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BIOCHEMICAL PROFILE, SEROTYPES, GENOTYPES AND RESISTANCE PHENOTYPES OF *VIBRIO CHOLERAE* O1 ISOLATED FROM CHAD, 2010-2020

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ABSTRACT
Chad experienced five cholera epidemics in 15 provinces out of 23 and 39 health districts out of 61 from 2010 to 2020. Four epidemic foci were identified based on the onset, the spread of epidemics and the surface-water hydrology viz. the Lake Chad basin, Chari-Logone basin, Mayo-Kébbi basin and the city of N'Djamena. Stools were collected into sterile vials containing alkaline peptone water. *Vibrio cholerae* O1 was isolated using TCBS and Muller-Hinton agar and identified by agglutination tests, API®20E and API®20NE galleries. Their identities were confirmed using Vitek2TM and PFGE automated systems and RT-PCR. Chad recorded 25827 cases of cholera and 802 deaths in five years, with a mortality rate of 3.10%. *Vibrio cholerae* O1, all of El Tor biotypes, were detected in 56% of stools of which and consist in 71.03% Ogawa and 29% Inaba serotypes. The susceptibility profiles of the strains to antibiotics varied from one antibiotic to another. Resistance rates of 100% were observed with trimethoprim-sulfamethoxazole and vibriostatic compound O / 129. The most active antibiotics are doxycycline (99.45%), tetracycline (99%), norfloxacin (78.14%) and ciprofloxacin (76%). As well, 71 out 72 isolated strains (98.61%) carriers of the virulence genes encoding for the synthesis of cholera toxin (ctxB and ctxA) as confirmed by molecular analyses. Overall, doxycycline, alternatively tetracycline, ciprofloxacin and norfloxacin should be endorse for the treatment of cholera in Chad.

KEYWORDS: *Vibrio cholerae* O1, serotype, genotype, biochemical, Resistance phenotype, Chad.

1.0. INTRODCUTION

Vibrio cholerae is responsible for major outbreaks of cholera associated with socio-economic consequences make it a serious public health problem that requires epidemiological surveillance in both humans and animals.[1]

The seventh cholera pandemic started in 1961 and emerged in Equatorial Africa (Guinea) in August 1970. The disease then spread from West to East through the coast and the inner continent to gradually reach all neighboring countries, then Chad.[2]

From 1971 to 2019, Chad registered a total 44 cholera epidemics cumulating in 85475 cases and 6475 deaths with a mortality rate of 13.2%. The spread of the disease was linked to sociocultural behaviors.[3]

The first *Vibrio cholerae* O1 serotype Ogawa was isolated in 1971.[3] In 1991, *Vibrio cholerae* O1, serotype Ogawa, biotype El Tor was isolated for the first in the Laboratory of the Central Hospital of N'Djamena, Chad. It was later isolated in 1994 from stools of cholera patients coming from different towns in the country during a smaller epidemic.[4] The outbreaks foci in the southern of Chad are humid area that exhibit classic or intermediate epidemiological characteristics (backwater, rivers, areas flooded by rainwater, unsanitary food and drink, lack of hygiene, etc.).[5] The 2019 epidemic preceded that of 2017 with 797 cases and 29 deaths

(5.83%). In 2010, after heavy rainfall causing flooding, cholera epidemic erupted in two foci; the north and south of N'Djamena, Chad. Of note, the epidemic was raging in neighboring countries: Niger, Nigeria, and Cameroon a week before its onset in Chad.

Owing the recurrence of the cholera epidemic in Africa and more particularly in Chad, it is imperative to understand the epidemiological characteristics of the causal agent, the spread of the disease at the individual and collective level, public sanitation, the landscape of urban sites as well as the source of water supply.[6,7]

This study aimed to assess the prevalence, biochemical profile, serogroup, serotype and genotypes of *Vibrio cholera* involved in people with cholera from 2010 to 2019 and to determine their antibiotic susceptibility profile. This work is an awareness-raising tool for adequate prevention strategy and establishment of effective antibiotic therapy against recurrent cholera epidemics in Chad.

2.0. MATERIAL AND METHODS

2.1. Framework, period and type of study

This study is a transversal retrospective study focused on the etiological diagnostic of cholera carried out over five consecutive years of cholera epidemics viz. 2010, 2011, 2014, 2017 and 2019 in Chad (Figure 1). The investigations were conducted and executed by the Bacteriology Unit, National Reference Laboratory, University Hospital Center (CHU-RN) and the Vibrio Research Unit of the Institute Pasteur of Paris and included field trips in affected regions.

2.2. Inclusion criteria

The cholera cases that meet criteria defined by the Directive for the Prevention and Control of Cholera established by the Integrated Epidemic Surveillance Service (SSEI) in August 2010 were selected:

- In an area where the disease is unknown: patient aged 5 years or over with severe dehydration or succumbs to an acute episode of watery diarrhea.

- In an area where there is an epidemic of cholera: patient aged 5 years or over with an acute episode of watery diarrhea with or without vomiting.

2.3. Stool collection

Stools were collected from patients in different areas where cases were reported. They were placed in sterile flasks containing 5 ml of sterile alkaline peptone water (EPA) (Bio-Rad®) and transferred in ice packs-refrigerated container to the Bacteriology Unit, National Reference Laboratory, University Hospital Center (CHU-RN) for analyzes.

2.3.1. Examination of stool

Samples of the stool were directly examined to detect motile germs (search for mobility in midge flight). The Gram staining of a stool smear was used to search for the curved Gram-negative bacilli.

2.3.2. Data collection and processing

A data collection sheet was used to collection information on the origin, sex, age and suspected origin of the contamination (the source of drinking water, food and the method of life of each person admitted) prior to stool collection and culture.

MapInfo software was used to map cholera epidemiological study areas. The data collected was computed and analyzed using Word 2013 and Excel 2013 software.

2.4. Culture, antibiogram and molecular biology

Three different methods were used for the detection of *Vibrio*: microbiological method and the molecular characterization of *Vibrio cholerae* O 1 isolates by polymerase chain reaction (PCR) and pulsed field electrophoresis (PFGE).

2.4.1. Microbiological method

To isolate *Vibrio*, 1 ml of stool in a vial of 4 ml of alkaline peptone water was enriching for 3 hours at 37 ° C in a bacteriological oven and subcultured on Thiosulfate Bile Citrate Sucrose agar (TCBS: T-MAST, DM218D) and Muller-Hinton agar (MH: Bio-Rad®) for 18 to 24 hours at 37 ° C as described by Dodin and Fournier (1992). After incubation time, the yellow sucrose-positive colonies and the bright colonies on MH agar suspected to be *Vibrio cholerae* were subjected to Gram staining and oxidase tests; then well isolated colonies on MH were cultured on API®20E (Bio-Mérieux 20100) and API®20NE (Bio-Mérieux 20050) galleries to reveal their biochemical characteristics.

The differentiation between the El Tor biotype and the classical biotype was achieved using the following tests:

- sensitivity to polymyxin B: with the classic biotype, the zone of inhibition around the 50 unit polymyxin B disc was 12-15 millimeters; with the El Tor biotype it was narrower and only measures 1-2 millimeters (resistant).

- The reaction of Voges-Proskauer (VP). Usually the El Tor biotype is positive and strains of the classic biotype are negative.

Agglutination tests were carried out with the polyvalent anti-O1 sera (Deben Diagnostics Ltd 293831), mono specific Inaba (Deben Diangostics Ltd 293824) and monospecific Ogawa (Deben Diangostics Ltd 293848).

The antibiogram was performed using standard techniques (disk diffusion method or Kirby-Bauer technique).

The search for resistance or sensitivity to 2,4-diamino-6,7-diisopropylteridine, also called vibriostatic

compound O / 129 was carried out to differentiate *Vibrio cholerae* from *Plesiomonas* and *Aeromonas*:
- the sensitivity of vibriostatic: with *Vibrio cholerae* and *Plesiomonas*, the zone of inhibition around the disc of 50 unit of vibriostatic (discs loaded with 0.5 mg) was 15 millimeters (sensitive);

- with the *Aeromonas*, it was narrower and only measures 1-2 millimeters (resistant). The medium used to perform the antibiotic susceptibility test was Mueller-Hinton agar (MH). For each medium, a volume of 25 ml is used for a 90 mm diameter Petri dish giving a thickness of 4 mm according to the work of the Antibiogram Standardization Group of the French Society of Medical Microbiology (SFMM) and the European reference technique known as ASFT-EUCAST (Antibiotic Susceptibility Testing - European Committee on Antimicrobial Susceptibility).[8,9] (table 1).

The isolated strains were then sent to the National Reference Center for vibrios and cholera, Cholera and Vibrios Unit (France) for confirmation. The strains were stored at - 80 ° C in brain heart broth (BCC) (Bio-Rad®) at 10% glycerol according to the instructions National Reference Center for Vibrios and Cholera, Cholera and Vibrio Unit of the 'Institut Pasteur de Paris / France for molecular characterization.[10]

Table 1.0: Antibiotic Drugs used for drug susceptibility testing.

Category	Class	Anti-infective	Dose/disc
Antibiotic (Bio-Rad)	Beta-Lactamines	Amoxicillin (AMX)	25 µg
		Ampicillin (AMP)	10 µg
		Amoxicillin + clavulanic acid (AMC)	20/10 µg
	Fluroquinolone	Ciprofloxacin (CIP)	5 µg
		Norfloxacin (Nor)	5 µg
	Quinolone	Nalidixic acid (NA)	30 µg
	Cyclines	Tetracyclin (TET)	30 µg
		Doxycyclin (DO)	30 UI
	Phenicoles	Chloramphénicol (CHL)	30 µg
	Sulfamides	Triméthoprim-sulfamethoxazole (SXT)	1,25 /23,75 µg
	Macrolides	Erythromycin (Ery)	15 µg
	Polymyxin B	Polymyxin B	50 UI
		Composed vibriostatic O/129	150 µg

2.4.2. Molecular characterization of *Vibrio cholerae* O 1 isolates

The molecular characterization of the isolated *V. cholerae* O 1 strains was carried out at the National Reference Center for Vibrios and Cholera at the Institute Pasteur in Paris / France. The manipulations were carried out according to the instructions of the CNRVC.

Polymerase C chain Reaction (PCR)

The search for pathogenicity factors by PCR was systematically performed for the confirmation of all strains of *V. cholerae* O 1 sent to the CNRVC of IPP / F. PCR amplifies *in vitro* a specific region of a given nucleic acid in order to obtain a sufficient quantity to detect and study it. In this case, the ctxA and ctxB genes, encoding the production of cholera toxin, were systematically searched in all isolated strains of *Vibrio cholerae* by PCR.

The DNA extraction was done using the heating method. DNA was extracted using the InstaGene Matrix kit (Bio Rad laboratories, Marnes La Coquette, France) according to the manufacturer's recommendations. Briefly, a loop full of colonies from a 24-hour culture of the bacteria on MH media was collected and triturated in 1 ml of sterile buffered physiological water previously distributed in the 1.5 ml microtubes. The mixture was vortexed, then centrifugation was performed for 1 min at 12000 rpm and the supernatant was removed. Then, 200 µL of InstaGene matrix were added to the pellet and the whole was mixed by several pipetting and incubated in a water bath at 56 ° C for 30 min. High speed vortexing of the microtube was performed for 10 sec and the microtube was placed in a heat block at 100 ° C for 8 min. The microtube was further vortexed at high speed for 10 sec and centrifuged at 12000 rpm for 3 min. Finally, 1 µL of supernatant containing bacterial DNA was used for 50 µL of PCR reaction.

The reaction mixture for the PCRs consists of 1 µL of diluted DNA template (concentration of 0.1 to 0.2 µg / 50 µL) and a PCR master mix with a volume of 49 µL for a final reaction mixture of 50 µL. A control of the PCR master mix was performed by adding 1 µL of sterile distilled water (H2O) to the PCR master mix.

Regarding the amplification of the ctxA and ctxB genes, the PCR master mix consisted of 33.5 µL of sterile distilled water (H_2O), 5 µL of 1X amplification buffer, 5 µL of deoxy ribonucleotide triphosphate solution (dNTPs) or 200 µM final for each dNTP, 2.5 µL of each primer at 1 µM and 0.5 µL of Taq DNA polymerase at 2.5 U / 50 µL. Internal controls were also used including a negative control with 1 µL of DNA from a non-O1 / non-O139 *V. cholerae* strain (ctxA negative) and a positive control with 1 µL of DNA from a strain of *V. cholerae* O1 or O139 (ctxA positive) having an amplification product of 564 base pairs (bp).

PCR was performed by initial denaturation at 95°C for 5 min, followed by 25 cycles of amplification. Each cycle consists of denaturation at 95°C for 1 min, hybridization at 60°C for 1 min, polymerization at 72°C for 1 min. Finally, a final elongation at 72°C for 10 min was performed.

Pulsed field electrophoresis (PFGE)

This type of electrophoresis was developed by Schwartz and Cantor in 1984 in order to separate large DNA molecules (> 50 kb) that conventional agarose gel electrophoresis cannot resolve, even by minimizing the agarose concentration (below 0.4% the gels are impossible to handle). The porosity of a conventional agarose gel is less than one micron while the length of a fully stretched 50 kb DNA molecule is about 18 microns. The speed of migration of DNA molecules larger than 20 kb is no longer affected by the filtration effect, it is constant whatever the size of the molecule.

The principle of pulsed field electrophoresis consists of alternating the orientation of the electric field over time. Each change in electric field reorients the DNA molecule in the gel, increasing the likelihood that the DNA molecule will be oriented to pass through the mesh of the gel. This probability depends on the size of the molecule and the speed of migration of a DNA fragment in the gel varies in the opposite direction of its size. Pulsed field electrophoresis thus makes it possible to separate DNA fragments ranging in size from less than 1 kb to around ten mega bases. For this type of electrophoresis, it is not possible to use DNAs purified by conventional techniques because these techniques break them into fragments of a size less than 100 kb. To avoid mechanical breakage of DNA molecules, cells are embedded in agarose blocks. The electrophoresis conditions depend on the size area to be solved.

Gel preparation

1.5% (w / v) agarose gel (Seakem ME Agarose for gel electrophoresis) was prepared in an Erlenmeyer flask containing 300 ml of 0.5X TBE Buffer (100 mM Trizma base, 100 mM boric acid, 2 mM EDTA) and poured onto the electrophoresis gel support after positioning the comb and allowing it to solidify (at least 30 min).

Migration

The electrophoresis tank was filled with 0.5X TBE Buffer until complete immersion of the gel after removal of the comb. An aliquot of the PCR product was mixed with loading buffer (0.040% bromophenol, 7% glycerol, 6 mM EDTA), and 10 µl of this mixture was introduced into a well of agarose gel. A 100 bp molecular weight marker (GeneRuler, Fermentas) was used. The negatively charged nucleic acid molecules will migrate on the gel only by electric field crawling and their elongation occurs in the direction of the field. The program for the electrophoretic migration was as follows: initial pulse time 60 s, final pulse time 120 s, migration time 72 hours at 6 volts / cm, angle 120^{o}, temperature 14^{o} C. At the end of the migration, the gel was stained with ethidium bromide solution (BET, 1.5%) for 30 min. The visualization was made under a UV lamp with an image integrating computer software.[11]

3.0. RESULTS AND DISCUSSION

3.1. Origin spread of disease and environmental conditions

The geographical origin and direction of the spread of cholera epidemics from 2010 to 2019 are illustrated in Figure 1. From the week 25 (W25) of June 2010 to W51 of December 2011, Chad experienced two consecutive years of cholera epidemics with 24332 cases recorded and 710 deaths (mortality rate of 3%).[12] In fact, 2010 was a year of heavy rainfall triggering flooding across the country. The first cases of cholera were reported in a locality of Kiskra located 75 km from Bol in the Lake Chad region at week 28 (W28) (figure 1). This case probably occurred in the nomadic Arab community, after consumption of water from an old abandoned well in the Ferrik Maday near Kiskra, a locality close to the border with Niger. In fact, cases of acute gastroenteritis with vomiting and diarrhea were reported in the health district of Nguigmi in Niger (Figure 1) a week earlier (W27). This district shares borders with Bol (Chad). Two days earlier, the village of Kiskawa recorded cases. The disease spread and caused deaths likely due to the movement of local populations as part of their socio-economic and socio-cultural activities.

On July 31, 2010, the health authorities of Bol (Lake Chad) and the Health District of Nguigmi (Niger) exchanged information on the progression of cholera cases. On October 25, 2010, in Kanem Province, Mao health district, under Fayaye index of N'Djamena was affected by the cholera epidemic.[12] The high promiscuity between members of the same nomadic group in unfavorable hygienic conditions was certainly a predominant factor in the spread of the epidemic. The permanent contact between the breeders and the cattle could also have favored new contaminations.[13] In 1980, Dodin isolated *Vibrio* in the stools of camels in the Sahelian zone.[14] The disease will spread slowly in the border district with Niger between W32 and W36. It was really between W37 and W43, or three months, that the epidemic took on a worrying scale. Two new outbreaks foci were reported during that period, one in N'Djamena and another along the Logone on the Cameroon border. In N'Djamena, cases of gastroenteritis due to vomiting and diarrhea were recorded in the Walia quarter (9th Subdivision) at W36 on October 2, 2010. This quarter was completely flooded by rainwater. A week later, cases of acute gastroenteritis with vomiting and diarrhea were registered in Chagoua (7th subdivision), N'Djari and Diguel (8th subdivision) quarters following the consumption of raw vegetables and meat (Kilichi). Besides, Kilichi has been suggested as a source of food poisoning.[15]

In Kournari, a locality situated at 35 km in the south of N'Djamena, a man coming from the epidemic area of Bol, developed diarrhea with vomiting and subsequently died with three of his brothers. The 2010-2011 epidemics were significant due to its long epidemic duration (77 weeks), the number of affected health districts[17] and administrative regions.[8] They were grouped into 4 foci depending on the onset and the development of the epidemic and the surface-water hydrology (Figure 1): the Lake Chad basin (Bol, Massaguet, Massakory, N'gouri and Mao) with 32.14% of cases, the Chari-Logone basin (16.72%), the Lakes of Mayo-Kébbi basin (31.19%), the city of N'Djamena (19.51%). The high fatality rates associated with the hydrological basins could be explained by the proximity between towns and villages whose rivers, lakes, and lagoons constitute a repository of waste and wastewater, or to the interurban traffic of healthy carriers of the disease. Reliable data have shown that relatives of cholera victims washed corpses in the waters of the Logone rivers. Indeed, the two rivers of the country (Chari and Logone) communicated during floods with Lake Chad. In 8 administrative provinces divided into health delegations (Chari-Baguirmi, Guera, Hadjer-Lamis, Kanem, Lac, Mayo-Kebbi East, Mayo-Kebbi West and Tandjilé) 1434, 35, 503, 30, 1833, 1227, and 29 cases were recorded respectively with deaths: 34, 3, 14, 3, 72, 74, 23, 3 corresponding respectively to fatality rates of 2.37%, 8.57%, 2.78%, 3.92%, 4.08%, 1.87%, and 10.34%.

From week 25 (W25) of June 2010 to W51 of December 2011, 327 cholera stool cultures were performed on 24332 recorded cases and 710 deaths with a fatality of 2.91%.[16] Cases and deaths were also reported in week 1-52 during 2009 cholera outbreaks in Ethiopia with 31509 cases and 434 deaths; Democratic Republic of Congo (22899 cases and 237 deaths); Mozambique (19679 cases and 155 deaths); Kenya (11425 cases and 264 deaths) and Angola (2019 cases and 88 deaths) respectively.[17] The cholera epidemic has also claimed more than 150000 cases and 3333 deaths in Haiti during the same period.[18]

The 2014 epidemic began on October 20, 2014, a total of 151 cases and 10 deaths were recorded in three separate epidemic foci: the district of Léré, the island of Kinasserom and the area of responsibility of Kaiga on the border with Niger. Two cases of cholera were also recorded in N'Djamena following the outbreak in Kinasserom Island. The resurgence of cholera in these three outbreaks is independent and consecutive to major epidemics underway in neighboring countries, particularly in Nigeria, Cameroon and Niger. The 2014 epidemic affected three provinces (Lac, N'Djamena and Mayo-Kébbi West) and four Health Districts (Léré, Bol, N'Djamena Center and Liwa).[19]

The 2017 epidemic was focused in the Am-Timan province with 1254 cases and 80 deaths with a fatality rate of 6.37%. This restricted epidemic affected 14 of the 16 areas of under Am-Timan district.[20]

The 2019 cholera epidemic was of slighter importance as it affected only one the West Mayo-Kébbi province and two health districts with 90 cases and 2 deaths, for a fatality rate of 2.24%. In contrast, that of 2010 was the highest in terms of its incidence (6901 cases and 226 deaths, with a fatality rate of 3.27%) and its duration of 27 weeks. This shows that the therapeutic management recommended by the WHO, which favored oral and intravenous rehydration, was well followed in Chad by the reduction of death rates compared to previous epidemics. Of note, there is a significant difference in terms of intensity and morality rates between these epidemics.[21] In the Logone Ghana River, a locality located 110 km from South-West of N'Djamena (Karaska village), the source of contamination of the disease was sought in the guts of three species of fish (*Heterotus niloticus*, *Clarias* fry and *Tilapia*). This research enabled the isolation and identification of *Aeromonas hydrophila* from the intestines of *Heterotus niloticus*. This source of *Vibrio* contamination on food has also been reported elsewhere.[22]

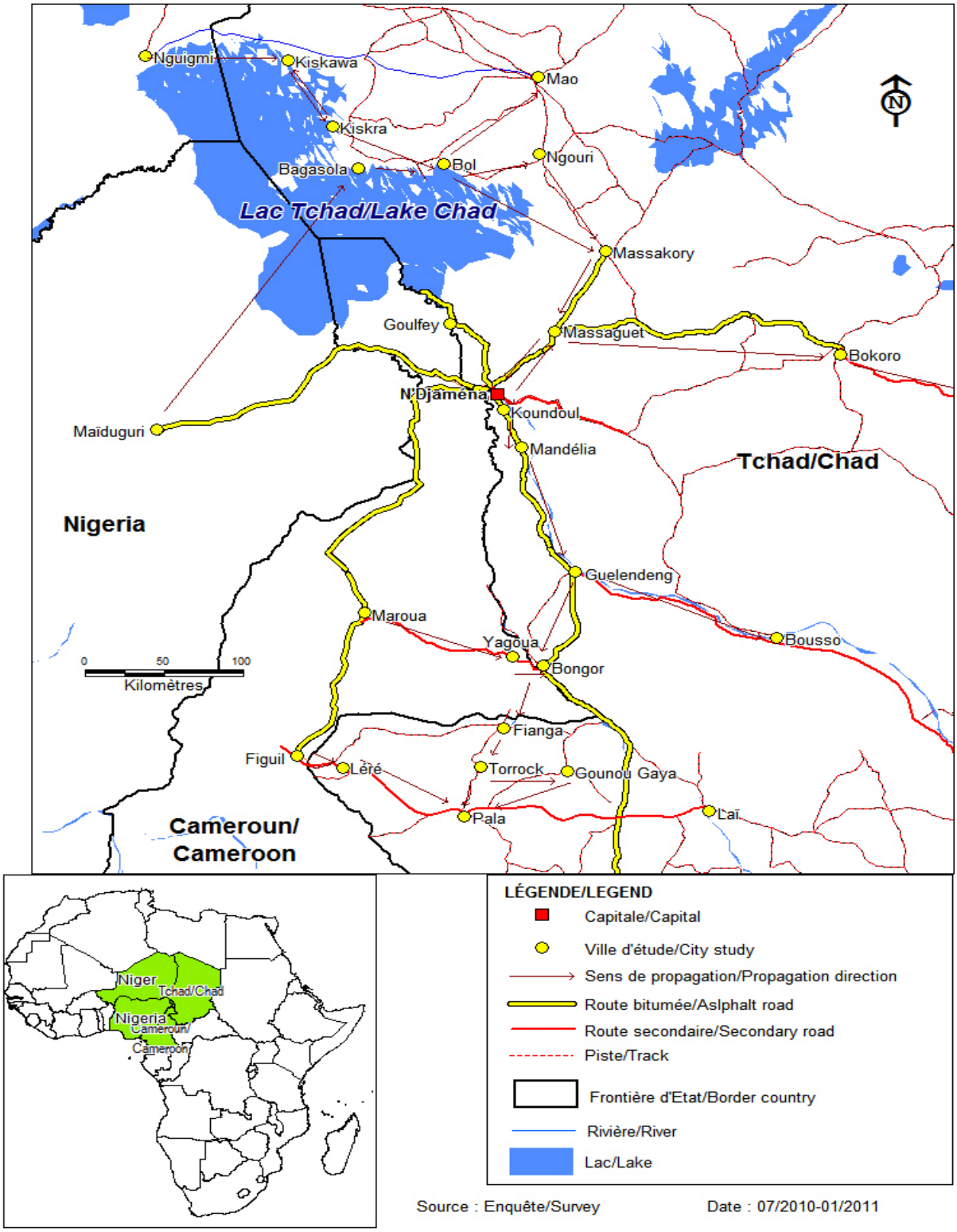


Figure a: 2010/Map of study areas of cholera.

3.2. Morphological characteristics

Vibrio cholerae O1 isolates produced yellow-orange pigments demonstrating the use of sucrose which turned TCBS medium from green to yellow-orange (Table 6 figure: c) and whitish translucent colonies were observed on Mueller-Hinton agar (Table 6, figure: d). The microscopic observation of the stools in a fresh state showed the monomorphic appearance and scaled cells with the presence of numerous motile germs which move in flight of midges. The smear observation after Gram staining showed the presence of the monomorphic flora made up of thin, curved Gram-negative bacilli.[23,24]

3.3. Biochemical profile

The isolated *Vibrio cholerae* O1 have for the most part biochemical characters: ONPG +, ADH, LDC +, ODC +,

Nitrate-reductase +, VP +, Glucose +, Sucrose +, Manose +, Gelatin + (Table 6, figures: d, e), indole +, Hydrogen sulfide (H2S) - and oxidase +.

All isolated strains were positive to the Voges-Proskauer (VP) reaction as previously reported.[25,26,27]

3.4. Distribution in Serogroup, Serotype and Biotypes of *Vibrio cholerae* O1 strains isolated in 2010-2019

Table 2 shows the distribution by serogroup, serotype and biotype of the strains of *Vibrio cholerae* O 1 isolated from 2010-2019.

A total of 183 *Vibrio cholerae* O1 were isolated at the Bacteriology Laboratory Unit of CHU-RN consisting in 130 Ogawa and 53 Inaba from 2010 to 2019. The determination of the Inaba, Ogawa or Hikojima serotype is still wrongly considered useful, even essential. This determination is of very limited interest since the study of the genetics of the *Vibrio cholerae* O1 lipopolysaccharide, which is the molecular support of the serotype, showed that a strain could easily pass from one serotype to another and therefore a change in serotype during an epidemic did not necessarily mean the advent of a new strain.[28]

Table 2.0: Distribution of strains of *Vibrio cholerae* O 1 isolated in 2010-2020.

Strain	Year	Number	Serogroup	Serotype		Biotype
Vibrio cholerae	2010	38	O 1	32 Ogawa	6 Inaba	El Tor
	2011	87	O 1	62 Ogawa	25 Inaba	El Tor
	2014	26	O 1	11 Ogawa	15 Inaba	El Tor
	2017	19	O 1	19 Ogawa	00	El Tor
	2019	13	O 1	00	13 Inaba	El Tor
Total	**5**	**183**	O 1	**130 Ogawa**	**53 Inaba**	El Tor

3.5. Prevalence of isolated *Vibrio* from 2010-2020 in Chad

From 2010 to 2019, Chad experienced five different cholera epidemics (2010, 2011, 2014, 2017 and 2019). During that period, 327 stool cultures were performed, of which 183 (56%) *Vibrio cholerae* O1 were isolated. Of the 183 *Vibrio cholerae* O1 isolated, 130 Ogawa serotypes and 53 Inaba serotypes were identified. The distribution of the 327 stool cultures over outbreaks was as follow: 71 (22%) in 2010, 106 (32.41%) in 2011, 81 (25%) in 2014, 43 (13.14%) in 2017 and 26 (8%) in 2019 with the number of isolated *Vibrio cholerae* O1 being respectively 38 (53.52%), 87 (82.07%), 26 (32.09%), 19 (44.18%) and 13 (50 %). In 2009 in Sudan, similar strains of *Vibrio cholerae* O1 were detected and described by the American Center for Disease Control and Prevention.[29] However, some of the stool specimens were cultured sterile, probably due to pre-treatment with antibiotics, poor handling and improper transportation of specimens in Chad. Therefore, it would be advisable to carry out a detailed evaluation to find out whether they are false negative or true negative.

3.6. Distribution of infection by age and sex

Of 327 stool cultures used to identify *Vibrio cholerae* O1, 30 stools (9.17%) originated from 0-5 years old children, 42 stools (13%) from 5-10 years old patients, 60 stools (18.34%) from 10 to 15 years old, 75 stools (23%) from 15-20 years and finally 120 stools (37%) from 20 years and over patients with respectively a rate of infections by *Vibrio cholerae* O1 of 10 (33.33%), 25 (59.52%), 44 (73.33%), 42 (56%) and 62 (52%) (Table 3). The 10 to 15 years age group was most affected (73.33%) followed by 5 to 10 years (59.52%). A prevalence of 33.33% was recorded for 0 to 5 years old children and is contrary to the national definition of cholera cases.[30] All age groups were affected without distinction of sex (Table 3).

Table 3.0: Distribution of infection by age and sex of patients.

Age (year)	Number of culture	*Vibrio cholerae* O1 infection		
		female	male	Total (%)
0-5	30	2	6	8 (4.37)
5-10	42	8	17	25 (13.66)
10-15	60	18	26	44 (24.04)
15-20	75	17	25	42 (23)
20 et +	120	19	45	64 (35)
Total	327	64	119	183 (100)

% = Percentage

3.7. Susceptibility of antibiotics to isolated Vibrio cholerae O1

Table 4 shows the results of the susceptibility test for *Vibrio cholerae* O1 isolates to antibiotics. A panel of 12 antibiotics and a vibriostatic O / 129 compound were tested against the *Vibrio cholerae* O:1 isolates. The isolates studied showed good sensitivity to Ampicillin, Doxycycline, Tetracycline, Ciprofloxacin and Norfloxacin. They were resistant to Amoxicillin, Trimethoprim-sulfamethoxazole, Nalidixic Acid, Chloramphenicol, and Polymixin B. Intermediate profiles were observed with Amoxicillin + Clavulanic acid and Erythromycin. Resistance to beta-lactams is probably due to the production of beta-lactamases of the penicillinase type. A study by Aucher et al., report that Ampicillin is generally inactive against the majority of *Vibrios*.[31] Overall, Quinolones (Ciprofloxacin, Nalidixic Acid and Norfloxacin), Tetracycline, Doxycycline and Chloramphenicol exhibited good activity.[32] However, *Vibrio* resistance to tetracycline have been reported.[33]

Strains isolated in 1991 and 1994 were sensitive to: Furans, Doxycycline, Chloramphenicol, Trimethoprim-sulfamethoxazole, Sulfonamides, Ampicillin, Tetracycline and Pefloxacin.[34] In the Kathmandu Valley

in Nepal, the strains of *Vibrio cholerae* O: 1 isolated by Kari and Tiwari showed very high sensitivity to Ciprofloxacin (97.84%), Norfloxacin (93.41%), Tetracycline (92.34 %), Erythromycin (92.34%), and resistant to Ampicillin (100%).[35] These results corroborate our study, except that our strains showed intermediate sensitivity to Erythromycin (56.28%) and Sensitive to Ampicillin (57.37%) (Table 4). This study showed that all the strains isolated were resistant to the vibriostatic compound O / 129. The resistance of *Vibrio cholerae* O1 is thought to be due to a plasmid which encoded resistance genes to the vibriostatic compound O / 129.[36] These results do not corroborate those of Massenet et al. which revealed that all the strains isolated during the epidemics of 1991 and 1994 were sensitive to this same compound.[37] In Côte d'Ivoire, strains of non-O1 non-O139 *Vibrio* isolated from the waters of Grand-Lahou showed resistance at 66.7% to Ampicillin and sensitivity to the vibriostatic compound O / 129.[38] The strains sent for confirmation to the the National Reference Center for Vibrios and Cholera, Cholera and Vibrios Unit, Institute Pasteur of Paris (IPCNRVC, UCV) showed an average sensitivity to 6 (40%) antibiotics (Ampicillin, Doxycycline, Tetracycline, Ciprofloxacin, Pefloxacin and Cefolatin), resistance to 5 (33.33%) antibiotics (Trimethoprim-sulfamethoxazole, nalidixic acid, Polymixin B, Sulfonamides and Nitrifurans) and Intermediate 4 (26.66%) antibiotics (Erythoxromacenicolin, Norlorophycine and Ofloxacin). The strong resistance observed to amoxicillin is probably due to a low-level of penicillinase (amoxicillin + clavulanic acid) (Table 5). Strains isolated in Tanzania and the Democratic Republic of Congo appear to be less resistant.[39, 40] However, we found a level of resistance similar to our strains in 86% of Angolan strains.[41] The resistance of *Vibrio cholerae* strains documented so far to ampicillin is mediated by a plasmid concurrently encoding for many other antibiotics.[42, 43, 44] In fact, penicillinases carried by the plasmids have been found in most Gram-negative bacilli, the best known of which are TEM1 and SHV1.[45] These results corroborate ours (Table 4) except that some antibiotics (Pefloxaine, Ofloxacin, Cefalotine, Nitrifurans) were not tested in our study. The strains isolated from 1971 to 2019 present an identical resistance and sensitivity profile to antibiotics, which argues that, the same strains are responsible for cholera endemics since.[46, 47] It would be interesting to compare the antibiogram of strains isolated in other African countries since 1983 to those that shows an increase in strains resistant to the vibriostatic compound O / 129, to sulfonamides and to cyclins.[48] However, only a detailed study of several molecular markers would confirm or refute the hypothesis of the existence of a *Vibrio cholera* strain which is endemic in Chad.

Table 4: Evaluation of the sensitivity of strains of *Vibrio cholerae* O: 1 with respect to antibiotic.

Anti-infective	Dose/disc (µg, UI)	Efficiency			Ø (mm)
		R (%)	S (%)	I (%)	
Tétracyclin	30	2 (1.09)	181 (99)	0 (0)	24-29
Doxycyclin	30	0 (0)	182 (99.45)	1 (0.54)	≥19
Ciprofloxacin	5	33 (18.03)	139 (76)	11 (6.01)	24-27
Norfloxacin	5	35 (19.12)	143 (78.14)	5 (3).	20-25
Nalidixic acid	30	175 (97)	7 (38.25)	1 (0.54)	24-28
Erythromycin	15	67 (37)	3 (2)	103(56.28)	15-25
Trimethoprim-sulfamethoxazole	1,25 /23,75	183 (100)	0 (0)	0 (0)	23-32
Ampicillin	10	70 (38.25)	105 (57.37)	8 (4.37)	15-21
Amoxicillin	25	127 (69.39)	0(0)	56 (31)	≥21
Clavulanic acid +Amoxicillin	20/10	16 (9)	0 (0)	167(91.25)	≥20
Polymixin B	50	156 (85.24)	0 ()	27 (15)	12-15
Chloramphenicol	30	136 (74.31)	0 (0)	47(26)	21-27
Composed vibriostatic O/129	150	183 (100)	0 (0)	0 (0)	≥15

S = sensible ; R = resistance ; I = intermédiate ; % = percentage ; Ø = diameter ; µg = microgram ; UI : international unit.

3.8. Molecular characterization of *Vibrio cholerae* O 1 isolates from Chad

Of 72 strains of *Vibrio cholerae* O 1 sent to the National Reference Center for Vibrios and Cholera (CNRVC) at the Institut Pasteur of Paris, 45 (61.64%) were confirmed positive by Pulsed Field Electrophoresis, 26 (36, 11%) by PCR, ie a total of 71 (99%) positive by these two methods. This shows that molecular methods offer better specificity than phenotypic methods for identifying *Vibrio cholerae* O1 or discriminating them from other species. A *Vibrio cholerae* O1 strain (Re.266), serotype Ogawa sent by the laboratory of CHU-RN was neither confirmed by PCR nor by PFGE at CNRVC / IPPF. 71 (98.61%) strains were detected by restriction primers (CTX2, CTX3 and CTX7, CTX9B) and confirmed by molecular methods (PCR and PFGE) as carriers of the virulence genes encoding for the synthesis of cholera toxin (ctxB and ctxA). All strains were confirmed to be resistant to nalidixic acid (Nal, MIC> 256); 23 (32.39%) and sensitive to Ciprofloxacin (MIC = 0.5); 48 (67.60%) were also sensitive to Ciprofloxacin (MIC = 0.38).

The mechanism of resistance to nalidixic acid is manifested by mutations that occur in specific regions of four enzymes: DNA gyrase A, DNA gyrase B, topoisomerase IV C, and topoisomerase IV E. This complementary study focused on mutations in the determinants of quinolone resistance (Nal) of the regions (QRDRs) of gyrase A and B. The regions (QRDR) of the gyrases at the locus of these genes are amplified by PCR and sequenced. DNA sequences have been translated into protein sequences. The sequences of these strains were compared with those of certain strains sensitive to Ciprofloxacin in order to look for amino acid substitutions conferring resistance. Several substitutions which were not present in all strains sensitive to Ciprofloxacin were detected, and those which involved a marked change in the chemical nature of the amino acid at the position where the substitution took place were identified as presumed mutation conferring resistance. Cholera toxin virulence genes were detected in 98.61% by PCR and PFGE.

The strains identified by biochemical methods in Chad were detected by restriction primers (CTX2, CTX3 and CTX7, CTX9B) and confirmed by molecular methods (PCR and PFGE) as carriers of the virulence genes encoding the synthesis of the cholera toxin (ctxA and ctxB). Moreover, Iftekhar Bin Naser, al (2019) and Rakoto Aslon et al (2001) and Faruque (1998) have shown that the transfer of plasmids takes place by two types of determinants:

- The tet A type which results in a high level of resistance to oxytetracycline and tetracycline but low to monocycline and doxycycline;

- The tet B type, which is expressed by a high level of cross resistance to all cyclins, suggests that our study strains carry the tet B determinant (Table 5).[49, 50]

The recurrent cholera epidemics and pandemic in Africa and around the world are believed to be due to the pathogenicity and virulence of these cholera toxin-producing genes, which argues for the persistence of the same strain responsible for these scourges.

The application of molecular methods allows to carry out epidemiological surveillance of strains (circulation of strains, diversification of populations, possible introduction of a new strain, origin of imported cases, etc.). The specificity and sensitivity of the PCR technique, compared to the conventional culture method, for the determination of pathogenic Vibrios in fishery products have been demonstrated in several studies.[51, 52]

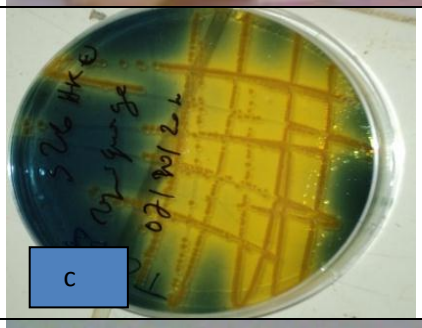
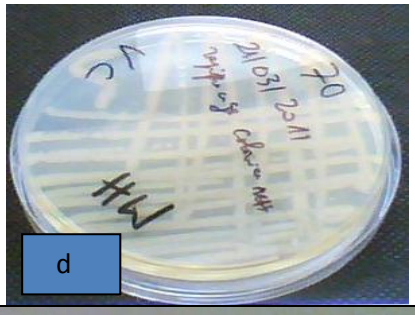
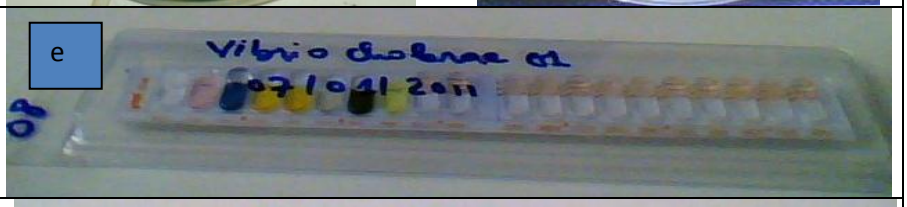
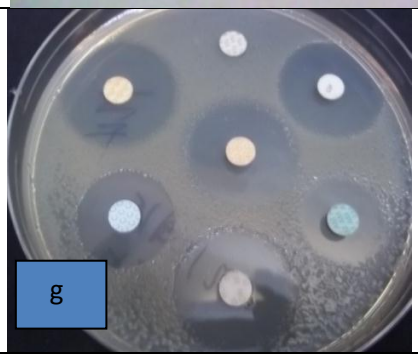
Table 5.0: Phénotypic and génotypic characterization *Vibrio cholerae* O 1 strains isolated in Chad from 2010-2020.

Strain	LCHU-RN and CNRVC results						Additional CNRVC studies									
	CHU-RN	CNRVC	Serotype		Genotype		PCR	PFGE	Seq ctxB7	CMI Nal>256	CMI Cip = 0.5	CMI Cip = 0.38	gyrA	gyrB	parC	parE
			Og	In	ctxA	ctxB										
***V.cholerae* O1**	n=72	n=71	54	18	71	71	26	45	71	71	23	48	46	-	46	-

n= number of strains ; LCHU-RN = Laboratory University Center Hospital of the National Reference of N'Djamena ; CNRVC = National Reférence Center for Vibrios and cholera in Paris France ; PCR = Polymerase Chain Reaction ; Og = Ogawa ; In = Inaba ; PFGE = Pulsed Field Électrophoresis ; Seq = sequence ; CMI = Minimum Inibitory Concentration. CtxA et ctxB = cholera toxin A et B ; gyrA and gyrB = gyrase subunit A et B, respectively ; Nal = Nalidixic acid ; CIP = Ciprofloxacin ; parC et ParE = topoisomérase IV subunit C et E, respectively

NB : (-) = négatif result

Table 6.0: Macroscopic and microscopic characteristics of *Vibrio cholera* 01.

1	b : Character of the setools of the des cholera patients : the stools have the appearance of rice water (Photo : *Bessimbaye.N*, 2010)	
2	c : Yellow opaque sucrose+ colonies on TCBS medium : *Vibrio cholerae* O1 d : Whitiss colonies of *Vibrio cholerae* O1 on MH medium (Photo : *Bessimbaye.N*, 2011).	 
3	e : Biochemical identification *Vibrio cholerae O 1* on API® 20NE (Photo : *Bessimbaye.N*, 2011).	
4	f : **:** Biochemical identification *Vibrio cholerae O 1* on API® 20E (Photo : *Bessimbaye.N*, 2011).	
5	g: : areas of antibiotic inhibition (Photo : *Bessimbaye.N*, 2011). h : agglutination serums of strains of *Vibrio cholerae* O 1 (Photo: *Bessimbaye.N*, 2010).	 

CONCLUSION

For 44 years, Chad has been the repeated target of cholera epidemics of varying magnitude and mortality: 1971 (fatality rate 29.3%), 2010-2011 (fatality rate 3%) and 2019 (fatality rate 2.24%). This shows that the therapeutic management proposed by WHO which favored oral and intravenous rehydration was well followed from 2010 until 2019. We also note an increase in the number of strains resistant to the vibriostatic compound O / 129, to trimethoprim-sulfamethoxazole, and to nalidixic acid from 1971 to 2010 and from 2010 to 2019. The most active antibiotics against *Vibrio cholera* O1 isolates are doxycycline, tetracycline and ampicillin. The strains detected were confirmed by molecular methods to carry the virulence genes encoding for the synthesis of cholera toxin (ctxB and ctxA). Overall, the characteristics of the isolated germs since 1971 suggest that the same strain is persisting since and is responsible for recurrent epidemics in Chad.

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