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## Original Article

# Risk of Vibrio Transmission Linked to Consumption and Contact with Water in Benin.

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## ABSTRACT

**Background:** Vibrio infections have increased in Benin, and this phenomenon is expected to increase due to climate change, increased consumption of contaminated water and the number of people who are immunocompromised.

**The aim of the work:** The objective of this study was to evaluate the risk of Vibrio transmission linked to the use of contaminated water in Benin.

**Methodology:** Water samples [n = 220] were analyzed to isolate Vibrio strains using their biochemical and cultural characteristics. The species were identified by the Polymerase Chain Reaction technique by monitoring the search for genes encoding the cholera toxin of *Vibrio cholerae* [ctxA and ctxB] and the direct thermostable and thermostable hemolysins linked to *Vibrio parahaemolyticus* [tdh and trh].

**Results:** Among the 220 collected samples, the biochemical tests revealed 86 strains of Vibrio species; *Vibrio cholerae* [35%], *Vibrio parahaemolyticus* [18.60%] and *Vibrio alginolyticus* [13.95%] were identified using molecular tool. The presence of genes encoding the main virulence factors of the strains studied. Thus 6.67%, 10% and 3.33% of the strains of *Vibrio cholerae* respectively contain the toxins ctxA, ctxB and the couple ctxA and ctxB. Likewise, the *Vibrio parahaemolyticus* strains contain 12.5% tdh toxins and 31.25% [tdh and trh]. The search for genes [tdh and trh] in *Vibrio alginolyticus* was also negative.

**Conclusion:** Epidemics can be triggered by natural or fabricated events that contaminate drinking water or compromise access to safe drinking water and sanitation. The incidence of vibriosis is increasing, perhaps in part because of the spread of Vibrio species promoted by climate change and increasing water temperature.

**Keywords:** Water; Vibrio species; Polymerase Chain Reaction; Virulence Genes; Bacterial Resistance.

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\* Main subject and any subcategories have been classified according to the research topic.

## INTRODUCTION

Cholera, a disease of wars, famines and natural disasters that seemed to belong to history, is unfortunately still relevant around the world [1]. This disease is caused by ingesting food or water contaminated with the bacillus *Vibrio cholerae*, belonging to the genus *Vibrio*. In 1970, the Seventh Cholera Pandemic first hit Africa [2]. Since 2000, the incidence of cholera has gradually increased with a cumulative number of cases increased by 43% [3].

In Benin, the cholera epidemic appeared in 1970. Since then, cholera epidemics have become almost annual and generally occur at the end of the rainy season [4]. Between 2004 and 2013, epidemiological surveillance notified 5,432 cases with 48 deaths [i.e. a case fatality rate of 0.9%].

Although from 2012 to 2014 there was a decrease in the number of notified cases, in 2016 there was an upsurge in cholera where Benin recorded 874 cases including 13 deaths, i.e. a fatality of 1.96% [5].

The *Vibrio* transmission factors are well identified and mainly concerns water and food in endemic countries. The marine environment is cited as a potential vector for infections of non-cholera vibrios. This includes seafood [fish, shellfish and crustaceans], swimming and more generally all contact with the coastal environment. The density of these vibrios in the environment changes depending on various environmental factors such as water temperature, salinity, pH, turbidity, and chlorophyll A. However, only 12 of these species are considered pathogenic for human while the others were cultivated mainly from different species of aquatic animals and marine environment [6-8].

Currently, non-cholera *Vibrio* species [spp.] play an important role as causative agents of sporadic cases of cholera-like illness and isolated epidemics associated with the consumption of contaminated water [9]. Recent work in India and Bangladesh has shown that there are epidemics caused by non-cholera *Vibrio* spp. isolated in an aquatic environment [9]. However, due to its continual expansion, both inside and outside hospitals, each study of the virulence factors of *Vibrio* spp. analyzing a particular situation makes it possible to not only follow and understand its evolution but also to define control strategies at the hospital or community level. Indeed, the pathogenic *Vibrio* species are not always so. The majority of environmental strains lack the colonizing factors necessary for adhesion and penetration of toxins and/or other virulence determinants necessary to initiate disease. Nevertheless, little is known about the presence of *Vibrio* in bodies of water in Benin. However, the lagoon regions of Benin are

considered cholera endemic areas. Studies have shown that pathogens, *Vibrio cholerae* 01 in particular, can be viable and potentially pathogenic but in a dormant state during periods of unfavorable conditions in the aquatic environment [10]. Thus, this study aimed at evaluating the risk of *Vibrio* transmission linked to uses of potentially contaminated water in Benin.

## MATERIAL AND METHODS

### Study area

The study was carried out in 11 cholera endemic communes in Benin [11]. These are Abomey-Calavi, Cotonou, Porto-Novo, Seme-Kpodji, Dassa-Zoumé, Savalou, Djougou, Sô-Ava Parakou, Athiémé and Aguégoué [Figure 1].

### Sampling and samples collection

The sample size was determined using Schwartz's formula with a 95% confidence level and a 5% margin of error and a prevalence of *Vibrio cholerae* in water [14.8%] according to Madoroba and Momba [12]. This gave us a minimum height of 195 samples. A 13.4% increase was made and gave a final size of 220 samples. Per targeted endemic commune, 20 water samples were collected from June to October 2018. A total of 220 water samples were collected using the Rodier technique [13]. Briefly, 500 ml of water was taken at about 0.5 cm from the water surface in sterile glass vials.

### Physico-chemistry parameter of sampled waters

The pH and the temperature of these samples were measured with respectively an electronic pH meter [HI 96107 instruments from Hanna] and a digital thermometer [VWR EU 620-2132 NA 98000-162]. After collection, samples were transported to the laboratory in coolers containing thermal accumulators [~4°C].

### Isolation of *Vibrio* spp from water samples

The isolation was carried out according to the method of Rodier *et al.* [13]. Each water sample [10 ml] was enriched in an alkaline nutrient broth [alkaline peptone water at 30 g / l of NaCl] for 24 hours at 37°C. The haze that will develop on the surface of this medium will be seeded on Thiosulfate Citrate Bile Sucrose [TCBS, Bio-Rad®]. After 24 h of incubation at 37°C, each presumptive *Vibrio* characteristic colonies was isolated and sub cultured on 2% NaCl alkaline nutrient agar [Biorad], then incubated 24 h at 37°C to obtain pure strains. Identification was completed according to standard bacteriological methods [oxidase test, TSI test,

serogrouping and hemolysis test on blood agar medium].

### Molecular identification of *Vibrio*'s strains and detection of virulence genes

The total DNA isolated *Vibrio* strains was extract from bacterial culture using heating method [14]. Thus, a 24 h old *Vibrio* culture was suspended on sterile distilled water [500 µl] heated in a dry bath [95°C for 15 min] then centrifuged [12,000 rpm for 5 min]. To a volume of supernatant, an equivalent volume of fresh ethanol [4°C] was added in ice. After 15 min, the precipitate was recovered by centrifugation [12000 rpm for 5 min] and suspended in 50 µl of DNase free pure water. The isolated *Vibrio* species were confirmed molecularly using previously describes methods [15-18].

### Detection of Virulence Genes among the isolated *Vibrio*'s strains

Further characterization was performed to investigate the presence genes encoding virulence factors on *V. cholerae* [ctxA and ctxB] and *V. parahaemolyticus* [tdh and trh] using appropriate primers sequences [19-21]. Positive control, for the confirmation and characterization of virulence factors in *Vibrio cholerae* were obtain from the National Laboratory of Benin and Research and Training Unit on Ecology and the Control of Infectious Diseases [URF-ECMI] of the DRC. The primers used in this study and the target genes are summarized in [Table 1].

### Data analysis and processing

The averages were calculated from the results using the Excel 2016 spreadsheet. Graph-Pad Prism 8 was used to make graphs. Following identification, a simple correspondence factorial analysis was performed to determine the correlation between species [CFA] with the "CA" function of the "Facto Mine R" package [22] using the software R 3.4.0 [23].

## RESULTS

### Physico-chemistry parameter of water samples

The average values of the temperature and the pH of the water obtained from the lakes, lagoons and estuaries are presented in [Table 2].

The average pH values recorded were between 6.11±0.03 and 7.52±0. The lowest pH [acidic pH] was obtained in the communes of Athiémé [6.11 ± 0.03] and Sèmè-Podji [6.34±0.03] while the highest pH [alkaline] was recorded in the town of Savalou. Likewise, the average

temperature values recorded were between 27.85±0.1 [Savalou] and 32.91±1.7°C [Parakou].

### Microbial contamination of the water samples according to the collection places

The results of the incidence of vibrio's and other strains in the different communes are summarized in [Figure 2 and 3]. Thus, it appear that the collected water samples were contaminated by *Citrobacter freundii* [17.73%], *Vibrio* spp [32.73%], *Proteus* spp [20.45%], *Salmonella* spp [28.18%] and *Escherichia coli* [23.18%]. The lowest rate of *Vibrio* spp [15%] was obtained in the communes of Athiémé and Sèmè-Podji while a high rate of *Vibrio* spp [65%] was obtained in the commune of Savalou. In addition, *Salmonella* sp. was highly identified [60%] in the samples collected in the municipality of Aguégué. Concerning the *Escherichia coli* strains, they were highly isolated from the samples collected in the northern [85% for Parakou and 55% for Djougou] part of the country.

### Molecular Identification of *Vibrio* species

Of the 86 strains of *Vibrio* isolated from sampled waters, 13.95% belong to the species *V. alginolyticus*, 18.60% to the species *V. parahaemolyticus*, 35% to the species *V. cholerae* and 32.56% to the species *Vibrio* spp. [Table 3] shows the distribution of *Vibrio* species identified by PCR according to the municipalities.

The correlation between identified species seems weak except between *V. alginolyticus* and *V. parahaemolyticus* [Figure 4]. Analyzing this figure, the first two axes explain 70% of the total variability. On the first axis of the correlation circle: the variables *V. alginolyticus* and *V. parahaemolyticus* have a strong correlation with this axis.

The two variables contribute 97% to the formation of this axis and are very well represented. On the first axis of the individuals' cloud, an axis is strongly determined by the commune of Djougou that contributes 65% to its formation. Globally, Djougou is the city for which the variables *V. alginolyticus* and *V. parahaemolyticus* have the highest values. In the samples of Savalou, Parakou and Abomey-Calavi, high values for *Vibrio cholerae* and *Vibrio* spp. were observed while at Porto-Novo, the values for the same strains were low. This explain why Porto-Novo is opposed to Savalou, Abomey-Calavi on axis 2 [Figure 5].

### Distribution of virulence factors in *Vibrio* species.

The genes encoding major virulence factors, cholera toxin of *V. cholerae* [ctxA and ctxB] and the direct and thermostable heat-stable hemolysins of *V. parahaemo-*

*lyticus* [tdh and trh] were detected in strains of *V. cholerae* and *V. parahaemolyticus* which we characterized by PCR. Thus, the characterized *Vibrio cholerae* strains harbor the gene encoding for ctxA [6.67%], ctxB [10%] and the couple ctxA-ctxB [3.33%] toxins [Figure 6].

Likewise, the 12.5% *V. parahaemolyticus* strains contain the encoding for tdh toxins and 31.25% of them harbor both tdh and trh [Figure 7]. The search for genes [tdh and trh] in *V. alginolyticus* was also negative.

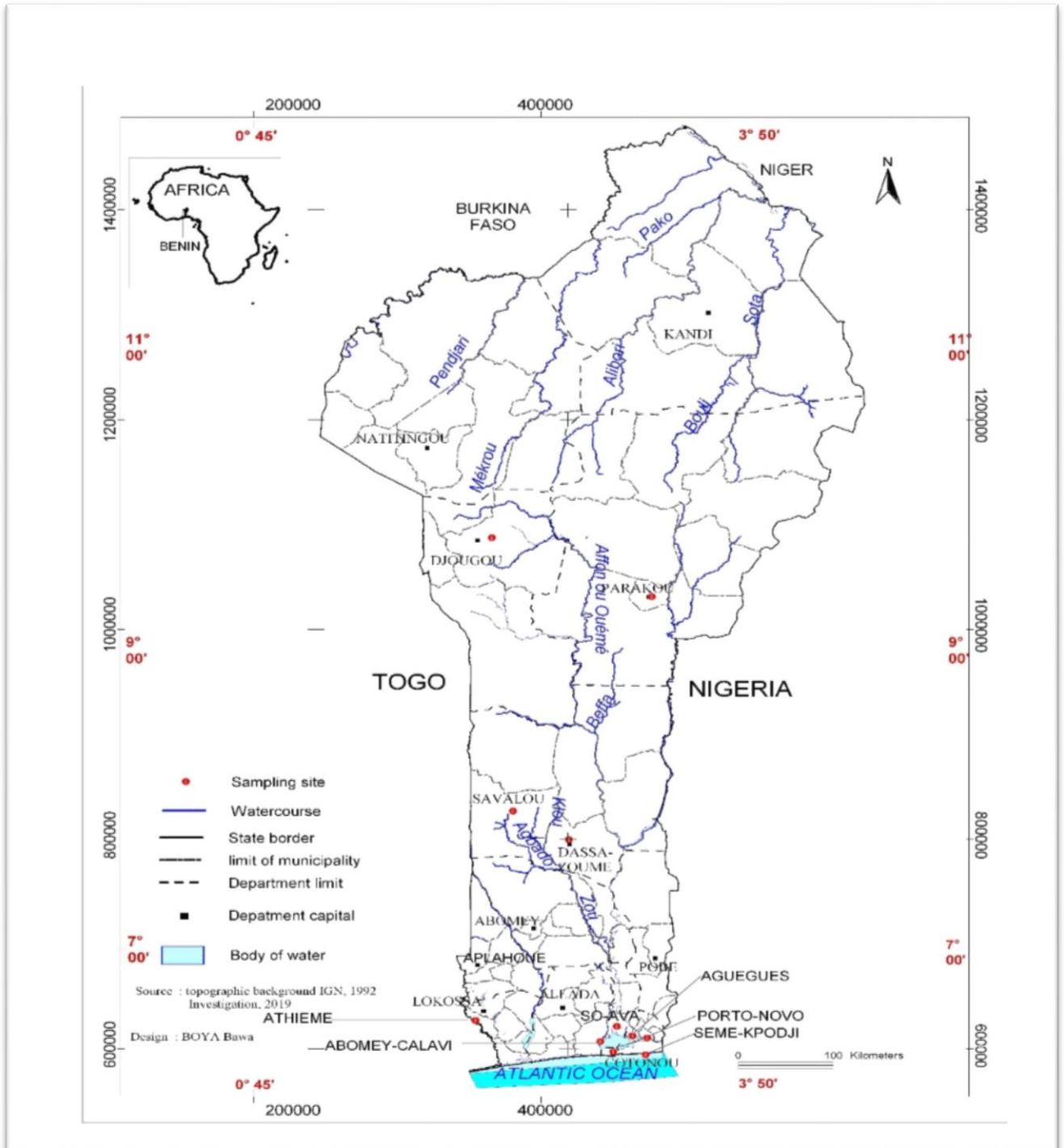


Figure [1]: Map showing the sample's areas

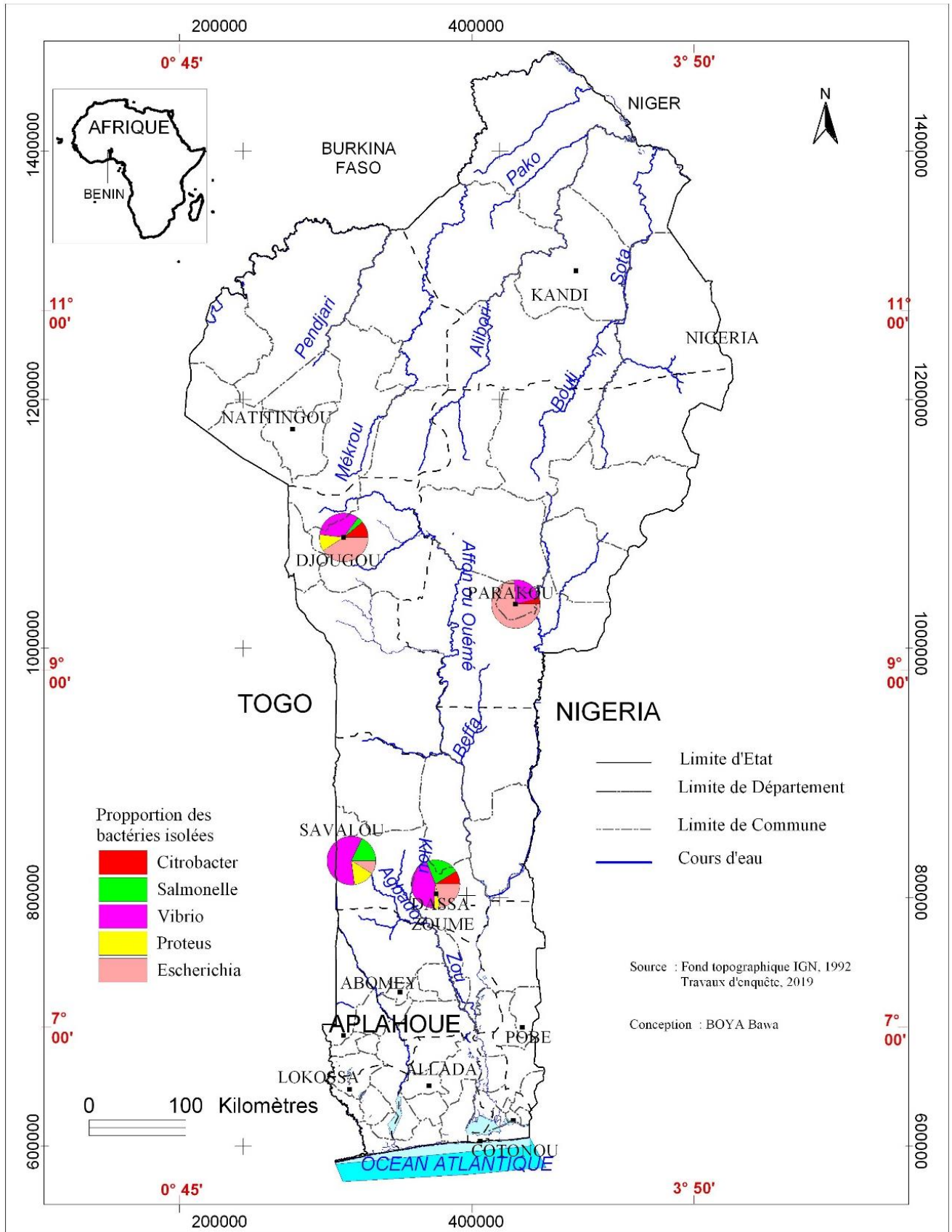


Figure [2]: Distribution of strains identified by biochemical tests in northern Benin

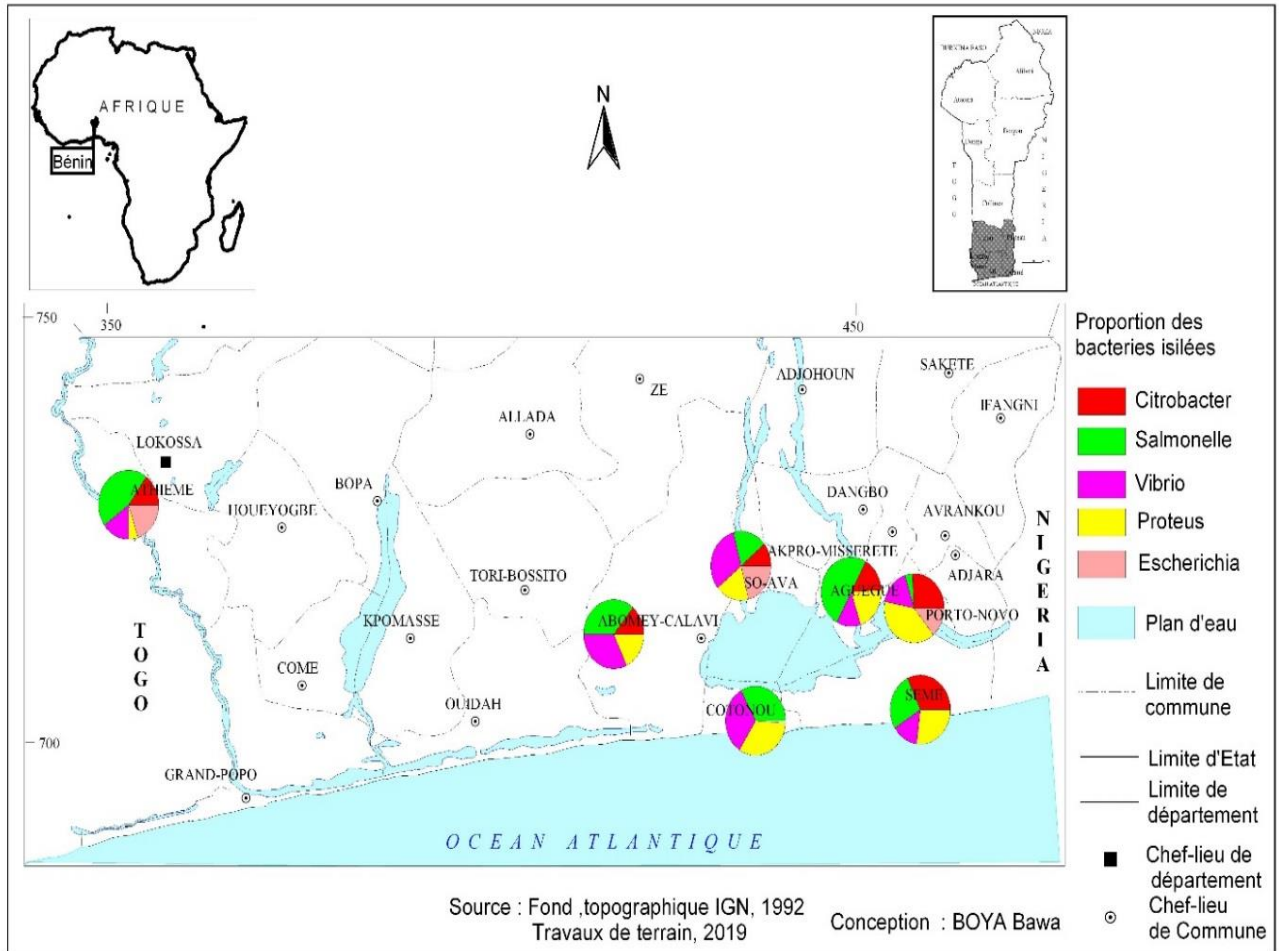


Figure [3]: Distribution of strains identified by biochemical tests in southern Benin

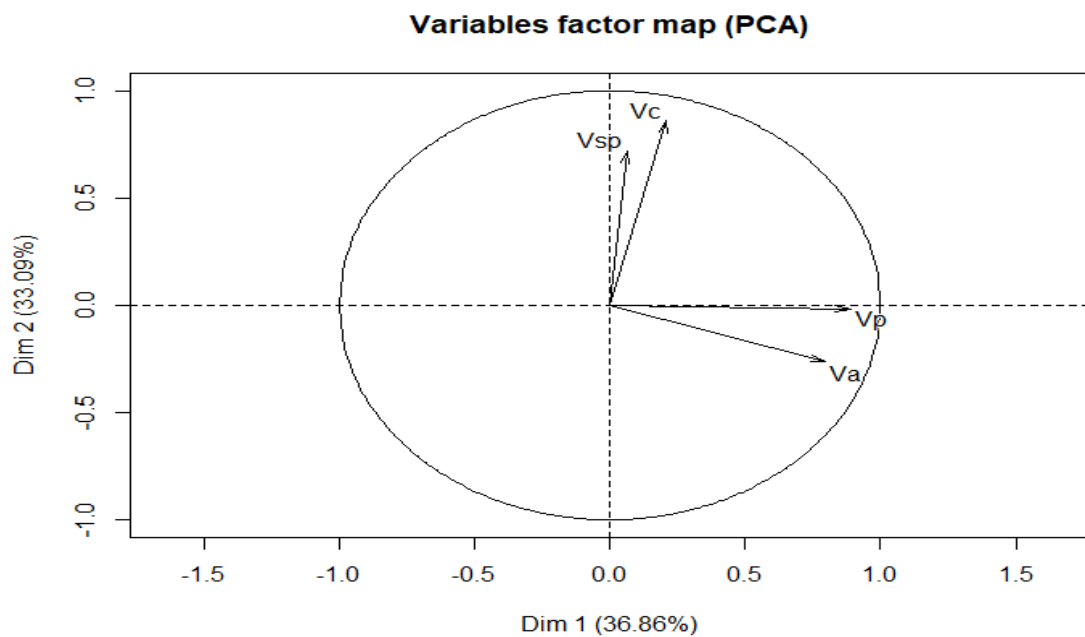


Figure [4]: Grouping of species identified according to their similarity



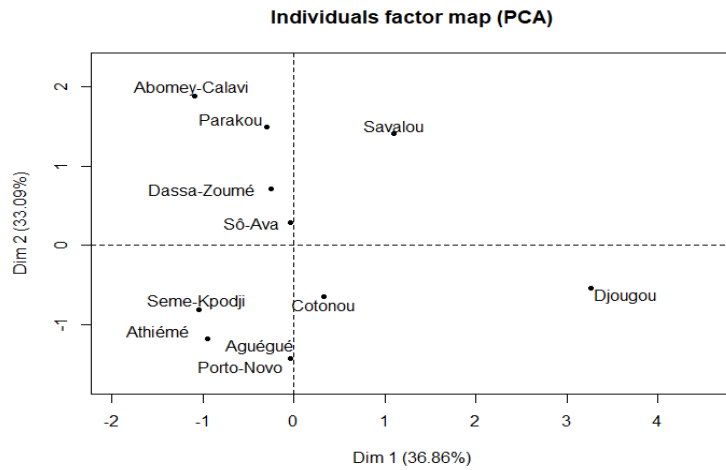


Figure [5]: Grouping of genera and species identified according to the municipalities

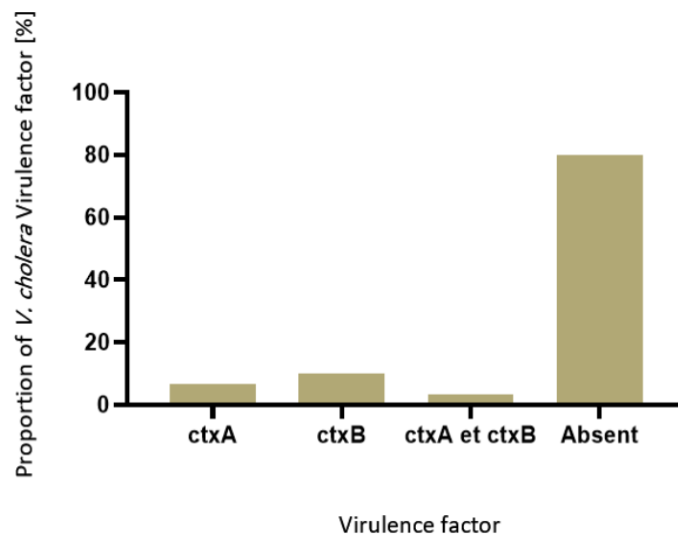


Figure [6]: Percentage of *Vibrio cholerae* species carrying genes encoding cholera toxin.

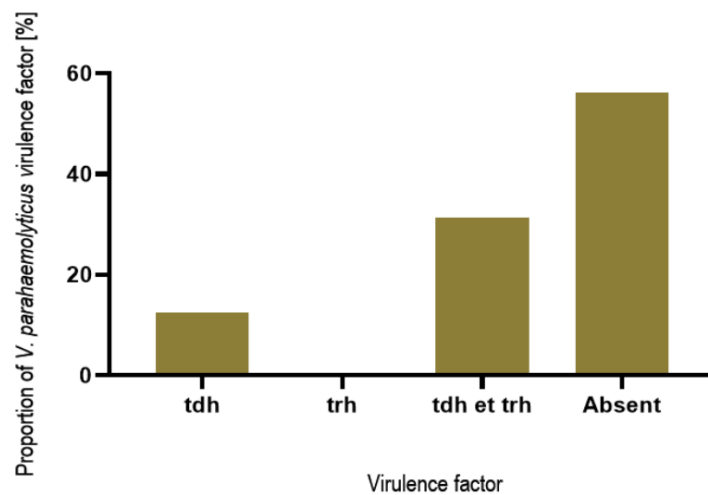


Figure [7]: Percentage of *Vibrio parahaemolyticus* species harboring genes encoding the hemolysins tdh and trh.

**Table [1]:** List of used primers for the molecular characterization of *Vibrio* strains

| Targets genes | Primers          | Sequence [5'-3']  | Size       |
|---------------|------------------|---|------------|
| 16S/23S ISR   | VCM-F            | 5'-TTAAGCSTTTTCRCTGAGAATG- 3'                                       | 295-310 pb |
|               | VCM-R            | 5'-AGTCACTTAACCATACAACCC G- 3'                                      |            |
| <i>Pr72H</i>  | VP32             | 5'-CGAATCCTTGAACATACGCAGC- 3'                                       | 320-387 pb |
|               | VP33             | 5'-TGCGAATTCGATAGGGTGTTA ACC-3'                                     |            |
| <i>ToxR</i>   | ToxR 4<br>ToxR 7 | 5'-GTCTTCTGACGCAATCGTTG-3'<br>5'-ATACGAGTGGTTGCTGTCATG- 3'          | 368 pb     |
| <i>ctxA</i>   | CTX2<br>CTX3     | 5'-CGGGCAGATTCTAGACCTCCTG- 3'<br>5'-CGATGATCTTGGAGCATTCCCAC- 3'     | 564 pb     |
| <i>ctxB</i>   | CTX7<br>CTX9B    | 5'-GGTTGCTTCTCATCATCGAACCAC- 3'<br>5'-GATACACATAATAGAATTAAGGATG- 3' | 460 pb     |
| <i>tdh</i>    | L.tdh<br>R.tdh   | 5'-GTAAAGGTCTCTGACTTTTGGAC- 3'<br>5'-TGGAATAGAACCCTTCATCTTACC- 3'   | 269 pb     |
| <i>trh</i>    | L.trh<br>R.trh   | 5'-TTGGCTTCGATATTTTCAGTA TCT- 3'<br>5'CATAACAAACATATGCCCATTTCCG-3'  | 500 pb     |

**Table [2]:** Physicochemical parameters of water samples

| Communes      | Parameters |             |
|---------------|------------|-------------|
|               | pH         | Temperature |
| Abomey-Calavi | 7.13±0.2   | 31.4±2      |
| Aguégoué      | 7.17±0.03  | 28.35±0.5   |
| Athiémé       | 6.11±0.03  | 28±0        |
| Cotonou       | 7.2±0.9    | 29±1.4      |
| Sô-Ava        | 7.33±0     | 29.3±0      |
| Porto-Novu    | 7.30±0.2   | 28.7±0      |
| Sèmè-Podji    | 6.34±0.3   | 30.09±2.5   |
| Parakou       | 7.25±0.4   | 32.91±1.7   |
| Djougou       | 7.21±0.3   | 29.14±0.9   |
| Savalou       | 7.52±0     | 27.85±0.1   |
| Dassa         | 7.24±0.2   | 31.96±1.4   |

**Table [3].** Distribution of *Vibrio* species identified by PCR according to the municipalities

| Communes      | Species [%]        |                            |                         |                   |
|---------------|--------------------|----------------------------|-------------------------|-------------------|
|               | <i>V. cholerae</i> | <i>V. parahaemolyticus</i> | <i>V. alginolyticus</i> | <i>Vibrio spp</i> |
| Abomey-Calavi | 16,67              | 0                          | 0                       | 17.86             |
| Aguégoué      | 0                  | 0                          | 8.33                    | 7.14              |
| Athiémé       | 0                  | 0                          | 8.33                    | 7.14              |
| Cotonou       | 6.67               | 6.25                       | 16.67                   | 7.14              |
| Dassa-Zoumé   | 20.00              | 12.5                       | 0                       | 0                 |
| Djougou       | 10.00              | 37.5                       | 25                      | 7.14              |
| Parakou       | 13.33              | 12.5                       | 0                       | 17.86             |
| Porto-Novu    | 3.33               | 12.5                       | 8.33                    | 0                 |
| Savalou       | 16.67              | 12.5                       | 16.67                   | 17.86             |
| Sème-Kpodji   | 6.67               | 6.25                       | 0                       | 0                 |
| Sô-Ava        | 6.67               | 0                          | 16.67                   | 17.86             |

## DISCUSSION

The physico-chemical characteristics measured show a variation from one municipality to another. Issola *et al.* [24] have reported the variation in temperature we observed in our study. Those authors explain that the spatial variations in water temperature originate in border environments. In addition, the high values observed for the temperature measurement can be explained by the effect of global warming on the coastal lagoon as no source of hot residual water discharge has been recorded. This explanation refers to the study by Boko *et al.* [25] on the issue of climate change in Benin, stressing that the temperature rose by 0.9°C after 2010.

Indeed, the average depth of the lagoon, of the order of 0.5 to 4 m, the mixing of the waters due to the winds, fishing activities, navigation on the lagoon water. In addition, the sun's rays have the ability to pass through this small thickness of water to heat it evenly [26-27].

The average pH of 7.52 of the waters shows that these waters are basic. These values are of the same order as those observed in the surface waters of the lagoons of in Brazil from 1984 to 2000 [28].

The lower pH values observed during our study reflect the direct effects of acidic water. Indeed, as Kouassi [26] showed, the spatial and temporal variations in salinity, depending on the relative importance of continental and oceanic inputs, conditioning the seasonal pH variation. Thus, periods of high salinity correspond to rather basic waters and to sequences of strong continental influences weakly acidic waters. This shows that the oceanic influence on lagoons and lakes is preponderant over the continental influence for most of the year.

*Vibrio* spp is one of microorganisms that primary habitats are aquatic ecosystems [29]. In this study, 32.73% water samples was positive for *Vibrio* spp. The prevalence of *Vibrio* spp observed in this study is higher than those reported in previous studies [6 and 20%] conducted in Burkina Faso [30] and Tanzania [31]. Thus, *Vibrio* spp isolated from water in this study is often the leading cause of human diarrhea.

A waterborne infection, cholera is spread by ingesting food or water contaminated with the bacteria [32]. In this study, the presence of *Salmonella* spp. [28.18%] and *Escherichia coli* [23.18%] in the water samples indicates continuous fecal [Human and animal] contamination [13].

Human droppings may be predominant because during samples collection, it was observed areas of peoples frequently defecate in the wild. It is noted that the latrines and waste dumps are located in the immediate environment of some lakes, lagoon, and rivers sampled. Under these conditions, the contamination of the waters of lakes, lagoons and rivers by rejected excreta is favored by runoff and infiltration of rainwater [33].

Three species including *Vibrio alginolyticus*, *Vibrio Parahaemolyticus*, and *Vibrio cholerae* non O1 [35%] were molecularly identified. There are 32.56% of unidentified *Vibrio* strains. This work showed that the biochemically identified strains of *V. parahaemolyticus* were not all confirmed by PCR. Of the 28 biochemically identified isolates, 12 were identified as *V. alginolyticus*, 16 were identified as *Vibrio parahaemolyticus* by PCR. It is therefore evident that the PCR technique used offers better specificity than the phenotypic methods for differentiating these species of vibrios. The specificity and sensitivity of the PCR technique, compared to the conventional culture method, for the determination of pathogenic vibrios have been reported by several studies [15, 21, 34-36].

The genes encoding the cholera toxin [ctxA and ctxB] were detected in the strains isolated during our study. Our results are contrary to those obtained in Côte d'Ivoire [37]. Those authors found that *V. cholerae* non-O1 and non-O139 do not produce ctxA gene. In contrast, over 95% of strains belonging to non-O1 / non-O139 serogroups do not produce CT or TCP [38].

A study conducted by Theophilo *et al.* [38] detected ctxA gene in only 4.5% of their strains, which is in agreement with the toxigenic profile of *V. cholerae*.

It has been suggested that most strains of *V. cholerae*, especially those from the environment, lack the genes necessary to produce CT and the possibility of genetic exchange in the environment allows the potential emergence of new toxigenic clones [39]. The horizontal gene transfer plays an important role in increasing the genetic variability of bacterial species and confers new phenotypes, such as virulence, on the recipient [40-41].

The emergence of new toxigenic strains of *Vibrio cholerae* and their selective enrichment during cholera epidemics constitute essential mechanisms for the survival and development of *Vibrio cholerae* and genetic elements that ensure the transfer of virulence genes [42].

Strains of *Vibrio cholerae* may produce unknown virulence factors [39].

Faruque *et al.* [39] observed that non-O1 non-O139 *V. cholerae* strains colonize and can cause fluid accumulation in rabbits despite the absence of the genes encoding TCP and CT. However, we cannot conclude that the non-O1 *Vibrio cholerae* species that we have isolated do not possess the *ctx* genes, are not potentially pathogenic and do not pose a risk to human health.

In fact, approximately 70% of non-O1 *Vibrio cholerae* strains have a polysaccharide capsule, made up entirely of sugars that increases the ability of bacteria to resist phagocytosis and to cause sepsis in immunosuppressed subjects [43-45].

In our study, the prevalence of *V. parahaemolyticus* in water is 5%, which is similar to the figure of 5% reported by Bouchriti *et al.* [46]. The genes encoding major virulence factors the direct thermostable and delayed thermostable hemolysins of *Vibrio parahaemolyticus* [*tdh* and *trh*] were detected in a few strains isolated during our study. Hemolysis, which is due to direct heat-stable hemolysin TDH, distinguishes positive Kanagawa [virulent] strains from negative Kanagawa strains [ $\alpha$ -virulent]. However, the existence of negative Kanagawa strains has been shown which have occasionally been associated with outbreaks of gastroenteritis [47].

*Vibrio alginolyticus* is one of the most common and frequent species of vibrios, living freely in water and sediments, even under unfavorable conditions the latter retain their virulence. They are opportunistic pathogens whose pathogenicity is considered similar to that of *Vibrio parahaemolyticus* [48]. In our study, the strains of *Vibrio alginolyticus* were isolated during the rainy season [June – October] at a prevalence of 4.55% of the water; this could be attributed to the favorable environmental conditions, in particular the increase in temperature and salinity that promote the multiplication and transmission of this germ.

All the *V. cholerae* non-O1 strains isolated from water produced a zone of hemolysis on sheep blood agar which is in fact due to hemodigestion [protease and lecithinase], but it is only the biovar El Tor which is hemolytic.

## Conclusion

Our study identified 51 strains of *Vibrio* isolated from the waters and revealed that they belong to three species of *Vibrio*. Two of those species [*V. cholerae* and *V. parahaemolyticus*] are associated with gastrointestinal diseases. In addition, genes encoding the main virulence factors were detected in strains of *V. cholerae*. However, it was detected in *V. parahaemolyticus*. However, our results

revealed considerable contamination of water with *Vibrio* spp., which could represent a risk to human health.

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## Data Availability Statement

The data are available from the corresponding author upon request.

## Declaration of Interests

The authors declare there is no competing interests about the publication of this manuscript.

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The background of the cover is a light blue gradient. It features a faint, semi-transparent graphic of a stethoscope on the left side, a globe in the center, and a white ECG (heart rate) line running across the bottom. The overall theme is medical and global.

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