



Resistance Profile and Molecular Characterization of Toxigenic *Vibrio cholerae* Strains Responsible for the 2016 to 2018 Epidemic in Republic of Benin (West Africa)

**Eliane Assiba Olorun-Kosun Akpo¹, Tamegnon Victorien Dougnon^{1*},
Rosalie Sacheli², Alidehou Jerrold Agbankpe¹,
Olivia Mariette Yégbandé Hounbégnon¹, Pierrette Melin²
and Honoré Sourou Bankolé¹**

¹Research Unit in Applied Microbiology and Pharmacology of Natural Substances, Research Laboratory in Applied Biology, Polytechnic School of Abomey-Calavi, University of Abomey-Calavi, Abomey-Calavi, Benin.

²CHU of Liège – University of Liège Clinical Microbiology B-23 Sart Tilman Avenue de l'Hôpital 4000 LIEGE, Belgique.

Authors' contributions

This work was carried out in collaboration among all authors. Authors EAOKA, TVD, AJA and HSB wrote the protocol. Authors EAOKA, TVD, RS and OMYH processed the samples. Authors EAOKA, TVD and AJA did the statistical analyses. Authors EAOKA, TVD, AJA wrote the draft of the manuscript. Author RS, PM and HSB reviewed the manuscript. All authors read and approved the final version of the manuscript.

Article Information

DOI: 10.9734/MRJI/2021/v31i630322

Editor(s):

(1) Dr. Zahid Anwar, University of Gujrat, Pakistan.

Reviewers:

(1) Naveen Dhingra, Mody University of Science and Technology, India.

(2) Ashutosh Kumar, Andhra University, India.

Complete Peer review History: <https://www.sdiarticle4.com/review-history/72136>

Original Research Article

**Received 03 June 2021
Accepted 09 August 2021
Published 20 August 2021**

ABSTRACT

Aims: This study aims to characterize toxigenic *Vibrio cholerae* strains for improved cholera surveillance in Benin.

Methodology: 304 diarrheal stool samples were collected from people with watery diarrhea of unknown etiology and vomiting during epidemics from 2016 to 2018 in Benin. Toxigenic *Vibrio*

*Corresponding author: E-mail: dougnon.victorien@gmail.com;

cholerae strains were isolated and then biochemical tests, serogrouping and serotyping were performed. Antibiotic susceptibility tests were performed using the disc diffusion method and E-tests. Multiplex and real-time PCR were used to identify and detect virulence genes (*CtxA*, *OmpW* and *TcpA*).

Results: The results showed a 21.71% prevalence of toxigenic *Vibrio cholera* in Benin. All strains were *Vibrio cholerae* O1 serotype Inaba (100%) and showed a high sensitivity to doxycycline (96.97%), chloramphenicol (95.45%) and ciprofloxacin (90.91%). However, antibiotic resistance was observed, especially for erythromycin (74.24%), Trimethoprim-sulfamethoxazole (71.21%) and ampicillin (43.94%). The *CtxA* and *TcpA* virulence genes were respectively detected in 100% and 96.97% of the toxigenic strains of *V. cholerae* isolated. While the *OmpW* gene was identified on all the toxigenic strains of *Vibrio cholerae* isolated.

Conclusion: *Vibrio cholerae* strains isolated from patients suspected of cholera were highly virulent and resistant to antimicrobials.

Keywords: Toxigenic *Vibrio cholerae* O1; Inaba serotype; Resistance profile; Virulence genes, Benin.

1. INTRODUCTION

Cholera is an epidemic diarrheal disease. It rages throughout the world with waves of endemic diseases in sub-Saharan Africa or Asia in the form of vast epidemics such as the one that reached Haiti at the end of October 2010 or Yemen in 2017, illustrating the very current threat that this scourge poses to public health [1]. Cholera is a key indicator of the lack of social development [2]. Previously, the global burden of cholera was estimated at 2.8 million cases per year, with 91,000 deaths [3]. In 2017, 1,227,391 cases including 5,654 deaths were reported by WHO [4]. However, WHO estimates that the actual figures are higher, due to under-reporting and other deficiencies in the surveillance systems. The gap between the reported figures and the estimated burden of the disease could be attributed, among others, to weak surveillance and laboratory systems. In addition, the two most affected continents are Africa and Asia with 94% of total cases, with 98% of deaths reported in Africa [5]. In Africa, 55,000 cases of cholera with 1,190 deaths were reported in the Democratic Republic of Congo, representing a 2.1% case-fatality rate [6]. In Benin, between 2004 and 2013, the country's epidemiological surveillance reported 5432 cases with 48 deaths, that is, a case-fatality rate of 0.9% [7].

Vibrio cholerae is the causative agent of cholera. It is a highly mobile Gram-negative bacterium with modest nutritional needs, of which humans and in some cases the environment is the reservoir [8,9]. This bacterium has more than 200 serogroups. Only serogroups O1 and O139 cause epidemics [10]. Contamination occurs through ingestion of food or water contaminated with bacteria. The development of cholera often

begins with stomach cramps, vomiting and diarrhea, and if left untreated it can progress to fluid loss. Water loss caused by cholera can be as much as one liter per hour, leading to severe dehydration, metabolic acidosis and therefore shock and possibly death [11]. Cholera treatment relies mainly on oral or intravenous rehydration to compensate for digestive losses of water and electrolytes. In severe cases, antibiotic therapy is indicated to reduce the duration and severity of the disease, as well as subsequent transmission to others [12]. However, antibiotic-resistant strains are increasingly being found [13]. Smith et al. [14] conducted a study on the characterization of *Vibrio cholerae* O1 strains from Ivory Coast, Democratic Republic of Congo, Guinea-Bissau, Mozambique and Namibia. All isolates were of Ogawa serotype. Widespread resistance to nalidixic acid, chloramphenicol and cotrimoxazole was observed in all isolates from the five countries [14]. In addition, resistance to trimethoprim-sulfamethoxazole, ampicillin and nalidixic acid was documented by Eibach et al. [15] in Ghana.

The bacteriological diagnosis of cholera is relatively easy due to the abundance of *Vibrio cholerae* in the stools. It relies mainly on the isolation and identification of the bacteria. The isolation of *Vibrio cholerae* strains is not sufficient to confirm the presence of cholera. It is therefore necessary to determine its serogroup. For a better knowledge of the pathogenic agent, further investigations are needed in order to evaluate the pathogenicity of *Vibrio cholerae* strains by looking for virulence genes. But in Benin, due to the lack of appropriate resources in routine laboratories, the diagnosis of cholera is limited to conventional methods of bacterial isolation without any molecular

investigations. The molecular characteristics of *Vibrio cholerae* in Benin are not known. The present study is therefore a contribution to a better knowledge of the positivity rate of the disease but also of the antibiotic resistance profile and the characteristics of the *Vibrio cholerae* strains circulating in Benin.

2. MATERIALS AND METHODS

2.1 Sample Collection

Sampling was carried out each year (2016 to 2018) during the epidemic period of cholera in Benin. Sampling was exhaustive and Samples were from patients with suspected cholera. These are fecal samples, which were collected in stool containers for stool culture at the sampling sites of health facilities. The patient's name, department, site, date and time of collection have been recorded on the patient information sheet. The samples were sent within 24 hours to the national public health laboratory in Cotonou for the various bacteriological analyzes.

2.2 Bacteriological Analyses of Samples

Enrichment of each stool sample was performed in 4mL of 3% alkaline peptone water. After 6 hours incubation at 37°C, the enrichment broth was inoculated at the same time on TCBS Agar plates, Columbia supplemented with 5% sheep blood and Mueller Hinton agar. The inoculated media were then incubated at 37°C for 24 hours [13,16].

Four characteristic colonies of *Vibrio cholerae* were randomly selected on Mueller Hinton agar for these different tests. These colonies were tested for catalase and cytochrome oxidase. The API 20E gallery system was then used to confirm the identity of the strains. Serogroups and serotypes of *Vibrio cholerae* strains were respectively determined by the agglutination method from young colonies obtained on Columbia blood agar. For serogrouping, either polyvalent *Vibrio cholerae* O1 antiserum or *Vibrio cholerae* O139 Bengal antiserum was used according to the manufacturer's instructions. *Vibrio cholerae* O1 antiserum Inaba or *Vibrio cholerae* O1 antiserum Ogawa was used for serotyping [13,16].

2.3 Confirmation of *Vibrio cholerae* Strains by MALDI-TOF Mass Spectrometry

With the help of a tip, a portion of the colony cultured first on blood Columbia medium was collected and deposited on the Maldi plate. An extended direct deposit was performed by adding 1µl of 70% formic acid to the sample on a MALDI target plate (MSP 96 BC ground steel target; Bruker Daltonics, Germany). Then, 1µl of saturated cyano-4-hydroxycinnamic acid solution was added. Mass spectra acquisition was done using a Microflex Flex Control V3.4 system (Bruker Daltonics, Germany). A Bruker Bacterial Test Standard (BTS255343; Bruker Daltonics, Germany) was used for instrument calibration. The acquisition was done at 24h post culture. Each microorganism tested was spotted twice on the same target slide. Measurement was performed with MALDI Flex control V3.4 (Bruker Daltonics, Germany) following the settings suggested by the manufacturer using automated collecting spectra. The spectra of each duplicated spot were compared with those in the reference library (BD 8326 or version V 9.0) and analyzed with MALDI Biotyper software (Bruker Daltonics, Germany), with log scores ranging from 0 (no similarity) to 3 (perfect match). The mass spectra-based identification was considered reliable only if at least one out of the two spots resulted in log scores of ≥ 2 [17].

2.4 Evaluation of Resistance of *Vibrio cholerae* Strains to Conventional Antibiotics

The antibiogram was performed according to CLSI criteria and procedures [18]. Antibiotics from different families were tested with disks following the Kirby–Bauer method, namely Ciprofloxacin (5 µg), Trimethoprim-sulfamethoxazole (25 µg), Doxycyclin (30 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Ampicillin (10 µg). A reference strain of *Vibrio cholerae* (NCTC 8021) was used for quality assurance of the test. Inhibition zone diameter values were measured and interpreted according to the recommendations [19].

E-tests were also used to determine the minimum inhibitory concentration (MIC) in agar medium [20]. Antibiotics from different families were tested.

2.5 DNA Extraction

The DNA of *Vibrio cholerae* O1 isolates was extracted using the Maxwell® 16 (Promega, ref: As 1020, USA) device and the Promega kit. The extraction was done following manufacturer's instructions. Positive and negative controls were treated under the same conditions than samples. The extracted DNA was used for PCR amplification.

2.6 Multiplex PCR for the Detection of *CtxA*, *OmpW* and *TcpA*

The reaction medium was a total volume of 50 μ L containing 0.4 μ L deoxyribonucleotides (Promega Corporation, Wisconsin, United States), 0.25 μ L Taq Polymerase (Applied Biosystems, California, United States), 7 μ L MgCl₂ (Applied Biosystems, California, United States), 0.5 μ L Forward (F) Primers, 0.5 μ L Reverse (R) Primers (IDT Belgium), 24.35 μ L water and 10 μ L bacterial DNA. A positive control expressing all the desired genes and a negative control (water) were used. Multiplex PCRs were performed by combining the primers for all three genes in the same PCR mixture. The thermocycling conditions consisted of an initial denaturation at 94 °C for 4 min followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C and 1 min extension at 72 °C. A final extension was performed at 72 °C for 5 min [21]. The PCR products were then treated by electrophoresis on a 2% agarose gel. A molecular weight marker was used and the DNA fragments were revealed under an ultraviolet trans-illuminator. The gel was then photographed

by a Biodoc it imaging system, (Biorad, USA). Table 1. shows the sequences of the primers used to amplify the three described genes.

2.7 Real Time PCR for the Detection of *CtxA* Gene

5 μ L of extracted sample DNA was added to a 20 μ L master mix. 0.25 μ M of each primer (Eurogentec, Belgium), 0.1 μ M of *CtxA* probe, (Eurogentec Bnelgium), 2.5 μ L of an internal positive control detection set of probes and primers (Diacontrol DNA DICD-CY-L100, Diagenode, Belgium) and 5 μ L of 2 \times PCR LC 480 probes master mix (Roche, Switzerland). A Light Cycler 480 II (Roche, Switzerland) was used for amplification using an initial denaturation and polymerase activation step at 94 ° C for 2 min, followed by 45 cycles of denaturation at 94 ° C for 10 s, and a combined annealing and extension step at 63 ° C for 30 s [23]. Data analysis was done using the LC480 software (version 1.5.1.62 SP2). Primers and probes used here were described by Blackstone et al. [24].

3. RESULTS

3.1 Distribution of Samples According to their Origin

As part of this study, we received samples only in 9 departments out of the 12 in Benin. The Littoral department is mainly represented (38.2%), followed by Ouémé (23.4%) and Atlantic (19.7%) (Fi. 1).

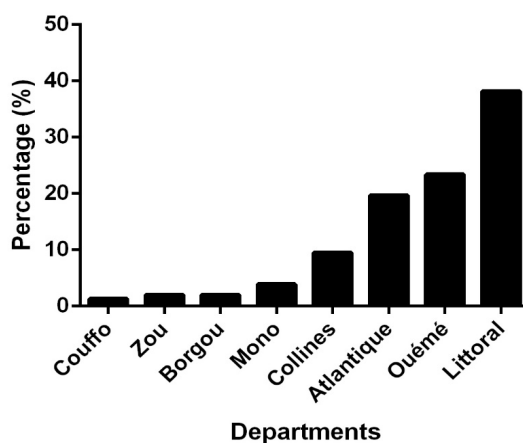


Fig. 1. Distribution of samples according to their origin

3.2 Appearance of Stool Samples

Of the 304 stool samples collected, more than half (70.4%) looked like rice water. Besides the appearance of rice water, three other different aspects were noted: greenish liquid (13.8%), yellowish liquid (8.6%) and brownish liquid (7.2%) (Fig. 2).

3.3 Result of Bacteriological Diagnosis

Of the 304 stool samples analyzed, 66 were positive for culture and identification of *Vibrio cholerae*. That is to say a prevalence of positivity of 21.71%.

3.3.1 Distribution of patients infected with *V. cholerae* by age group and sex

Patients primarily affected by cholera were aged 20-29 (30.30%), followed by patients aged 10-19 (24.20%), patients aged 30-39 (16.70%), 40 to 49 years (12.10%) and 0 to 9 years (9.10%). As for patients aged 50 to 59 (7.60%), they were less affected by the disease (Figure 3). But this difference in proportion between the different age groups of infected patients is not statistically significant ($p = 0.05$). The majority (58%) of infected patients were female with a sex ratio of 175/129.

Table 1. Sequence of primers used to search for virulence genes [22]

Primers		Sequences	Amplicon size
CtxA	F	GGTCTTATGCCAGAGGACAG	219pb
	R	GTTGGGTGCAGTGGCTATAAC	
TcpA	F	ATTCTTGGTGATCTCATGATAAGG	297pb
	R	TTAATTCACCACAAATATCTGCC	
OmpW	F	GAACTTATAACCCACCCGCG	588pb
	R	CACCAAGAAGGTGACTTTATTGTG	

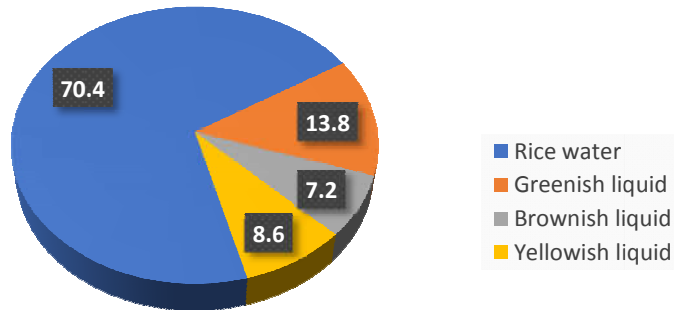


Fig. 2. Distribution of samples according to their appearance

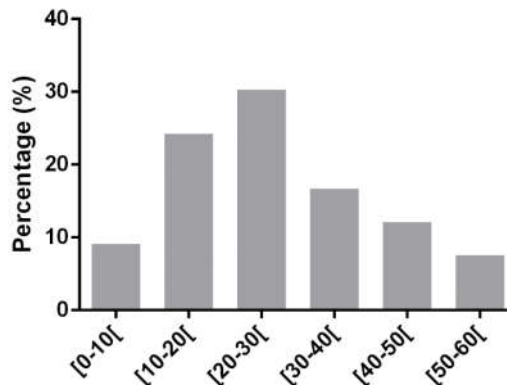


Fig. 3. Distribution of patients infected with *V. cholerae* by age group

3.3.2 Distribution of patients infected with *V. cholera* according to clinical signs

In patients with cholera, diarrhea and vomiting were the most common clinical signs observed in 100% and 59.1%, respectively (Fig. 4). However, it should be noted that this difference in proportion is not statistically significant ($p > 0.05$).

3.3.3 Serogrouping and serotyping of *V. cholerae* strains

Serogrouping and serotyping of the 66 strains of *V. cholerae* obtained showed that all the strains were serogroup O1 and serotype (Table 2).

3.3.4 Resistance of toxigenic *Vibrio cholerae* strains to the antibiotics used

The 66 strains of *Vibrio cholerae* isolated were highly resistant to erythromycin (74.24%; n

= 49), to trimethoprim-sulfamethoxazole (71.21%; n = 47) and to ampicillin (43.94%; n= 29). However, they were mainly sensitive to doxycycline (96.97%; n = 64), chloramphenicol (95.45%; n = 63) and ciprofloxacin (90.91%; n = 60) (Fig. 5). Through these resistances, 16 antibiotic resistance profiles were identified within the 66 strains of *Vibrio cholerae* isolated. The most represented resistance profile was: sensitivity to doxycycline-resistance to ampicillin-resistance to erythromycin-sensitivity to ciprofloxacin-sensitivity to chloramphenicol-resistance to trimethoprim-sulfamethoxazole with 22.73% (15/66), followed by: sensitivity to doxycycline-sensitivity to ampicillin-resistance to erythromycin-sensitivity to ciprofloxacin-sensitivity to chloramphenicol-resistance to trimethoprim-sulfamethoxazole with 21.21% (14/66) (Table 3).

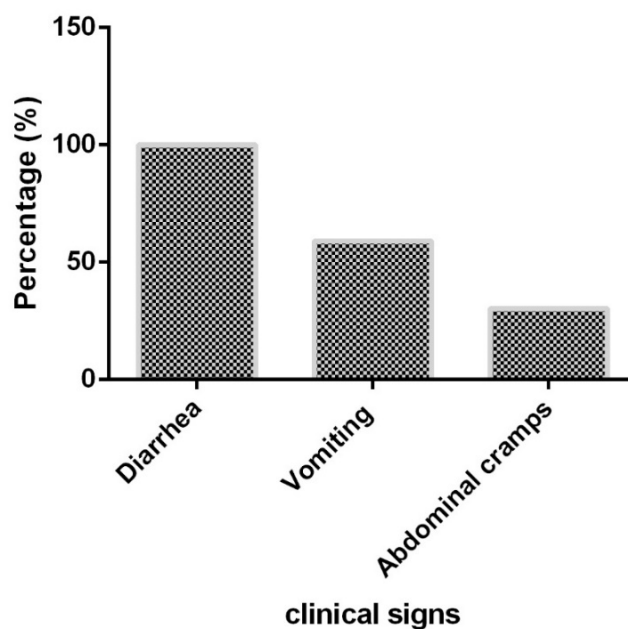


Fig. 4. Distribution of patients infected with *V. cholera* according to clinical signs

Table 2. Serogrouping and serotyping results

Serogroups	Serotypes	Number	Percentage (%)
O1	Inaba	66	100.0
	Ogawa	0	0.0
	Hikojima	0	0.0
O139	-	0	0.0
Total		66	100.0

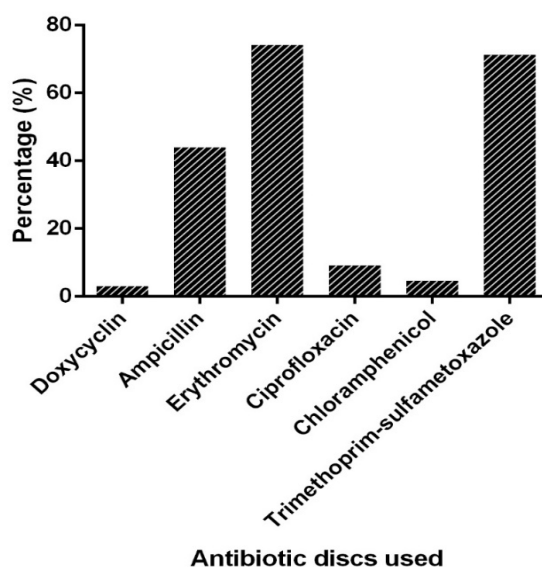


Fig. 5. Percentage of resistance of toxigenic strains of *Vibrio cholerae* to the antibiotics used

Table 3. Resistance profile of toxigenic *Vibrio cholerae* strains isolated

Profil	Phenotypes						Number
Profile 1	D ^S	AM ^S	E ^S	CIP ^S	C ^S	SXT ^S	01
Profile 2	D ^S	AM ^S	E ^R	CIP ^S	C ^S	SXT ^S	08
Profile 3	D ^S	AM ^S	E ^S	CIP ^R	C ^S	SXT ^S	01
Profile 4	D ^S	AM ^S	E ^S	CIP ^S	C ^S	SXT ^R	09
Profile 5	D ^S	AM ^S	E ^R	CIP ^S	C ^S	SXT ^R	14
Profile 6	D ^S	AM ^S	E ^S	CIP ^R	C ^S	SXT ^R	01
Profile 7	D ^S	AM ^S	E ^R	CIP ^R	C ^S	SXT ^R	02
Profile 8	D ^S	AM ^R	E ^S	CIP ^S	C ^S	SXT ^S	02
Profile 9	D ^S	AM ^R	E ^R	CIP ^S	C ^S	SXT ^S	05
Profile 10	D ^S	AM ^R	E ^R	CIP ^R	C ^S	SXT ^S	01
Profile 11	D ^S	AM ^R	E ^R	CIP ^S	C ^R	SXT ^S	01
Profile 12	D ^S	AM ^R	E ^S	CIP ^S	C ^S	SXT ^R	03
Profile 13	D ^S	AM ^R	E ^R	CIP ^S	C ^S	SXT ^R	15
Profile 14	D ^S	AM ^R	E ^R	CIP ^R	C ^S	SXT ^R	01
Profile 15	D ^R	AM ^S	E ^R	CIP ^S	C ^R	SXT ^R	01
Profile 16	D ^R	AM ^R	E ^R	CIP ^S	C ^R	SXT ^R	01
Total							66

D: Doxycyclin; AM: Ampicillin; E: Erythromycin; CIP: Ciprofloxacin; C: Chloramphenicol; SXT: Trimethoprim-sulfamethoxazole; S: Sensitive; R: Resistant

3.4 Detection of Virulence Genes

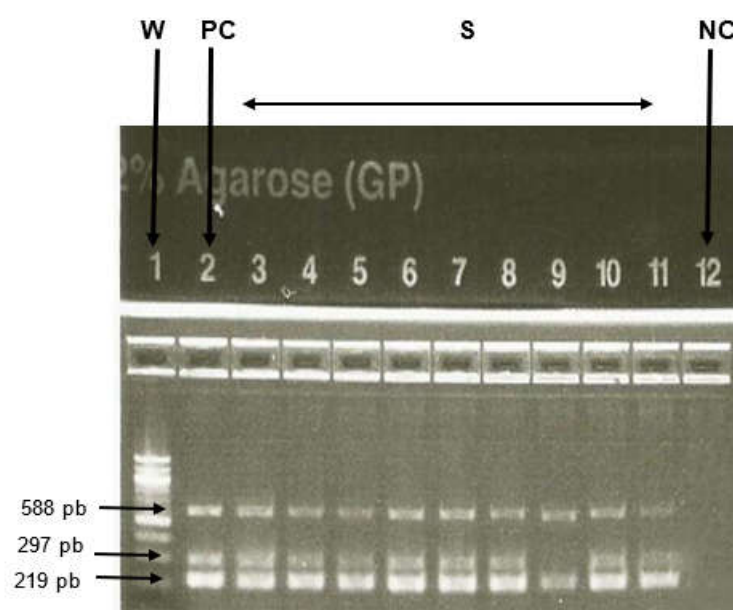
A multiplex PCR was carried out, targeting first the ubiquitous *OmpW* present in the strains of toxigenic *V. cholerae*, but also targeting the regions of two genes associated with the virulence of the genes of *V. cholera*, *CtxA* and *TcpA*. *CtxA* and *OmpW* were identified in 100% (66/66) of the strains of toxigenic *Vibrio cholerae* isolated, while the *TcpA* gene was identified in

96.97% (64/66) of the strains of toxigenic *Vibrio cholerae* (Table 4).

It should be noted that the detection of the *CtxA* gene was additionally performed by real-time PCR. The *CtxA* gene was identified in 100% (66/66) of *Vibrio cholerae* strains. The results of electrophoretic migration on agarose gel are shown for some samples in the figure below (Fig. 6).

Table 4. Percentage of detection of virulence genes in the genome of toxigenic strains of *V. cholerae* isolated

Genes	Results	
	Positive	Negative
<i>CtxA</i>	66 (100%)	0
<i>TcpA</i>	64 (96.97%)	2 (3.03%)
<i>OmpW</i>	66 (100%)	0

**Fig. 6. Photograph of 2% agarose gel after electrophoresis of amplification products**
W: Weight marker PC: Positive Control NC: Negative Control S: Samples

4. DISCUSSION

Cholera is a major public health problem in Africa, particularly in Benin. The present study was carried out on 304 diarrheal stool samples taken from patients who were suspected of having cholera after clinical examination.

More than half (70.4%) of the stool samples were liquid and had a rice water appearance. These observations raise the suspicion of cholera. According to the WHO, the rice water appearance of diarrheal stool samples is characteristic of the severe form of cholera [24]. However, macroscopic examination is not sufficient to affirm the presence of *Vibrio cholerae* in the stool sample [4]. Identification of *Vibrio cholerae* in diarrheal stool samples is, therefore, essential to confirm cholera [5].

The present study showed that 21.71% (66/304) of stool samples were positive for *Vibrio cholerae*

in analyzed geographic areas of Benin. This prevalence confirms the public health problem of cholera in Benin. The relatively high positivity rate obtained is believed to be related to the high population density in vulnerable areas which is associated with poor quality informal housing and insalubrity. These poor hygiene practices lead to the contamination of drinking water or food sources, then favoring the transmission of the disease [25]. While in Africa, Sauvageot *et al.* [26] reported a prevalence of 19% close to ours, in Togo, Ivory Coast, Uganda and Mozambique. The same authors reported higher rates (34%) and (47%) respectively observed in Conakry Guinea and in Goma in the Democratic Republic of Congo [26]. These data reinforce the alarming epidemiological situation of cholera in Africa.

Serological characterization revealed that all isolates (100%) belonged to the O1 serogroup Inaba serotype. Similar results were also obtained in other parts of Africa. Indeed, in

Kenya between 2007 and 2010, in a study of *Vibrio cholerae* O1 strains isolated, it was reported that the Inaba serotype was dominant (88.2%) compared to the Ogawa serotype (11.8%) [27]. Contrary to our results, recent works carried out in West Africa had shown that *Vibrio cholerae* O1 isolates consisted of both Inaba and Ogawa serotypes. In the study of Eibach et al. [15], conducted in Ghana, *Vibrio cholerae* O1 serotype Ogawa was predominant (95.7%) while Musa et al. [28] in Nigeria and Abana et al. [29] in Ghana had isolated only *Vibrio cholerae* O1 Ogawa from stool samples. These results suggest a change in the presence of Ogawa and Inaba serotypes following the geographical area [30,31].

Patients in the 20-30 age group were mostly affected (30.3%) by cholera. This result corroborates those of Makoutodé et al. [32] in Benin and Kyelem et al. [33] in Burkina-Faso who found that patients in the 15-29 age groups were the most affected by cholera in the proportions of 46% and 51.1% respectively. This age group of the population would be more mobile and active and therefore more exposed to the infection.

The majority (58%) of patients infected with cholera were female. This result is also in line with the data from the 2008 epidemic in Benin during which the sex ratio was 0.8 male to one female. Furthermore, this result is comparable to that of Cissé [34] who found that 55% of women were affected during the cholera epidemic in the cercle de Nara (Koulikoro region) in Mali. This feminine predominance would be linked to the fact that women are the primary actors in the healthiness, that is, care of children's body, care of the sick, maintenance of toilets and handling of soiled food, which could make easy their contamination. On the other hand, according to the work of Kyelem et al. [33] in Burkina-Faso and Touré [35] in Mali, male patients were more affected with the respective frequencies of 55.04% and 57%.

In addition, diarrhea (100%) and vomiting (59.1%) were the most common clinical signs among infected patients. Ndour et al. [36] in Senegal made the same observations: diarrhea (95%) and vomiting (78%). These findings confirm the reliability of such symptomatology in the manifestation of cholera.

When we look at the geographical origin of infected patients, the Littoral and Atlantic

departments come first in the proportions of 50% and 21.2% respectively. These data are in line with the heavy epidemiological history of the cities of these departments with regard to cholera. Indeed, Makoutodé et al. [32] reported that insufficient sanitation conditions and poor waste management in the city of Cotonou, in the Littoral department, have favored the development of cholera epidemics. The high rates of contamination noted in these departments also corroborate UNICEF data in Benin, which reports that the coastal departments of Littoral, Atlantic, Ouémé and Mono recorded major cholera epidemics, according to which between 2004 and 2013, nearly 60% of cholera cases were reported in these departments and mainly in Littoral and Atlantic almost every year [37]. These observations may be explained by the fact that cholera occurs mainly in areas with a combination of several risk factors including problems of sanitation and access to drinking water, high population density and lack of hygiene [38].

In combination with rehydration therapy, some antibiotics can help in addition by reducing the duration of illness, the excretion of *Vibrio cholerae* in feces and deaths by about 50% [8,39,40]. The knowledge of the susceptibility profile of the epidemic strains was therefore essential to recommend effective and good quality antibiotics for the treatment of affected patients. This study showed that the isolates were highly resistant (74.24%) to erythromycin, erythromycin being one of the administered antibiotics in the treatment of cholera for pregnant women and children [41]. These results suggest that erythromycin should no longer be indicated for the treatment of cholera caused by the current circulating strains of *Vibrio cholerae* O1, Inaba. High levels of resistance were also observed (71.21%) and (43.94%) respectively to Trimethoprim + Sulfamethoxazole (Cotrimoxazole) and Ampicillin. This makes them unreliable for the treatment of cholera in Benin.

The antibiotics tested in this study, although recommended by WHO [37] for the treatment of cholera, were not active on all strains. The high resistance to erythromycin could be explained by the increased use of this broad-spectrum antibiotic in human infectious therapy. Similarly, the high resistance of *Vibrio cholerae* strains to Trimethoprim + Sulfamethoxazole (71.21%) could be justified by the fact that this drug is commonly prescribed in human medicine to treat

a wide range of infections including urinary tract and skin infections and respiratory and gastrointestinal tract infections, including diarrhea [42]. Thus, this observed ineffectiveness could be related either to the emergence of resistant strains due to the easy access to antibiotics [40], or to the acquisition of resistance factors by these circulating strains because they were isolated in an environment where multidrug-resistant enterobacteria are also prevalent. This phenomenon of resistance poses a major problem in the management of cholera related to these strains. It is therefore important that susceptibility testing is routinely performed to guide antibiotic therapy [41].

In addition, high susceptibility to doxycycline (96.97%), chloramphenicol (95.45%) and ciprofloxacin (90.91%) was observed. Doxycycline is the first-choice antibiotic in the treatment of cholera for adult in Benin. However, special attention must be paid to the emergence of resistant strains for these drugs.

OmpW gene which is a membrane protein, ubiquitously expressed in *Vibrio cholerae* was searched for by multiplex PCR.

Different virulence factors have been suggested to be involved in the pathogenicity of *Vibrio cholerae*. Diagnosis therefore included the identification of known virulence factors in these isolates. The two major proteins involved in the pathogenicity of *Vibrio cholerae* are, on the one hand, the cholera toxin (CT: cholera toxin) encoded by the *CtxA* and *CtxB* genes and, on the other hand, the co-regulatory factor of the toxin encoded by the *TcpA* gene (Toxin coregulated pilus). The *CtxA* and *CtxB* genes are respectively carrier of the catalytic activity and responsible for binding to intestinal cells. The *TcpA* gene, as it is concerned, colonizes the small intestine [43]. This technique was used by several authors for the detection of virulence genes associated with *Vibrio cholerae*. The virulence of *Vibrio cholerae* strains is thus associated with two processes: colonization of the small intestine of patients and production of cholera toxin.

The *CtxA*, *TcpA* and *OmpW* (588 genes were simultaneously identified in 100% (66/66), 96.97% (64/66) and 100% (66/66) of *Vibrio cholerae* strains by multiplex PCR, respectively. Detection of the *CtxA* gene was also performed by real-time PCR and was identified in 100% (66/66) of *Vibrio cholerae* strains. *Vibrio cholerae*

strains currently isolated are all highly pathogenic. The toxins produced by these strains are capable of inducing profuse, watery diarrhea, which can be fatal [27]. Strains of *V. cholera* that do not produce the toxin, were not identified in this study.

5. CONCLUSION

This study helped to establish a positivity rate of 21.71% of *Vibrio cholerae* strains from September 2016 to December 2018 in Benin. The circulating strains were all *Vibrio cholerae* O1 serotype Inaba and showed a high susceptibility to doxycycline. However, antibiotic resistance was observed, especially for erythromycin and ampicillin. The virulence of isolated *Vibrio cholerae* strains was demonstrated by the detection of *CtxA* and *TcpA* genes. Note that the *OmpW* gene was detected in all positive strains confirming the identity of the strains as *Vibrio cholerae*. The study thus confirmed that the phenomenon of bacterial resistance to antibiotics in Benin is increasing, as well as the circulation of virulent strains that deserve to be effectively eradicated. The great question remains to find appropriate measures to reduce this relatively high prevalence and the problem of strains resistance. Environmental factors are very determining and must be taken into account in the implementation of strategies to reduce cholera-related health risks.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

Informed consent was sought and obtained from patients or parents/guardians of patients who were sampled in this study.

ETHICAL APPROVAL

In Benin, ethics approval was not required during cholera epidemics. When a case is suspected,

sampling is done automatically for epidemiological surveillance, as it was approved as part of the Ministry of Health's routine diagnostic algorithm.

ACKNOWLEDGEMENTS

The authors are grateful all of colleagues of Bacteriology Laboratory of the Ministry of Health of Benin and of the Research Unit in Applied Microbiology and Pharmacology of Natural Substances, University of Abomey-Calavi for their involvement in this study. The authors are also grateful to Dr Charles Hornel Koudokpon for the review of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

- Institut Pasteur. Cholera: *Vibrio cholerae* bacteria responsible for cholera. Surveillance and public health. National Reference Center for Vibrios and Cholera and Enteric Pathogenic Bacteria Unit; 2018. Available: <https://www.pasteur.fr/fr/centre-medical/fiches-maladies/cholera>. French
- WHO. 10 facts about cholera. Updated Global Burden of Cholera in Endemic Countries. French; 2016.
- Ali M, Nelson AR, Lopez AL, Sack DA. Updated Global Burden of Cholera in Endemic Countries. *PLOS Neglected Tropical Diseases*. 2015;9(6):e0003832. DOI: 10.1371/journal.pntd.0003832.
- WHO. Cholera vaccines. *Weekly Epidemiological Record*, position paper. 2017; 34.
- Quilici M-L. Bacteriological diagnosis of cholera. National reference center for vibrios and cholera Laboratory of enteric pathogenic bacteria Institut Pasteur. *Tropical Diseases (2). Revue Francophone Des Laboratoires*. 2011; 31:51- 65. French
- WHO. Cholera. Vaccine Preventable Disease Surveillance Standards. *WHO_SurveillanceVaccinePreventable_02_Cholera_French_R1*. 2019; 13. French
- Legba CS, Yabi I, Azonhe T, Osseni A-A. Analysis of the Determinants of Cholera in the City of Djougou, Benin. *European Scientific Journal*. 2017;13(18). DOI: 10.19044/esj.2017.v13n18p171. French
- WHO. Communicable diseases epidemiological profile. Disease Control in Emergency Humanitarian Action (WFD) Global Alert and Response Department (GAR); 2010. French
- Weil AA, Ryan ET. Cholera: recent updates. *Current Opinion in Infectious Diseases*. 2018 ;31(5):455-461 DOI: 10.1097/QCO.0000000000000474
- Clemens JD, Nair GB, Ahmed T, Qadri F, Holmgren J. Cholera. *The Lancet*. 2017; 390(10101):1539–1549. DOI:10.1016/s0140-6736(17)30559-7.
- Lucien MAB, Schaad N, Steenland MW, Mintz ED, Emmanuel R, Freeman N, Boncy J, Adrien P, Joseph GA, Katz MA. Identifying the most sensitive and specific sign and symptom combinations for cholera: results from an analysis of laboratory-based surveillance data from Haiti, 2012-2013. *Am J Trop Med Hyg*. 2015;92(4):758-764. DOI: 10.4269/ajtmh.14-0429. Epub 2015 Mar 2. PMID: 25732682; PMCID: PMC4385769.
- Hounmanou YM, Mdegela RH, Dougnon TV, Mhongole OJ, Mayila ES, Malakalinga J, Makingi G, Dalsgaard A. Toxigenic *Vibrio cholerae* O1 in vegetables and fish raised in wastewater irrigated fields and stabilization ponds during a non-cholera outbreak period in Morogoro, Tanzania: an environmental health study. *BMC Res Notes*. 2016;9(1):466. DOI: 10.1186/s13104-016-2283-0.
- Morita D, Morita M, Alam M, Mukhopadhyay AK, Johura FT, Sultana M, Monira S, Ahmed N, Chowdhury G, Dutta S, Ramamurthy T, Samanta P, Takahashi E, Okamoto K, Izumiya H, Ohnishi M. Whole-Genome Analysis of Clinical *Vibrio cholerae* O1 in Kolkata, India, and Dhaka, Bangladesh, Reveals Two Lineages of Circulating Strains, Indicating Variation in Genomic Attributes. *mBio*. 2020;11(6):e01227-20. DOI: 10.1128/mBio.01227-20.
- Smith AM, Njanpop-Lafourcade BM, Mengel MA, Gessner BD, Sauvageot D, Bidjada B, Miwanda BN, Saliou DM, N'Douba AK, Langa JP, Ismail H, Tau N, Sooka A, Keddy KH. Comparative Characterization of *Vibrio cholerae* O1 from Five Sub-Saharan African Countries Using Various Phenotypic and Genotypic

- Techniques. PLoS One. 2015;10(11): e0142989.
DOI: 10.1371/journal.pone.0142989.
15. Eibach D, Herrera-León S, Gil H, Hogan B, Ehlkes L, Adjabeng M, Kreuels B, Nagel M, Opore D, Fobil J N, May J. Molecular Epidemiology and Antibiotic Susceptibility of *Vibrio cholerae* Associated with a Large Cholera Outbreak in Ghana in 2014. PLOS Neglected Tropical Diseases. 2016;10(5): e0004751.
DOI:10.1371/journal.pntd.
 16. Feglo PK, Sewurah M. Characterization of highly virulent multidrug resistant *Vibrio cholerae* isolated from a large cholera outbreak in Ghana. BMC Res Notes. 2018;11(1):45.
DOI: 10.1186/s13104-017-2923-z.
 17. Paauw A, Trip H, Niemcewicz M, Sellek R, Heng JM, Mars-Groenendijk RH, de Jong AL, Majchrzykiewicz-Koehorst JA, Olsen JS, Tsvitvadze E. OmpU as a biomarker for rapid discrimination between toxigenic and epidemic *Vibrio cholerae* O1/O139 and non-epidemic *Vibrio cholerae* in a modified MALDI-TOF MS assay. BMC Microbiol. 2014;14:158.
DOI: 10.1186/1471-2180-14-158.
 18. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; seventeenth informational supplement. M100. 2007 ; 27(1):182.
 19. Antibigram Committee of the French Society of Microbiology. Recommendations; 2018.
 20. Joly-Guillou ML. Intérêt du E-test dans le suivi de l'antibiothérapie. Réanimation. 2006;15:237-240.
DOI : 10.1016/j.reaurg.2006.02.003.
 21. Mehrabadi JF, Morsali P, Nejad HR, Imani Fooladi AA. Detection of toxigenic *Vibrio cholerae* with new multiplex PCR. J Infect Public Health. 2012;5(3):263-7. doi: 10.1016/j.jiph.2012.02.004.
 22. Imani FA, Iman ID, Hosseini DR, Karami A, Marashi SM. Design of a multiplex PCR method for detection of toxigenic-pathogenic in *Vibrio cholerae*. Asian Pac J Trop Med. 2013;6(2):115-8.
DOI: 10.1016/S1995-7645(13)60005-X.
 23. Blackstone GM, Nordstrom JL, Bowen MD, Meyer RF, Imbro P, DePaola A. Use of a real time PCR assay for detection of the *ctxA* gene of *Vibrio cholerae* in an environmental survey of Mobile Bay. J Microbiol Methods. 2007;68(2):254-9.
DOI: 10.1016/j.mimet.2006.08.006.
 24. WHO. Cholera. Weekly Epidemiological Record. 2018;38:489-497.
 25. Mengel MA, Delrieu I, Heyerdahl L, Gessner BD. Cholera Outbreaks in Africa. Current Topics in Microbiology and Immunology. 2014;117-144.
DOI:10.1007/82_2014_369
 26. Sauvageot D, Njanpop-Lafourcade BM, Akilimali L, Anne JC, Bidjada P, Bompangue D, Bwire G, et al. Cholera incidence and mortality in Sub-Saharan African sites during Multi-country Surveillance. PLOS Neglected Tropical Diseases. 2016;10(5):e0004679.
DOI:10.1371/journal.pntd.0004679.
 27. Mercy N, Mohamed AA, Zipporah N, Chowdhury G, Pazhani GP, Ramamurthy T, Boga HI, Kariuki SM, Joseph O. Phenotypic and genetic characterization of *Vibrio cholera* O1 isolated from various regions of Kenya between 2007 and 2010. Pan African Medical Journal. 2014; 19.
DOI:10.11604/pamj.2014.19.8.2496
 28. Musa AG, Abubakar SB, Abubakar M, Galadima BG. Circulating serotypes and antimicrobial susceptibility pattern of *Vibrio cholerae* isolates from insurgency-stricken Maiduguri, north eastern Nigeria. Sub-Saharan African Journal of Medicine. 2019;6(1):21-26.
 29. Abana D, Gyamfi E, Dogbe M, Opoku G, Opore D, Boateng G, Mosi L. Investigating the virulence genes and antibiotic susceptibility patterns of *Vibrio cholerae* O1 in environmental and clinical isolates in Accra, Ghana. BMC Infectious Diseases. 2019;19(1).
DOI:10.1186/s12879-019-3714-z.
 30. Alam MT, Ray SS, Chun CN, Chowdhury ZG, Rashid MH, Madsen Beau De Rochars VE, Ali A. Major Shift of Toxigenic *V. cholerae* O1 from Ogawa to Inaba Serotype Isolated from Clinical and Environmental Samples in Haiti. PLOS Neglected Tropical Diseases. 2016;10(10):e0005045.
DOI:10.1371/journal.pntd.0005045
 31. Bundi M, Shah MM, Odoyo E, Kathiiko C, Wandera E, Miring'u G, Guyo S, Langat D, Morita K, Ichinose Y. Characterization of *Vibrio cholerae* O1 isolates responsible for cholera outbreaks in Kenya between 1975 and 2017. Microbiology and Immunology; 2019.
DOI:10.1111/1348-0421.12731.

32. Makoutodé M, Diallo F, Mongbo V, Guévar E, Bazira L. The response to the 2008 cholera epidemic in Cotonou (Benin). *Public Health (Bucur)*. 2010;22:425–35. French
33. Kyelem C, Bougouma A, Thiombiano R, Salou-Kagoné I, Sangaré L, Ouédraogo R. Cholera epidemic in Burkina Faso in 2005: epidemiological and diagnostic aspects. *Pan African Medical Journal*. 2011; 8(1). DOI: 10.4314 / pamj.v8i1.71047. French
34. Cissé A. Epidemiological aspects of cholera in the cercle of nara (koulikoro region) from November to December 2005. Doctoral thesis in Medicine, Faculty of Medicine, Pharmacy and Odontostomatology, University of Bamako, Mali, 2008;245 . French
35. Touré MOS. Epidemiological profile of cholera in the health district of Mopti in 2011 Faculty of Medicine and Odontostomatology Thesis of Medicine / FMOS Public health and infectious diseases. 2013;206. French
36. Ndour CT, Manga NM, Ká R, Dia-Badiane NM, Fortez L, Seydi M, Soumaré M, Sow AI, Diop BM, Sow PS 2006. Cholera epidemic of 2004 in Dakar, Senegal: epidemiologic, clinical and therapeutic aspects. *Med Trop (Mars)*. 2006;66(1):33-8. ID: mdl-16615613
37. Unicef. Cholera epidemiology and response. Unicef Factsheet; 2013. DOI: 10.4314 / pamj.v8i1.71047. French
38. Tubaya D. Study of risk factors for cholera. University of Lubumbashi - Diploma of in-depth studies in public health, option epidemiology and preventive medicine. 2007;189. French
39. Leibovici-Weissman Y, Neuberger A, Bitterman R, Sinclair D, Salam MA, Paul M. Antimicrobial drugs for treating cholera. *Cochrane Database Syst Rev*. 2014;2014 (6): CD008625. DOI: 10.1002 / 14651858.CD008625.pub2.
40. Zereen F, Akter S, Sobur MA, Hossain MT, Rahman MT. Molecular detection of *Vibrio cholerae* from human stool collected from SK Hospital, Mymensingh and their antibiogram. *Journal of Advanced veterinary and animal research*. 2019;6(4): 451–455 Available:<http://doi.org/10.5455/javar.2019.f367>.
41. Rabaan AA. Cholera: an overview with reference to the Yemen epidemic. *Frontiers of Medicine*; 2018. DOI: 10.1007 / s11684-018-0631-2
42. Mwebia MB. Characterization of toxigenic *Vibrio cholerae* isolates from kenya, 2007-2010. Master of Science (Biotechnology) in the School of Pure and Applied Sciences of Kenyatta University. 2012;89.
43. Zhu J, Mekalanos JJ. Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. *Dev Cell*. 2003;5(4):647-56. DOI: 10.1016 / s1534-5807 (03) 00295-8. PMID: 14536065.

© 2021 Akpo et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:

The peer review history for this paper can be accessed here:
<https://www.sdiarticle4.com/review-history/72136>