a prototypical ICE (*ICEVchInd5Hotspot IV* gene) was not detected and/or observed which is similar to Boyd and Waldor,[41] Faruque *et al*.[42] reports and other investigators.[31,43-46] However, following previous studies, such occurrence shows that the environmental strains may be inclined to potential outbreak but no outbreak was reported during the study period. This implies that these observed strains are potential sources of future outbreaks as previously reported by various investigators.[31,44,45]Other related genes were not also detected during the study, one such is the *ICEVchInd5Hotspot IV* gene. The non-detection of *ICEVchInd5Hotspot IV* gene suggests that isolates possess a high possibility for lateral gene transfer as its presence or absence may be used to predict the evolution/emergence of environmental bacteria strains.[46]

Another rare and/or atypical ICE detected was *ICEVchBan8attB/Ban8attP* gene (Figure 6). Such detection depicts the integration of genomic island between the MGEs and isolates genome as previously reported by Boyd and Waldor,[41] and Taviani *et al*.[20] These observations also support the discriminatory and emerging tendency of the studied strains based on mobilome genes.

Another affirmative observation from the study was the detection of class 1 and 2 integron genes (*INT1, Sul1, Sul2*) amongst confirmed non-O1/non-O139*V. cholerae* strains (Figure7). This indicates mobility of gene cassettes or mobilization of integron and loss/acquisition of multiple gene cassettes. It also infers that the isolates were promoters of MGEs activities and multiple antibiotic resistance emergence which corroborates the reports of Igere *et al.*,[47]

Banerjee *et al*.,[10] Delavat *et al.*,[48] and Touchon *et al.*[49] It is suggestive that since these isolates harbor indices that promote MGEs activities, they may also promote evolutionary tendency, especially amongst environmental strains.

The cluster analysis further affirmed that isolates are different as each has differing mobilome genes (dynamics) as shown by the dendrogram of different groups/clades (Figure 8). It also suggests that strains with positive genes may be easily discriminated or differentiated from non-associated strains, especially amongst those strains that do not harbor any mobilome dynamics. In addition, those strains with closely related mobilome dynamics were observed to have clustered together revealing their possible relatedness.

***Limitation of the study***

One limiting aspect of the study was the absence in the detection of the *IncA/C* plasmids which is one of the components of mobilome. Details in the determination of antibiotic susceptibility test (AST) and detection of multiple antibiotic-resistant genes such as Chloramphenicol (*FlorR*),Sulphonamide resistance gene markers (*sul1, sul2*), Trimethoprim, and sulphamethoxazole (*TMP*), extended-spectrum beta-lactamase (*ESβL*s), cabapenem resistant *V. cholerae* detection, New Delhi metallo-β-lactamase, fluoroquinolone-resistant gene as well as other integrase genes which are potential markers of the *SXT/R391* families of the *ICE* were accessed.[30]

**Conclusions**

The study was conceptualized for genetic characterization of mobilome, MGEs as well as other mobile resistome of environmental non-O1/non-O139*V. cholerae* strains retrieved from water nexus using multiplex and simplex PCR techniques as a bacterial discrimination strategy. Following the emerging nature of various environmental bacteria in the water nexus, the application of mobilome typing becomes pertinent and the use of this technique has proven a potential for low-cost molecular typing or discrimination of emerging environmental bacterial strains. One important note from the observations in the study is that members of the SA-NAG *V. cholerae* studied thus far possess some peculiar characteristics and possible occurrence of genomic rearrangement which may herald their natural transformation and evolutionary tendency. This may also be used to affirm environmental isolates' emergence and/or re-emergence in any aquatic or coastal environment. The implementation and application of the mobilome typing strategy as applied in this study

would in the future encourage adroit control of potential environmental pathogens. It would also encourage the surveillance and monitoring of such emerging/evolving environmental bacteria strains. In addition, the basic microbiological status (both wild and evolving strains) of such environmental isolates of clinical relevance would be easily determined or examined.

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**Table 1.** Detection and analysis of the Mobilome, Primer sets for multiplex PCR 1, 2 and two simplex Primer pair(s).

| Gene Targeted | Primer pair | Nucleotide sequence (5' to 3') | GeneBank Accession No | Position of Nucleotide | Expected Amplicon Size (bp) | Ref |
|---|---|---|---|---|---|---|
| **VSP-II integrase** | | | NC_002505 | | | Spagnoletti et al., [25] |
| | VSPIIintF | CCGACAAAGAATACACTCTCTCTGATGG | | 548835–548862 | 170 | |
| | VSPIIintR | ACGTCTTTTCCTTGCCTCGGCAAGAG | | 548979–549004 | | |
| **Prototypical VSP-II** | | | NC_002505 | | | Spagnoletti et al.,[25] |
| | VSPIIcutF | TTATCTACGACCACACCAGACAGC | | 541391–541414 | 245 | |
| | VSPIIcutR | ATGGGCATAGCAAAGGCACTTACCCA | | 541610–541635 | | |
| **SXT/R391 ICEs integrase** | | | GQ463142 | | | Ceccarelli et al.,[32] |
| | ICEdetF | TCAGTTAGCTGGCTCGATGCCAGG | | 4256–4279 | 505 | |
| | ICEdetR | GCAGTACAGACACTAGGCGCTCTG | | 3775–3798 | | |
| **SXT Hotspot IV** | | | AY055428 | | | Ceccarelli et al.,[32]; Wozn et al.,[31] |
| | SXTdetF | ACTTGTCGAATACAACCGATCATGAGG | | 68416–68442 | 357 | |
| | SXTdetR | CAGCATCGGAAAATTGAGCTTCAAACTCG | | 68088–68116 | | |
| **ICEVchInd5 Hotspot IV** | | | GQ463142 | | | Ceccarelli et al.,[32]; Wozn et al.,[31] |
| | Ind5detF | TGCACATTGAGGCCCTGCAAGCAC | | 66514–66537 | 423 | |
| | Ind5detR | GTGCATTCACCAGCTCTAACGTCG | | 66115–66138 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **ICEVchMoz10 Hotspot IV** | | | NZ_ACHZ01000011 | | | Ceccarelli et al.,[32]; Wozn et al.,[31] |
| | Moz10detF | CGGAAGATGACGAAGACCGCCTAAGC | | 15980–16005 | 712 | |
| | Moz10detR | ATTTGCCTTCGAACAAAAGGGGCA | | 15294–15317 | | |
| **Multiplex 2** | | | | | | |
| **TLC phage** | | | NC_002505 | | | Chun et al.,[34]; Faruque et al.,[35] |
| | TLCdetF | AATCAACTCACGGGTGCAGACCTC | | 1575253–1575276 | 449 | |
| | TLCdetR | TCCGCCAAGAAGTGACGTTGTAGC | | 541610–541635 | | |
| **Kappa phage** | | | NZ_DS990136 | | | Chun et al.,[34]; Faruque et al.,[35] |
| | KdetF | CGTCCGTAACCTTAAAGATGGCAGC | | 1560575–1560599 | 230 | |
| | KdetR | TCGTATGTCCGTGAACTTGCCACC | | 1560781–1560804 | | |
| **GI 12** | | | NC_012668 | | | Chun et al.,[34]; Faruque et al.,[35] |
| | GI12detF | CTACGGTTGAGCCGCTCCATTTGTC | | 1629546–1629570 | 571 | |
| | GI12detR | GTGCCTTCTAAATTGACCAAACGCGGCA | | 1630072– | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | 1630099 | | |
| **GI 14** | | | NC_012667 | | | Chun et al.,[34]; Faruque et al.,[35] |
| | GI14detF | AGACGAGTATCTAGTAAACGCCAAACC | | 940275–940301 | 142 | |
| | GI14detR | CTTTGCTTGCACTGGCAACCTCAG | | 940393–940416 | | |
| **GI 15** | | | NZ_ACHZ01000008 | | | Chun et al.,[34]; Faruque et al.,[35] |
| | GI15detF | CAGACCGCGAAGGAAAACGCTCTTTGC | | 53780–54937 | 348 | |
| | GI15detR | AGCGTCTCAGATGATGTCCGGCTG | | 54132–54155 | | |
| **attBBAN8 (an attachment site)** | | | | | | |
| | ban8attB-F | TTCTTGTTCAGCCGCGAGTTT | | | 614 | Taviani et al.,[20] |
| | ban8attB-R | GATCCCGCTCTTCTATGCCGT | | | | |
| **ICEVchBan8 integrase gene (attachment site)** | | | | | | |
| | ban8int-F | CGGAGATTTCGTCAGGGCT | | | 211 | Taviani et al.,[20] |
| | ban8int-R | CGTGCGCCGTTAACCATTG | | | | |

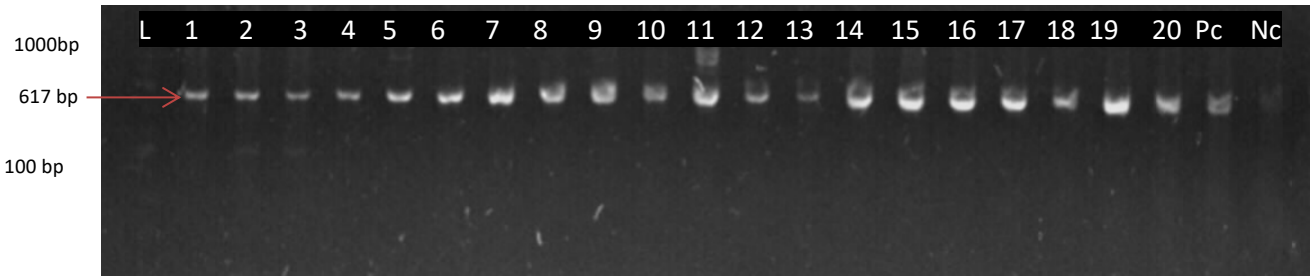



**Figure 1.** Gel photo of the Genus specific *16SrRNA gene* detection L represents a molecular marker of 1.2kb, Pc is a positive control *DSM 19130 V. cholerae*, Nc is a negative control *DSM 8224Plesiomonas shigelloides*, while numbers 1-20 are positive isolates.

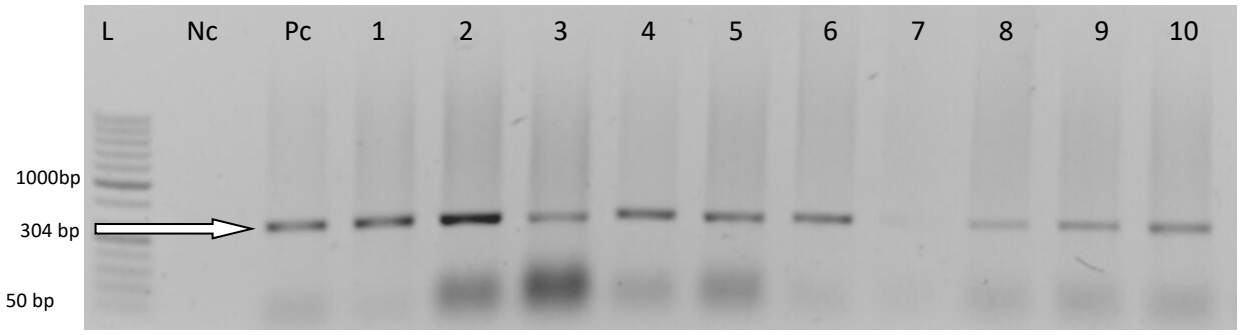



**Figure 2.** Photomicrogram of *OmpW* gene detection, L represents a molecular marker of 1.2kb, Pc is a positive control *DSM 19130 V. cholerae*, Nc is a negative control *DSM 8224Plesiomonas shigelloides*, while numbers 1-10 are positive isolates.

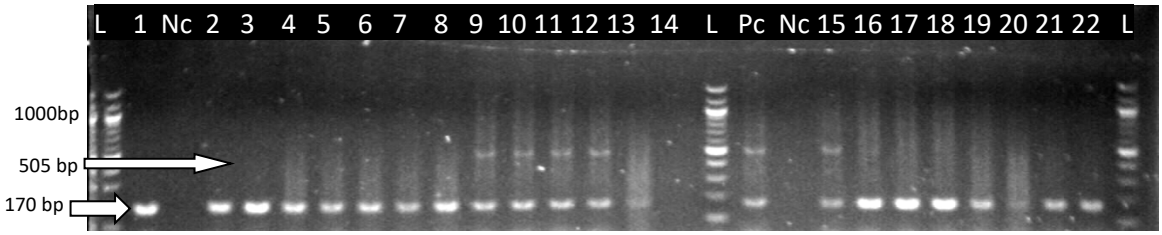



**Figure 3.** Multiplex 1 Mobilome typing gel photo of the detected genes; L represents a molecular marker of 1.2kb, Pc is a positive control reference strain, Nc is a negative control *DSM 8224Plesiomonas shigelloides*, while numbers 1-22 are isolates. All isolates were positive to *VSP II* integrase while 9,10,11,14,15 are positive to *SXT/R391ICEs* integrase.

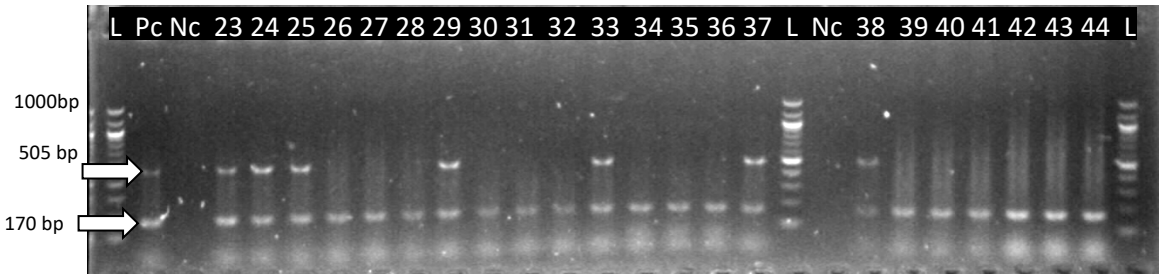



**Figure 4.**Multiplex 1 Mobilome typing gel photo of the detected genes; L represents a molecular marker of 1.2kb, Pc is a positive control reference strain, Nc is a negative control *DSM 8224Plesiomonas shigelloides*, while numbers 1-44 are isolates. All isolates were positive to *VSP II* integrase while,23,24,25,29,33,37,38 are positive to *SXT/R391 ICEs* integrase.

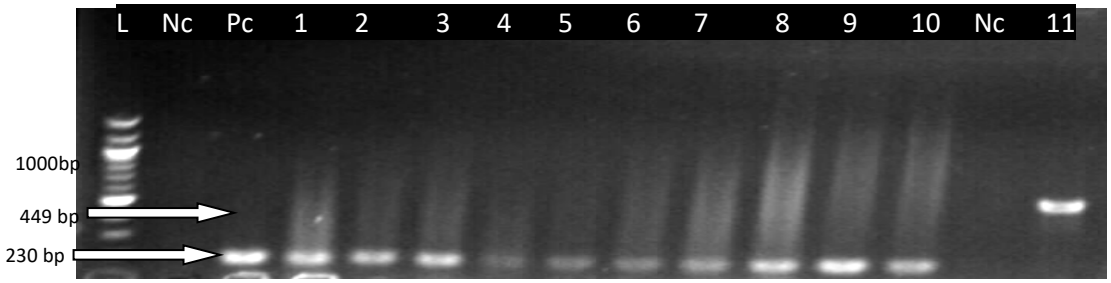



**Figure 5.** Multiplex 2 Mobilome typing gel photo of the *gene* detection L represents a molecular marker of 1.2kb, Pc is a positive control reference strain, Nc is a negative control *DSM 8224Plesiomonas shigelloides*, while numbers 1-11 are positive isolates. Isolate number 1-10 are positive to kappa phage while isolate number 11 is positive to TLC phage.

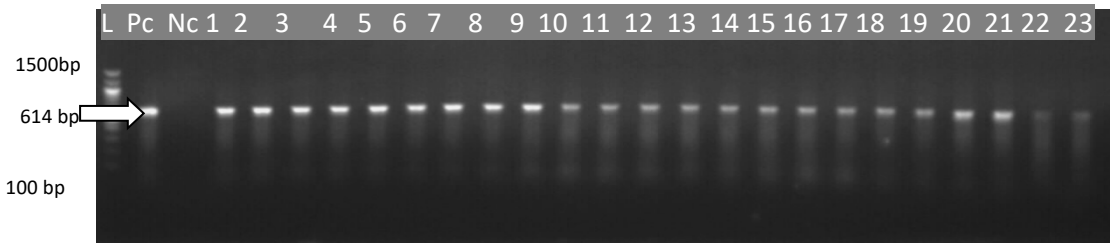



**Figure 6.** Microgram of rear integrase gene (*Ban8att gene*) with sites detected. L represents a molecular marker of 1.2kb, Pc is a positive control *DSM 19130 V. cholerae*, Nc is a negative control *DSM 8224Plesiomonas shigelloides*, while numbers 1-23 are positive isolates.

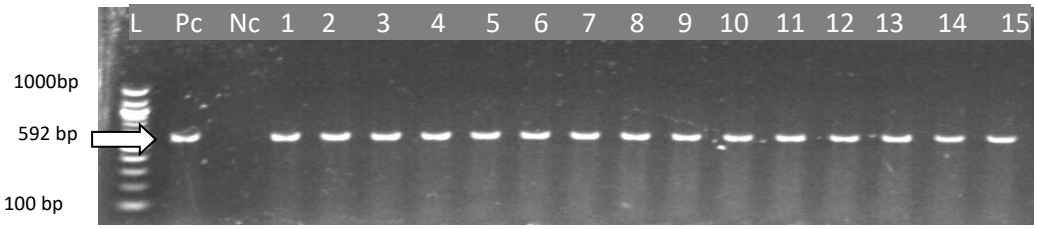



**Figure 7.** Microgram of rear integrase gene (*INT1* gene) with sites detected. L represents a molecular marker of 1.2kb, Pc is a positive control *DSM 19130 V. cholerae*, Nc is a negative control *DSM 8224Plesiomonas shigelloides*, while numbers 1-15 are positive isolates.

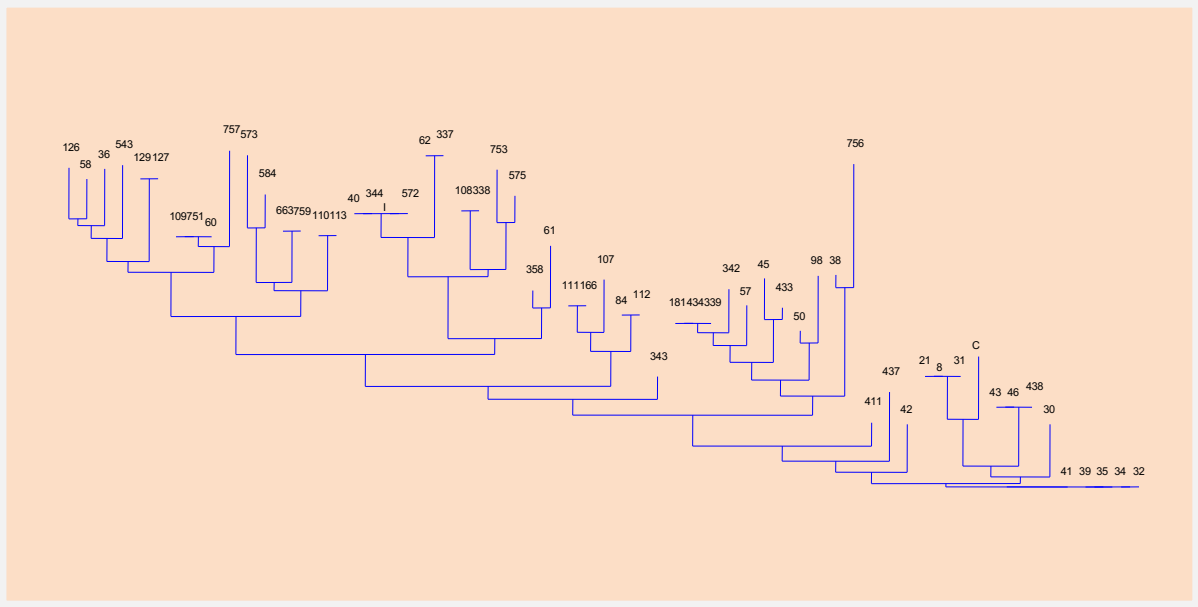
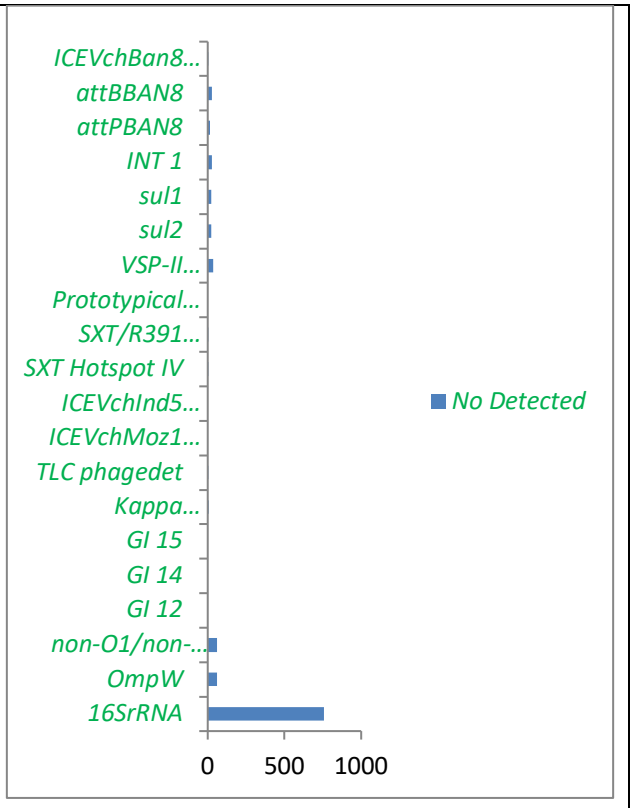



**Figure 8.** Dendrogram produced from the Neighbor joining (Euclidean paired group, kulczynski and final branching) by the multivar software of the past 3.14 version. The various isolates are discriminated or differentiated from the other as related isolates cluster while they each arise in their individual clade.

**Table 2.**The mobilome gene-typing/genotypes detected during study.

| Strain and Mobilome Genotype | Numbers Detected |
|---|---|
| 16SrRNA | 742/759 |
| OmpW | 61 (8.22%) |
| non-O1/non-O139 serogroup | 61 (8.22%) |
| GI 12 | 0 |
| GI 14 | 0 |
| GI 15 | 0 |
| Kappa phagedet | 7 (11.55) |
| TLC phagedet | 2 (3.3%) |
| ICEVchMoz10 Hotspot IV | 0 |
| ICEVchInd5 Hotspot IV | 0 |
| SXT Hotspot IV | 2 (3.3%) |
| SXT/R391 ICEs integrase | 4 (6.6%) |
| Prototypical VSP-II | 0 |
| VSP-II integrase | 34 (55.7%) |
| sul2 | 25 (41.0%) |
| sul1 | 21 (34.4%) |
| INT 1 | 29 (47.5%) |
| attPBAN8 | 14 (23.0%) |
| attBBAN8 | 29 (47.5%) |
| ICEVchBan8 integrase gene | 4 (6.6%) |




The above shows both a table and histogram representation of the mobilome genotypes observed during study and the numbers detected. No Detected means numbers detected,