Epidemiologic potentials and correlational analysis of *Vibrio* species and virulence toxins from water sources in greater Bushenyi districts, Uganda

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Adequate water supply is one of the public health issues among the population living in low-income settings. Vibriosis remain a significant health challenge drawing the attention of both healthcare planners and researchers in South West districts of Uganda. Intending to clamp down the disease cases in the safest water deprive locality, we investigated the virulent toxins as contaminants and epidemiologic potentials of *Vibrio* species recovered from surface waters in greater Bushenyi districts, Uganda. Surface water sources within 46 villages located in the study districts were obtained between June and October 2018. Standard microbiological and molecular methods were used to analyse samples. Our results showed that 981 presumptive isolates retrieved cell counts of 10–100 CFU/g, with, with (640) 65% confirmed as *Vibrio* genus using polymerase chain reaction, which is distributed as follows; *V. vulnificus* 46/640 (7.2%), *V. fluvialis* 30/594 (5.1), *V. parahaemolyticus* 21/564 (3.7), *V. cholera* 5/543 (0.9), *V. alginolyticus* 62/538 (11.5) and *V. mimicus* 20/476 (4.2). The virulence toxins observed were heat-stable enterotoxin (*stn*) 46 (82.10%), *V. vulnificus* virulence gene (*vcgCPI*) 40 (87.00%), extracellular haemolysin gene (*vfh*) 21 (70.00) and Heme utilization protein gene (*hupO*) 5 (16.70). The cluster analysis depicts *hupO* (4.46% n = 112); *vfh* (18.75%, n = 112); *vcgCPI* and *stn* (35.71%, & 41.07%, n = 112). The principal component analysis revealed the toxins (*hupO, vfh*) were correlated with the isolate recovered from Bohole water (BW) source, while (*vcgCPI, stn*) toxins are correlated with natural raw water (NRW) and open springs (OS) water sources isolates. Such observation indicates that surface waters sources are highly contaminated with an odds ratio of 1.00, 95% CI (70.48–90.5), attributed risk of (aR = 64.29) and relative risk of (RR = 73.91). In addition, it also implies that the surface waters sources have >1 risk of contamination with *vfh* and > six times of contamination with *hupO* (aR = 40, – 66). This is a call of utmost importance to the population, which depends on these water sources to undertake appropriate sanitation, personal hygienic practices and potential measures that ensure water quality.

The water niche is one of the essential nexuses of the ecosystem, which habitats numerous living organisms capable of causing diseases and spread pathogenic virulence toxins. Nevertheless, water forms the most extensive composition of the earth crust and utmost importance for every living thing. *Vibrio* spp. is among the most naturally occurring bacteria in surface water sources that are of human concern since they are implicated in vibriosis infections. Vibriosis is a generalised term used to describe elevated *Vibrio* spp. and associated infections in the intestine. While cholera is toxin induce sickness caused by *Vibrio cholerae* exotoxins released into the intestine. However, communications have shown a substantial proportion of environmental strains to be harmless and exist as commensals of marine microbiota. Although some are labelled opportunistic pathogens in humans and aquaculture, about 12 *Vibrio* species are recognised

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as pathogenic, causing human illness 2. These human pathogenic Vibrio spp. produce an array of virulence
genes or toxins linked to mild to fatal illnesses 3. For instant, toxins including Cholera toxin, cytolyisin VvhA, metal-
loprotease Vvp, flagella and RtxA toxin.

Vibrio Cholerae, the etiological agent of cholerac, produces chola toxin (CTX, Clx or CT) and toxin-coreg-
pulated pilus (TCP) as the primary virulence determinants for pathogenicity. CT encodes the ctxA and ctxB
genes located in the integrated prophage CTX `responsible for the severe loss of water and electrolyte` diarrhoea
infection. TCP encode for tcpA, utilised to colonies small intestinal epithelium by the bacterium `regulated by
the tcp regulon during expression in vivo`

Skin infections and severe gastrointestinal disorders associated with V. parahaemolyticus pathogenicity are
linked to the expression of thermostable direct hemolysin (tdh) and a gene associated with thermostable direct
hemolysin (trh) 5-9, as well the production of cytotoxic and enterotoxic effects 10. Vibrio Vulnificus and flavilus are
referred to as emerging pathogens of humans. VNdifificus is encoded the virulence-correlated gene (vgs) implicated
to cause wound infections, gastroenteritis or “primary septicemia” 11-13. However, the degree of virulence of V. vul-
ificus is related to the origin of the strain; thus, clinical strains are more virulent than environmental isolates 14.

Vibrio flavilus produce several compelling toxins, such as the stable heat enterotoxin (ctx) 15, cell elongation
factor or components like Chinese hamster ovary (CHO) or CHO cell-killing factor, lipase, cytotoxin, hemolysin
and protease 16-18 although their roles in pathogenesis are not well established. Vibrio flavilus virulence manifest
as hemorrhagic cellulates and cerebritis 14, peritonitis 16, acute otitis 16, biliary tract infection 17, bacteraeia 18
and ocular infections 18. Other virulence factors associated with flavilus include but are not limited to the V. flavilus
protease gene (vfp), heme utilisation protein gene (hupO), extracellular haemolysin gene (vfpH), and heme utilisa-
tion protein gene (hupO) 17.

The virulence toxR in cholerae and flavilus is specifically implicated in the bile resistance and the initial phase
of vibriosis disease establishment. Among the human pathogens include halophilic Vibrios, V. alginolyticus, and V.
metchnikovi as well as Vibrio mimicus via the production of virulence. Other species such as Vibrio alginolyticus
have been reported as probiotics for shrimp aquaculture 19. Vibrio parahaemolyticus Shrimp-associated gastro-
enteritis has been noted with Vibrio spp., and some of the pathogens are Vibrio cholera, Vibrio parahaemolyticus, and Vibrio Vulnificus 25 implicated in the bloody occurred diarrhoea in some patients.

The chola toxin A subunit 25 and the El Tor toxin/cytolsin/haemolysin is activated by the proteolytic effect metal-
loprotease of Vibrio cholerae, commonly called haemagglutinin/protease (Hap) seen in cholerae pathogenesis.

Though the percentage of the tap water available for use by different communities has increased, that of the
districts of greater Bushenyi is still far below the percentage that will meet the people's minimum needs. The
social, economic, and cultural bond between the local communities and the available ponds, lakes, springs and
streams continue to increase strength. It becomes challenging to isolate the people and these waters for any sig-
nificant organised study, especially in times of epidemic. There may be epileptic reports and studies at the national
level about Vibrio associated diseases but grass root coordinated surveillance to inform policy updates that can
lead to effective control of this disease is grossly inadequate and, in some cases, lacking. There is a sustained
significant upsurge in the reports of Vibrio associated and diarrheagenic disease conditions both in the districts
of greater Bushenyi and the country at large. This indicates that the national and local control mechanisms may
be wanting in their expected capacity to clamp down these diseases conditions that have continued to ravage
the ordinary citizens in remote hard to reach areas where the significant population still fall sick, get worst and
die without access to medical services and interventions34,35.

Nevertheless, new pathogenic strains and virulence continue to emerge in endemic, pandemic and spreading
to another region. It is worrisome that the precise role of most Vibrio spp., pathogenicity determinants in
producing the clinical manifestations remains unclear.

These clearly calls for coordinated local and national response to make society a better place to be. Based on
these premises, this study was designed to investigate the epidemiologic potentials of Vibrio species virulence
virulence toxins recovered from surface waters in greater Bushenyi districts, Uganda. The correlations association between
the level of virulence toxins contamination and water sources were analysed.

**Materials and methods**

**Study locations.** The surface waters sources used in the four districts of the Western region of Uganda,
including Bushenyi, Mitooma, Rubirizi and Sheema, were sampled for epidemiologic potentials of six pathogenic
Vibrio species and virulence toxins. According to WHO, standard classification for drinking-water quality36. The
surface waters used such as tap water, groundwater (borehole, open spring, ground running water, raw water,
well water), Lakes, and fish pond were sampled from each of the 19 points in Bushenyi, 8 points in Mitooma, 11 points in Riburizi, and 8 points in Sheema districts as shown in Fig. 1A–D. Focus group discussion was organised with stockholders that assisted in getting to the study’s sampling identification. The group comprising (investigators, interpreters, microbiologists, district health officers, village local chairperson (LCIs)) were selected base on their relevance to this study. To be more specific, the investigators outlined the purpose and objectives of the study; the district health officer assisted in suggesting the sampling points that fit the purpose of the study. The microbiologist provided the advice that ensured that sampling was aseptic. The LCIs confirmed that the water for sampling is present in the 46 villages and nominated the village health teams (VHTs). After the focus group discussion, the VHTs nominated by the LCIs took the investigators to the sampling points as shown in Fig. 1A–D.

The maps of the study areas in (Bushenyi; Mitooma; Rubirizi; and Sheema districts) was created using the open-source software QGIS desktop version 3.0. The United States Geographical Surveys (USGS) provided the Sentinel-2 image ID: L1C T35MRV A025815 20200601T082835 dated 2020/9/7, which was overlay on a shapefile for Uganda and highways, + a satellite image file was adjusted to show land vegetations.

Collection of samples, processing and enumeration of Vibrio spp.. A total of 46 villages was visited to obtain samples between June 2018 and October 2018. Using sterilised Nalgene glass bottles (1000 ml) water samples were salvaged for four months in different sampling points each of the four Districts (Bushenyi, Mitooma, Rubirizi and Sheema) of South West of Uganda and conveyed on an ice-cool box to the department of medical microbiology laboratory, Kampala International University, Western-campus, Uganda for analysis within 6 h. Ten-folded dilution was carried out on the water samples as described by, three series (10–1, 10–2 and 10–3) of which 1 ml of each dilution was spread plated onto thiosulphate citrate bile salts sucrose (TCBS) agar (Neogen, Lansing, MI 48912 USA) in triplicates for 24 h at 37 °C. The presumptive Vibrio spp., counted and expressed in colony-forming units per millilitres (CFU/ml) of water samples for the yellow and green colonies identified by colonial morphology and cultural characteristic of the colony. A single colony of the presumptive isolates was subsequently subcultured onto nutrient agar to ascertain purity; each sample’s pure culture was picked and stored in glycerol stock for further analysis at the Applied and Environmental Microbiology Research Group (AEMREG) laboratory, Department of Biochemistry and Microbiology, University of Fort Hare, South Africa.

Molecular confirmation of presumptive Vibrio genus and delineation into six pathogenic Vibrio species. The glycerol stocks were resuscitated using nutrient broth (Merck, Modderfontein, South Africa) and incubation for 24 h at 37 °C, while the genomic DNA of the 981 presumptive Vibrio spp., isolates were extracted following the boiling procedure described by with modifications. The fresh overnight cultured isolates were subcultured into a sterile 1.5 ml microfuge tube and centrifuged (HERMLE, Siemensstr-25, D-78564 Wehingen, Germany) at a speed of 13,000 rpm for 10 min. The cells pellet was washed twice with phosphate-buffered saline, suspended on 500 µl sterile distilled water, and then lysed to release the DNA by boiling at 100 °C for 10 min pre-heated heating block (Techne heating block Dri-Block, DB-3D; Gauteng, Pretoria, South Africa). Afterwards, the suspension was centrifuged for 5 min at 15,000 rpm, and the supernant was carefully pipetted into sterile Cryon tubes and stored at − 20 °C pending for use as a genomic DNA template PCR assays. The primer pair F-5′CGG TGA AAT GCG TAG AGA T-3′ and R-5′TTA CTA GCG ATT CCG AGT TC-3′ previously described by, was purchased from Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa and used to amplify 16s RNA genes of Vibrio spp. with the ampiclon size of 663 which was further delineated into the six pathogenic Vibrio species using the primers and condition in Table 1. The PCR reaction mixture of 25 µl (12 µl PCR master mix (New England BIOLABS), 1 µl of each forward and reverse primers, 6 µl of PCR grade water and 5 µl of genomic DNA template were amplified using BioRad T100 thermal Cycler Lasec. (621BR44012, Singapore). Afterwards, 4 µl of the amplicons were electrophoresed in 1.5%-agarose gel using the thermal tank (Labnet, Enduro Gel XL, USA) on staining with ethidium bromide (0.5 µl) and 0.5× Tris–borate EDTA (TBE) buffer with a controlled base size of 100-bp DNA ladder (New England BIOLABS, Madison, WI, USA). A 100 Volt and 60 min electrophoresis process was done, and the gels were visualised under the UV trans-illuminator (Alliance 4.7, UVItec), Merton, London, UK.

Evaluation of virulence genes signature of Vibrio spp. recovered isolates. The virulence genes signature distribution in the confirmed Vibrio spp. isolates using PCR technique as described by with modifications. The sets of primers indicating the targeted genes, sequence and conditions are presented in Table 2. The genomic DNA templates of confirmed isolates of Vibrio spp., including (62-Vibrio alginolyticus, 30-Vibrio fluvialis, 46-Vibrio vulnificus, 20-Vibrio parahaemolyticus, 21-Vibrio mimicus, 5-Vibrio cholerae) recovered from surface waters by adopting the earlier reported protocol described by. and the PCR reaction mixture was made up to a final volume of 25 µl while the amplified amplicons were electrophoresed and visualised as stated earlier.

Safety for research staff and environment. After the entire experiment, all specimens and isolates were decontaminated using autoclave at 121 °C, 15 PSI for 15 min. The decontaminated specimen and isolate were incinerated, and the ash was buried at the designated spot.

Statistical analysis. The result was entered into Microsoft excel. The distribution of toxins in the water sources was analysed using the violin box plot by considering Dunn’s post hoc test to compare the occurrence of virulence toxin across the surface waters sources. Using multi-cluster analysis and Spearman’s correlation
Table 1. PCR condition and primer sets used for the screening of *Vibrio* spp.

| Species | Primer | PCR primer sequence (5′–3′) | Amplicon size (bp) | PCR cycling condition | References |
|---------|--------|-----------------------------|-------------------|-----------------------|------------|
| *Vibrio* | 16S rRNA | CCGTTGAAATTCGCTGAGAT TTACTAGCGATGCGAGTTCT | 663 | Initial denaturation (94 °C for 5 min), denaturation (94 °C for 30 s), 35 cycles, annealing (52 °C for 30 s), extension (72 °C for 30 s), final extension (72 °C for 10 min) | 44 |
| *V. parahaemolyticus* | toxR | TGTACCGTTGAGACCCCTAA CACGTCTCTGACGCTGAG | 503 | Initial denaturation (94 °C for 5 min), denaturation (94 °C for 30 s), 35 cycles, annealing (55 °C for 30 s), extension (72 °C for 30 s), final extension (72 °C for 10 min) | 44 |
| *V. vulnificus* | vhA | ACTCAACATCTTGCGCAAG ACACGTTGTACGCGAG | 366 | Initial denaturation (94 °C for 5 min), denaturation (94 °C for 30 s), 35 cycles, annealing (55 °C for 30 s), extension (72 °C for 30 s), final extension (72 °C for 10 min) | 44 |
| *V. cholerae* | toxR | GAAAGCGTCTCTGATGC ATGAACTGCGGATGTTTAC | 275 | Initial denaturation (94 °C for 5 min), denaturation (94 °C for 30 s), 35 cycles, annealing (55 °C for 30 s), extension (72 °C for 30 s), final extension (72 °C for 10 min) | 44 |
| *V. fluvialis* | toxR | GGATACCGCGCTTGAGATG GTAAGCTGCGGACTGCGTGTG | 217 | Initial denaturation (94 °C for 5 min), denaturation (94 °C for 60 s), 35 cycles, annealing (57 °C for 60 s), extension (72 °C for 90 s), final extension (72 °C for 7 min) | 44 |
| *V. alginolyticus* | toxR | GAGACCGCAGAACGGAAGA CCGTCCGCGGATGCTGGTG | 338 | Initial denaturation (93 °C for 5 min), denaturation (93 °C for 60 s), 30 cycles, annealing (55 °C for 60 s), extension (72 °C for 60 s), final extension (72 °C for 7 min) | 44 |
| *V. mimicus* | VM-F | CAGTTGCTGCGGACGGGAAGA CTTGAGAGGCGCGGTCGAGC | 177 | Initial denaturation (93 °C for 5 min), denaturation (93 °C for 30 s), 30 cycles, annealing (55 °C for 1 min), extension (72 °C for 1.5 min), final extension (72 °C for 7 min) | 44 |

Table 2. PCR condition and primer sets used for the screening of *Vibrio* virulence toxins (genes).

| Virulence toxin (genes) | PCR primer sequence (5′–3′) | Amplicon size (bp) | PCR cycling condition | References |
|-------------------------|-----------------------------|-------------------|-----------------------|------------|
| tdhF | GGTCTACAAATGCTGACATC | 199 | Initial denaturation (93 °C for 5 min), denaturation (92 °C for 30 s), 30 cycles, annealing (55 °C for 60 s), extension (72 °C for 60 s), final extension (72 °C for 7 min) | 53 |
| tdhR | CCACTACCTCTCCTATAGC | 250 | | 53 |
| trhF | CATTCTCGCTCCTCATATGC | 278 | Initial denaturation (93 °C for 5 min), denaturation (94 °C for 40 s), 35 cycles, annealing (56 °C for 1 min), extension (72 °C for 60 s), final extension (72 °C for 7 min) | 53 |
| trhR | GGCTCAAAATGCTGACAGG | 278 | Initial denaturation (94 °C for 5 min), denaturation (94 °C for 40 s), 35 cycles, annealing (49 °C for 40 s), extension (72 °C for 60 s), final extension (72 °C for 7 min) | 53 |
| vgeCP1 | AGCTGCAGGATACGATCT | 278 | Initial denaturation (93 °C for 5 min), denaturation (94 °C for 60 s), 30 cycles, annealing (57 °C for 60 s), extension (72 °C for 60 s), final extension (72 °C for 7 min) | 54 |
| vgeP3 | CGCTTACGATCGTGGTG | 278 | Initial denaturation (94 °C for 5 min), denaturation (94 °C for 40 s), 35 cycles, annealing (49 °C for 40 s), extension (72 °C for 60 s), final extension (72 °C for 7 min) | 54 |
| vgeEP2 | CTCAATTGCGATCTATCT | 278 | Initial denaturation (94 °C for 5 min), denaturation (94 °C for 40 s), 35 cycles, annealing (49 °C for 40 s), extension (72 °C for 60 s), final extension (72 °C for 7 min) | 54 |
| vgeP3 | CGCTTACGATCGTGGTG | 278 | Initial denaturation (94 °C for 5 min), denaturation (94 °C for 40 s), 35 cycles, annealing (49 °C for 40 s), extension (72 °C for 60 s), final extension (72 °C for 7 min) | 54 |
| vh-F | GGGTGATGTTGCTGGTGAAG | 800 | Initial denaturation (94 °C for 15 min), denaturation (94 °C for 40 s), 35 cycles, annealing (50–60 °C for 40 s), extension (72 °C for 60 s), final extension (72 °C for 7 min) | 17 |
| vh-R | TCGTCTGAGCGCTCTGCTCTT | 600 | | 17 |
| hupO-F | ATACGCCAAGCAAGGTGGGAC | 1790 | Initial denaturation (93 °C for 15 min), denaturation (92 °C for 40 s), 35 cycles, annealing (50–62 °C for 60 s), extension (72 °C for 90 s), final extension (72 °C for 7 min) | 17 |
| hupO-R | ATTGAGATGTTAAACAGCCGTC | 375 | | 17 |
| vfpA-F | TACAAGCTCAGTTAAAAAGG | 1790 | | 17 |
| vfpA-R | GTAGGCGTCTGAGCCCTTCA | 375 | | 17 |

Table 3. Surface waters sources sampled in the districts. Author compilation as found in the districts.

| Surface waters sources | Uses |
|------------------------|------|
| Bohole water | Irrigation and domestic use |
| Fish pond | Fishing and farming |
| Ground running water | Car washing, farming, animal rearing and domestic use |
| Hot spring | Domestic use, spiritual and recreational purposes |
| Lake | Fishing, farming, irrigation, swimming, animal rearing, and domestic use |
| Natural raw water | Animal rearing, irrigation, national treatment and supply for domestic use |
| Open springs | Domestic uses, irrigation and farming |
| Tap water | Domestic uses, irrigation and farming |
| Well water | Domestic uses and farming |
coupled with Principal Component Analysis (PCA) was used to understand the correlations between the *Vibrio* spp., virulence toxins and surface water sources. All in RStudio version 3.5.1 software. Furthermore, the significant epidemiological prevalence and risk estimate of toxins contaminations at 95% confidence interval evaluated in WINPEPI software version 11.65. All statistical significant differences were recognised at p < 0.05.

**Ethical consideration.** The protocol for this study was reviewed by the research ethics committee of the Kampala International University, Western-Campus, Uganda, and obtained a clearance number of Nr.UG-REC-023/201919.

**Results**

**The Surface waters sources studied in the region.** South-Western Uganda is endowed with water resources and forest game reserved. The temperature ranges relatively between 19 and 24 °C through the year, even with the seasonal variations. We identified and studied nine primary types of surface waters sources used by the populaces in 46 villages of the greater Bushenyi districts. These surface waters were surveyed for the distribution and prevalence of *Vibrio* spp. and virulence toxins. The surface waters used by the general population across numerous purposes of life from domestic to agricultural uses and recreational/medical tourist attractions in the districts are highlighted in Table 3. The map of sample collection points in the four districts are shown in Fig. 1A–D.

The total *Vibrio* cell densities count from the surface waters used in the districts. The mean of presumptive *Vibrio* cell densities counts from the surface Waters from the districts are expressed in log10 CFU per gram. The results showed ranged values of 0.125–2.321 log10 in Bushenyi district; the sampling points have no even *Vibrio* cell counts. There are almost cell counts in all the sampling points in August and September except for few points. In the Mitooma district, the months has relatively moderate cell counts across the sampling points, with some months without cell counts in some sampling points. However, the cells count range from 0.753 to 2.474 log10. Also, in the Sheema district, the *Vibrio* cell counts range from 0.397 to 2.215 log10. There were relatively cell counts in all the sampling, only the month of September has cells count in the sampling point Nyakatomo Ibare open spring and no cell count in Kashebyi trading centre open spring. In the Rubirizi district, the cells count range from 0.301 to 2.426 log10. Rutoto borehole only has cells count in June, while the Butare town council well only has cells count for September and October. This implies that presumptive *Vibrio* species are high in some water in other months, as we see in the mean cell count in Fig. 2A–D.

The distribution of six pathogenic *Vibrio* spp. in surface waters in greater Bushenyi districts. Out of 981 presumptive isolates, (640) 65% were confirmed to be *Vibrio* genus using the polymerase chain reaction analysis as we reported and gel picture in Supplementary S1. The result of the distribution of the six pathogenic *Vibrio* species recovered from the water sources includes; *V. vulnificus* 46/640 (7.2%), *V. fluvialis* 30/594 (5.1%), *V. paraaeromlyticus* 21/564 (3.7%), *V. cholerae* 5/543 (0.9%), *V. alginolyticus* 62/538 (11.5%), *V. mimicus* 20/476 (4.2%) Table 4 and gel pictures in Fig. 3A–E.

Molecular identification and distribution of virulence toxins in the surface waters sources used in the region. Of the 316 *Vibrio* spp. strains screen for virulence toxins, 112 (35.44%) were positive to molecular PCR techniques. The gel pictures of the molecular characterisation of the majority of the virulence toxin are shown in Fig. 4. The results of the distributions of the toxins in the study surface waters reveal that Lake surface waters sources harbors virulence toxins 31 (27.68%) and natural raw waters sources 21 (18.75%) and open springs water sources 15 (13.39%) with the Hot Spring least of 1 (0.89). The occurrence of virulence toxins in the Surface waters sources varied significantly in distribution (BW and L, Dunn's post hoc, p = 0.0393, HS and L, p = 0.0054, HS and NRW, p = 0.0348). The amalgamated violin and box plots, violin expanse, displays the distribution by adding the mild and extreme outliers. The box plot displays the median at concentration ellipse of 25–75% percentiles in Fig. 5.

Prevalence and epidemiological significance of virulence toxins in the surface waters sources used in the region. The pathogenicity of Vibrioses is tied to the expression of the virulence toxins leading to the unending endemic infections in population and region. Among the twelve pathogenic endemic virulence toxins, heat-stable enterotoxin (*stn*) 46 (82.10%), *V. vulnificus* virulence genes (*vcgCPI*) 40 (87.00%) were found to be prevalent in surface waters sources, showing that the surface waters sources are highly contaminated with an odds ratio of 1.00, 95% CI (70.48–90.5), attributed risk of (aR = 64.29) and relative risk of (RR = 73.91). In addition, the Extracellular haemolysin gene *vfh* and Heme utilisation protein gene *hupO* prevalence was 21 (70.00) and 5 (16.70) implies that the surface waters sources has > 1 risk of contamination with *vfh* and > six times of contamination with *hupO* (aR = 40, -66). Nevertheless, all other virulence toxins investigated in this study showed a 0.00% prevalence and no contamination (aR = -100). The burden of risk of *vcgCPI* virulence toxin contaminations of the surface waters sources is of significant concern to the public (RR = 6.67) identified as shown in Table 5.

Using the multi-way cluster analysis, the pathogenic species virulence toxins was grouped according to their frequency of occurrence. Four clusters of virulence determinants were distinguished on the base of their frequency of occurrence (percentage number) and the surface water they were identified in. The calculations showed that the virulence toxins (*vcgEP2*, *vfpA*, *trd, trh, flaE,ompU, zothyla*) clustered into no. 1, (0.00%, n = 112); *hupO* clustered into 2, (4.46% n = 112); *vfh* clustered into no. 3, (18.75%, n = 112); and *vcgCPI* and *stn* clustered into no.
Figure 2. (A) Presumptive Vibrio counts from 19 selected sampling points in Bushenyi subcounties. The graph shows a plot of the seasonal average of log10 CFU/100 ml for Vibrio counts over the period of June 2019–October 2019. (B) Presumptive Vibrio counts from 11 selected sampling points in counties of Mitooma Districts. The graph shows a plot of the seasonal average of log10 CFU/100 ml for Vibrio counts over the period of June 2019–October 2019. (C) Presumptive Vibrio counts from 13 selected sampling points in counties of Sheema Districts. The graph shows a plot of the seasonal average of log10 CFU/100 ml for Vibrio counts over the period of June 2019–October 2019. (D) Presumptive Vibrio counts from 11 selected sampling points in counties of Rubirizi Districts. The graph shows a plot of the seasonal average of log10 CFU/100 ml for Vibrio counts over the period of June 2019–October 2019.
4, (35.71%, and 41.07%, n = 112) Fig. 6. But when converting results by only considering the number of specific virulence toxin contaminations in isolates, the frequency of occurrence was toxins vcgEP2, (0.00% n = 46), vfpA, (0.00% n = 30); trd, trh, flaE (0.00%, n = 21); ompU, zot, hylA (0.00% n = 5); hupO, (16.70% n = 5); vfh (70.00%, n = 21); vcgCPI and stn (82.10%, 87.00%, n = 46, 40) respectively. The Spearman’s coefficient (rho) indicated that statistically significant risk was posed by the virulence toxins stn (rho = 0.001) and/or vcgCPI (rho = 0.007), vfh (rho = 0.037) in surface water sources while for hupO (rho = 0.119) and in the case of other toxins the Spearman’s coefficient were statistically insignificant.

Correlations patterns between Vibrio spp. virulence toxins and surface water sources. We use principal component analysis PCA to study the multivariate association between the distribution of Virulence toxins and Surface waters sources. The results obtained from the PCA in Fig. 7 showed correlations between virulence toxins level contamination and Surface water sources of the isolated Vibrio spp. Interestingly, the Vibrios virulence toxins (trd, vfpA, trh, vcgEP, zot, flaE, ompU) show no correlation with the isolates recovered from the water sources. On the other hand, the toxins (hupO, vfh) are positively correlated with the isolate recovered from the bohole water (BW) source. Similarly, (vcgCPI, stn) toxins are positively correlated with natural raw water (NRW) and Open Springs (OS) water sources isolates.

To better understand the result, details of variables contained in PCA1 and PCA2, which explain the total correlation of 91.7%, were analysed further as recommended by scree plot of eigenvalues evaluation (p < 0.05). The...
Discussion

For the past decade, an array of several virulence determinants implicated in the pathogenesis of vibriosis have been reported both from clinical and environmental strains across the world. This study screened the prevalence and epidemiological significance of the virulence toxins stn, vgcCPI, vgcEP2, vfh, hupO, vfpA, trd, trh, flaE, ompU, and zot by PCR. The density of the *Vibrio* bacteria enumerated varies between months and water sources, with the highest densities recorded in September compared to other months (Fig. 2A–D). This may be due to changes in moisture and poor hygienic practices in some districts where poor probability orientation—also, the majority of the population living in the hard-to-reach region practice open defecation. The geographical location of the sampling sites has a significant contribution to the changing pattern of *Vibrio* densities observed in the respective months. However, the *Vibrio* densities obtained are sufficient to initiate an infection in humans, which is in harmony with the studies of various investigators. In addition, *Vibrio* species quantity (of about 2 × 10^5 to 3 × 10^7 CFU/ml) with a cultivation time spanning 4–96 h (approximately 15 h) is sufficient to cause acute gastroenteritis as previously reported by various investigators. Table 4 and Fig. 3 show that the pattern or profile of *Vibrio* strains recovered from the environmental samples depicts the magnitude of pathogenicity seen among *Vibrio* strains. Interestingly, the two major classes of *Vibrio* spp. and its associated infections were adequately represented among the isolates recovered. Thus, cholerae strain (ompW) and non-cholera strains (*V. vulnificus* (toxR), *V. fluvialis* (toxR), *V. parahaemolyticus* (toxR), *V. alginolyticus* (Vg ygb), *V. mimicus* (VM) were the observed prevalence strains.

Such *Vibrio* spp., infections could be worse in the immune-compromised individual; all the same, the infections are often self-limited. This is also similar to reports that the worst infection is observed in immunosuppressed patients. Major virulence toxins are expressed by *Vibrio* strains, including *V. cholerae*, *V. parahaemolyticus*, and *V. mimicus*. Interestingly, our result shows a very much high prevalence of 46/56 (82.10%) in the studied water sources as depicted in Table 4 (Figs. 4, 5), compared to the study of, which shows a relatively low frequency of 28.2% amongst Non-O1/non-O139 strains. Their study revealed 10.5% of toxicogenic *V. cholerae* O1 and 14.3% among O139 serogroups belonging to *Vibrio cholerae*, which are recovered from environmental samples in Europe. In Thailand, 10/21 of clinical isolates were recovered, and 26/193 (13.5%) in *V. fluvialis* of an environmental specimen of South Africa.

All the *Vibrio* strains studied show 35.44% positive to the virulence genes, and specifically, its result is as follows: Lake (27.68%), natural raw water sources (18.75%) and open Springs (13.39%), as shown in Table 5, indicating a zero-tolerance limit for vibriosis infections.

Results show that the virulence toxins include stn (82.10%), n = 56, vgcCPI (87%) n = 46, vfh (70%) n = 21, where the most highly prevalent. Whereas vgcEP2, hupO, vfpA, trd, trh, flaE, ompU, zot, hlyA were not detected in the surface waters in the region Fig. 6. Surprisingly, the results show a higher prevalence of *Vibrio* spp., virulence toxins or genes than the report in South Africa—1.0–13.5% in Europe, the surface waters in the region Fig. 6. Surprisingly, the results show a higher prevalence of *Vibrio* spp., virulence toxins or genes than the report in South Africa—1.0–13.5%. In Europe, stn/sto genes 28.2%, in Bangladesh tcpl, tcpA, ctxA, and zot (0.2–2%), hlyA, rtxA, hap, and TSS (82–99%) and T3SS (7–13%) genes. The results indicate the higher risk and potential public health threat of surface water contamination by *Vibrio* spp., virulence toxins. On the other hand, our result was opposite to the findings, where all the *Vibrio* strains were positive for genes vfh, hupO and vfpA negative for gene *stn* encoding the toxin NAG-ST enterotoxin.

The absence of thermostable direct haemolysin (tdh) and the trh related haemolysins (trh) virulence toxins responsible for the pathogenicity of *V. parahaemolyticus* is in agreement with the recent report of genes for cholerae toxin (ctx), thermostable direct hemolysin (tdh), or zonula occludens toxin (zot) as there were not detected in any of 116 isolates of seawater in Norway. Similar to the study of Lake isolates in Ohio US, and of ctxA, tcpA, and zot were not detected in the *V. cholerae* strains, while hlyA, rtxA, and rtxC were positive for water sample isolate in China. However, these toxins have been reported in a relatively low occurrence in environmental samples in Malaysian, in Turkey and in Atlantic coast in Spain, in Italy. Also, it has been reported that the highly cytotoxic and human gastrointestinal infecting *Vibrio parahaemolyticus* strains of environmental origin with no detection of the tdh or trh were observed. We are not surprised about the difference and absence of some virulence toxins in the *Vibrio* strains. Most virulence toxins predominates in clinical isolate sources of toxicogenic vibriosis, e.g. ctxAB or tdh and trh are predominant in a clinical strain of *V. cholera* and *V. parahaemolyticus*. Although Virulence toxinsgenes hupO, vfh and vfpA are often detected in *V. fluvialis* of both patient isolates, and seafood isolates strains, some *Vibrio* strains virulence can be prevalent irrespective of the origin.

The *Vibrio* strains may have acquired the virulence toxins *stn* by horizontal gene transfer or natural genetic exchange by Organism interactions in the ecosystems or human host. The significant role of *stn* is unclear in vibriosis pathogenesis (Table 5). However, the high frequency of concern is a threat to the population using the water sources.

The result of the virulence-correlated gene (vgc) of *Vulnicus*, vgcCPI for clinical (C-) genotypes and vgcEP2 environmental (E-) genotypes were observed to vgcEP2 40/46 (87%), and vgcCPI 0/46 (0%) is similar to the findings of 137 of E genotype and 53.1% 155 of C genotype Of the 292 isolates recovered from water samples, and also related to the study of 67/47 (8.1%) had the vgcC, and 68/74 vge (91.9%). However, it is surprising to find clinical genotypes highly prevalent in the surface waters in our study while there are no environmental isolates detected. Also, we cannot say if these *V. vulnificus* isolates indicate less or high virulent strains, as it has been reported that the vgcC gene linked to clinical isolates is potentially more...
A virulent vcgE linked to environmental isolates is less virulent. It also implies a difference to the report where almost an equal per cent of vcgE (46.9%) and vcgC (53.1%) were detected from oyster isolates and water areas surrounding oyster harvest. Consequently, it is of necessity to continuously monitor surface water source uses, although *Vulnificus* infection is frequent in aquaculture and rare in humans but can be fatal in immunocompromised persons, causing wound ulceration infections, gastroenteritis or septicemia.

### Table 4. Distribution of six human pathogenic *Vibrio* spp. by PCR, Risk estimate (relative risk and attributable risk), odds ratios in surface waters of greater Bushenyi Districts. CI confidence interval, LL lower limit, UL upper limit, RR relative risk, aR attributed risk.

| Human pathogenic *V. species* | Pathogens | 95% CI (LL–UL) prevalence | RR | aR | Odds ratios | p-value |
|-------------------------------|-----------|---------------------------|----|----|-------------|---------|
| *V. vulnificus*               | 46 (7.2)  | 5.37–9.39                 | 0.08 | – | –85.63 | 1 |
| *V. fluvialis*                | 30 (5.1)  | 3.5–7.04                  | 0.05 | – | 0.687 | 0.463 |
| *V. parahaemolyticus*         | 21 (3.7)  | 2.38–5.54                 | 0.04 | – | 0.499 | 0.039 |
| *V. cholerae*                 | 5 (0.9)   | 0.34–2.03                 | 0.01 | – | 0.12  | 0 |
| *V. alginolyticus*            | 62 (11.5) | 9.03–14.43                | 0.13 | – | 1.682 | 0.051 |
| *V. mimicus*                  | 20 (4.2)  | 2.66–6.30                 | 0.04 | – | 0.566 | 0.156 |

Figure 3. (A–E) Gel picture representing molecular confirmation of the pathogenic *Vibrio* species. For (A), Lane M: Molecular Marker (100 bp); Lane 1: Negative control; Lane 5, 6, 7 (*V. parahaemolyticus* (toxR) and *V. fluvialis* (toxR)); Lane 12, 13: *V. cholerae* (ompW). While (B–E), Lane L: Molecular Marker (100 bp); Lane N: Negative control and 1, positive control. (B) = *vulnificus* (vvhA), (C) = *fluvialis* (toxR), (D) = *alginolyticus* (Vg gyrB), (E) = *mimicus* (VM).

Figure 4. Gel picture representing molecular characterisation of virulence toxins. (A) (stn virulence toxins) Lane 1–11 positive, (B) (vfh virulence toxins) Lane 1–11 positive. Lane L: Molecular Marker (100 bp); Lane N: Negative control.
The result also showed a prevalence of 21/30 (70%) of vfh genes in the *Vibrio* flavilis strains, depicting health as significant as the virulence phenotypes were predominant in this species. *Vibrio* cholera and *Vibrio* vulnificus utilise 70% of vfp toxin acting as homologous precursor proteins of metalloproteases during pathogenicity in humans. Specifically, Vfh expressed by *Vibrio vulnificus* protease is implicated in proteolytic activity as well in haemagglutinating enhancing permeability and haemorrhagic activities. Also, the report showed that vfp virulence is predominant in *Vibrio* strains of clinical origin of and the expression of these toxins could be more virulent in their pathogenesis. Applying multi-way statistical computations provides a novel technique to interpret data on the prevalence of virulent toxins and contaminations levels associated with the water sources. Food and water research rarely adopt such calculations, except in few studies. However, this approach has not been untaken in literature; it gave us a more in-depth characteristic of *Vibrio* virulence toxin prevalence in the water samples. The multi-way computations enabled us to identify stn and vcgCPI as the most frequent toxins occurrence in sources terms of percentages. The PCA results indicate the prevalence of toxins in correlations to their sources; the analysed shown hupO and vfh are associated with bohole water (BW) source while vcgCPI, and stn, are positively correlated with

![Figure 5. Distribution of *Vibrio* spp. Virulence determinants varied significantly on the surface waters in the districts by (Dunn's post hoc, p ≤ 0.05). BW Bohole Water, FP fish pond, GRW ground running water, HS Hot Spring, L Lake, NRW natural raw water, OS open Springs, TW tap water, WW well water.](https://example.com/image.png)

**Table 5.** Prevalence distribution of virulence toxins of human pathogenic *Vibrio* spp. by PCR (relative risk and attributable risk), odds ratios in surface waters of each district. CI confidence interval, LL lower limit, UL upper limit, RR relative risk, aR attributed risk.

| Name of virulence toxins | Genes | Isolate tested | Virulence toxins | 95% CI of p LL; UL | RR | aR | Odds ratios | p-value |
|--------------------------|-------|----------------|-----------------|-------------------|----|----|-------------|---------|
| Heat stable enterotoxin  | stn   | 56             | 46 (82.10)      | 70.48–90.56       | 4.60 | 64.29 | 1.00         | -       |
| Virulence-correlated gene| vcgCPI| 46             | 40 (87.00)      | 74.83–94.54       | 6.67 | 73.91 | 0.45         | 1.000   |
| Virulence-correlated gene| vcgEP2| 46             | 0 (0.00)        | 0–6.3             | 0.00 | 0–100 | 0.00         | 0.000   |
| Extracellular haemolysin gene| vfh   | 30             | 21 (70.00)      | 15.73–47.97       | 2.33 | 40 | 0.51         | 0.917   |
| Heme utilization protein gene| hupO | 30             | 5 (16.70)       | 6.37–33.15        | 0.20 | 66 | 0.04         | 0.000   |
| Haemolysin toxin          | vfpA  | 30             | 0 (0.00)        | 0–9.50            | 0.00 | 0–100 | 0.00         | 0.000   |
| Thermostable direct hemolysin-related gene| trd | 21             | 0 (0.00)        | 0–13.29           | 0.00 | 0–100 | 0.00         | 0.000   |
| Thermostable direct hemolysin| trh | 21             | 0 (0.00)        | 0–13.29           | 0.00 | 0–100 | 0.00         | 0.000   |
| Flagellar genes           | flaE  | 21             | 0 (0.00)        | 0–13.29           | 0.00 | 0–100 | 0.00         | 0.000   |
| Outer membrane proteins   | ompU  | 5              | 0 (0.00)        | 0–45.07           | 0.00 | 0–100 | 0.00         | 0.001   |
| Zonula occludens toxin    | zot   | 5              | 0 (0.00)        | 0–45.07           | 0.00 | 0–100 | 0.00         | 0.001   |
| Protease gene             | hylA  | 5              | 0 (0.00)        | 0–45.07           | 0.00 | 0–100 | 0.00         | 0.001   |
| Total                     |       | 316            | 112 (35.44)     |                   |     |     |             |         |
natural raw water (NRW) and open springs (OS) water (Fig. 7). This study has expanded the baseline databases for *Vibrio* spp., and associated infections in this region. Therefore, the findings of this study have provided the basis for future studies lasting up to 3 years design to establish a trend.

**Conclusion**

In this study, the analysed *Vibrio* spp., recovered from the water sources used in the region of Uganda, were found to harbour virulence toxins of significant potential health concern. This is concerned with causing diseases associated explicitly with diarrhoeagenic infections, septicemia, and outbreaks of vibriosis in the region where there is an inadequate water supply or water treatment. The heat-stable enterotoxin (*stn*) and *V. vulnificus* virulence genes (*vcgCPI*) were the most frequently occurring toxins in Lakes and natural raw water in the region. The use

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**Figure 6.** Identified *Vibrio* spp. Virulence determinants contingent on their presence in Surface Waters.

**Figure 7.** PCA biplot for correlations between the characterised *Vibrio* spp. Virulence determinants and Surface Waters. *BW* bohole water, *FP* fish pond, *GRW* ground running water, *HS* hot spring, *L* Lake, *NRW* natural raw water, *OS* open springs, *TW* tap water, *WW* well water. A PCA biplot for correlating the water sources and the virulence toxins of *Vibrio* spp., the various colour shows their prevalence and dispersion.
of computational analysis turned out to be an effective tool in evaluating the distribution of Vibrio spp., virulence toxins in water used in the studied districts. The first study specifically evaluated the prevalence of V. vulnificus, V. fluvialis, V. parahaemolyticus, V. alginolyticus, and V. mimicus among Vibrio spp., and the associated virulence toxins from water sources in South West of Uganda to the best of our knowledge. Interestingly, the findings highlight the pathogenicity and epidemiological characteristic of virulence toxins to enhance surveillance data and its epidemic-causing potential. It provided scientific evidence for the prevalence and distribution of virulence toxins in water, which is of great importance to preventing vibriosis infections and outbreaks. Improved personal and environmental hygiene, including water sanitation practices, is highly recommended.

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