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Identification of *Clostridium botulinum* from Drinking and Food Processing Water Source in a Rural Area in Enugu, Nigeria

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Abstract: **Background**: *Clostridium botulinum* is responsible for the toxin-mediated disease, botulism. It is a food-borne disease, occurring globally with high mortality, especially in children. Gastrointestinal form, which is common in developing countries, is associated with ingestion of spore containing food. Many studies have reported animal botulism caused by ingestion of spore contaminated water. Such studies are scarce in humans. We here report incidental isolation of *Clostridium botulinum* in a river that is used for drinking and food processing in a community in Enugu Nigeria. **Method**: A descriptive study to ascertain the microbial content of Adada River that ran through Uzo-Uwani- and Igbo-Etiti Local Government Areas of Enugu State. The sampling sites were at the six differently determined geographical coordinates (stations 1-6) along the Aku bank of the Adada River flow. The sampling stations were selected according to the vegetation's cover and river use. Standard water sample processing for *Clostridium perfringens* was followed and the isolated organism was characterized phenotypically and using 16SrRNA gene sequencing. **Result**: *Clostridium botulinum* was isolated from station 2 in June 2016 (rainy season), and in February 2017 (dry season). The organism was a Gram-positive motile rod with oval subterminal spores which was confirmed using a 16S rRNA sequence. The sequence shows 96.8% identical to *Clostridium botulinum* strain AM1195 chromosome, complete genome (NCBI accession number CP013701). **Conclusion**: Such organism in the drinking water and water used for home food processing could cause gastrointestinal botulism, especially in children. There is a need to provide potable water for rural dwellers in Enugu, and for the concerned authorities to monitor the water used by local food processors.

Keywords: *gastrointestinal botulism, floppy child syndrome, contaminated drinking water, food poisoning, 16S rRNA gene*

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1. Introduction

Clostridium botulinum is an anaerobic spore-forming bacillus found in the soil and the environment [1]. It produces a toxin that is responsible for the clinical condition known as botulism [1]. The disease, which in the last decade has been frequently reported in Africa, involves blocking of acetylcholine release in the peripheral nervous system and resulting in muscle paralysis [1,2]. Death occurs in 5-10% of cases due to respiratory muscle arrest [3].

Food-borne botulism, the commonly described type, occurs by ingestion of food containing the preformed toxin. The implicated foods in Nigeria are homemade fish and vegetables [4]. In the rural area, however, data on food sources are lacking. Intestinal botulism is due to the proliferation of *Clostridium botulinum* in the intestine upon ingestion of the spores. This type is common in infants ('floppy child syndrome') and immunocompromised groups with poor mucosal immune protection [1].

In many rural areas in Nigeria, there is a scarcity of potable water [5,6]. The dwellers often depend on streams for drinking, food processing, and domestic work [6]. This could explain the frequent outbreak of water-borne infectious diseases in such areas [6,7]. Cholera, salmonellosis, and shigellosis are commonly diagnosed because of the availability of the test's facilities and the high index of suspicion [2,6]. Other rare diseases are often not diagnosed owing to a lack of testing capacity in many rural hospitals and a poor patient referral system in Nigeria [2]. Could gastrointestinal botulism be acquired from *Clostridium botulinum* spores contaminated water sources? There is a significant paucity of data in humans

than in animals on the possibility of acquiring botulism from contaminated water directly [8,9]. Many available studies described concerns with the potential danger of contaminated water in the food processing industry [10].

We isolated C*lostridium botulinum* from a river during our microbial water survey. This water is used by the villagers for drinking, food processing etc. Is this finding of public health importance? We here report the process that led to the isolation and characterization of the organism. This will inform the public and the policy makers on the need to provide potable water in the rural areas. The clinicians and the public health specialist will find this finding necessary in their decision, source tracing and in further research.

2. Materials and Methods

2.1. Study Area

The study area and sampled sites are all the-year-round sparkling clear Adada River that runs through Uzo-Uwani- and Igbo-Etiti Local Government Areas of Enugu State in Eastern Nigeria at approximately five kilometers North-West of Aku, a village located $6^0$40" N and $7^0$18" E on the geographical map ($6^0$42'7"N $7^0$19'56"E). It is also the original site of the defunct (now re-proposed) Adada Campus of Enugu State University of Science and Technology as well as the location of the defunct Federal Government military cadet training school in the 1960s. Presently, it is the site of the ongoing 2.8 billion Naira "Adada Dam" instituted by the government of the Federal Republic of Nigeria in 2011.

2.2. Sampling Sites/Stations

The sampling sites were at the six differently determined geographical coordinates (stations 1-6) along the Aku bank of the Adada River flow, The sampling stations were selected according to the vegetation's cover and river use. For this report on the isolation of *Clostridium botulinum*, only station two is here described. **Station 2** is a geographical coordinate $6^0$44'20"N $7^0$16'50"E. It was way downstream from station1, at the beginning of where the river water was diverted for an ongoing Adada River Dam construction; the vegetation is only still slightly virgin and disturbed by Fulani herdsmen that occasionally graze cattle along the banks of the river, and it is the camping site of the construction workers.

2.3. Water Sample Collection

Collection of water samples were between 10.00 am to 12.00 pm (by which human activities have resumed), and were done in two different sampling periods, June 2016 (rainy season); and the repeat in February 2017 (dry season), precisely at the same specified geographical coordinates.

At each of the six sampling stations, water samples were collected in duplicates at the same distance from the shore with clean pre-sterilized 500-ml bottles with stoppers. The bottles were aseptically opened five centimeters (5cm) below the water surface, first rinsed with the initial set of water samples, then filled with the required water sample, and the bottle aseptically closed. The samples were transported to the laboratory under ice and stored at about 4^0C until they were ready for use.

All analysis was done within six hours of collection. However, some physicochemical properties: odour (manual), taste (manual), and temperature (mercuric thermometer) were done at the source.

2.4. Sample Processing and Phenotypic Identification for Anaerobes

These samples were characterized for *Escherichia coli*, fecal coliform, and *Clostridium perfringens*. The preliminary sample processing was done at the Microbiology laboratory of Enugu State University College of Medicine.

The water samples were homogenized by gently tilting the collection bottle before vortexing for 30seconds. The sample dilution of 1:10 was made using sterile water for the enumeration of *C perfringens* spores. The sample dilutions were vortex mixed (30sec) before heat-shocked for 20min for 60°C and then placed on ice for 20min. The samples (1ml) were filtered through a 0.4um-pore-sized 47mm polycarbonate filter (Casella, India), and then placed on blood, and MacConkey media plates following the method of Armon and Payment [11]. The plate was incubated in an anaerobic jar containing an oxygen removing sachet (Oxoid Ltd, UK) at 35C for 48hrs). The above procedure was performed in triplicate. Sterile water was used as negative control while *Clostridium perfringens ATCC 13124* was used as the positive control.

Upon incubation, the colonies were characterized and stained using the Gram technique. Motility testing was performed by the hanging drop method. Catalase test was performed using 3mls of 3% hydrogen peroxide solution in a sterile tube. *Staphylococcus aureus* ATCC 25923 was used for positive control. A sterile stable oxidase reagent strip was used to perform an oxidase test with *Pseudomonas aeruginosa* ATCC 27853 serving as the positive control.

2.5. 16S rRNA Gene Sequencing

The identity of the isolates recovered from the media was determined using a 16rRNA gene sequence, which was performed at the Inqaba Biotechnology Laboratory, South Africa. The DNA was extracted from the isolate using Qiagen microbial DNA extraction kit (Qiagen, Germany).

The extracts and control were amplified as described by Wilson et al [12]. The amplicons were purified using the PCR DNA and gel band purification kits (Qiagen, Germany) according to the manufacturer's instructions. Purified products were sequenced using an automated sequencer ABI 3730 DNA Analyser (Life Technologies-Applied Biosystem) using Big Dye ® Terminator V3.1 cycle sequencing kit. The sequences of the PCR products were compared with known 16S rRNA gene sequences in the GenBank (http://www.ncbi.nlm.nih.gov) by multiple sequence alignment using the Blast program.

3. Result

In station 2, large whitish beta-hemolytic colonies with wavy outlines were observed on blood agar after 24hours of incubation. It was a non-lactose fermenter. The organism was a Gram positive, motile rod with oval subterminal spores. They were catalase and oxidase negative. Molecular analysis of the isolate using 16S rRNA oligonucleotide primer showed bands at about 1400 bp. The genomic sequence result of the molecular test for *Clostridium perfringens* (which turned out to be C. *botulinum*) *has* 96.8% identical to *Clostridium botulinum* strain AM1195 chromosome, complete genome (NCBI accession number CP013701)

TGCTGTATCTCGTATTCGGAGCTCTGCTGGGCG
TACGTGCGTAGTGGAGTTTAGTGGTATGTGAAT
CCCCGGGCTTACCTGGGGGCTGCATTCCAAACT
GGATATCTAGAGTGCAGGAGAGGAAAGCGGAA
TTCCTAGTGTAGCGGTGAAATGCGTAGAGATTA
GGAAGAACACCAGTGGCGAAGGCGGCTTTCTG
GACTGTAACTGACGCTGAGGCACGAAAGCGTG
GGTAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGGATACTAGGTGTAGGG
GGTATCAACTCCCCCTGTGCCGCAGTTAACACA
ATAAGTATCCCGCCTGGGGAGTACGGTCGCAA
GATTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCAGCGGAGCATGTGGTTTAATTCGAA
GCAACGCGAAGAACCTTACCTGGGACTTGACA
TCCCTTGCATAGCCTAGAGATAGGTGAAGCCCT
TCGGGGCATGGAGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGT
CCTGCAACGAGCGCAACCCTTGTTATTATTTGC
TACCATTAAGTTGAGCACTCTCATGAGACTGCC
TGGGTAACCAGGACGAAGGTGGGGATGACGTC
AAATCATCATGCCCCTTATGTCCAGGGCTACAC
ACGTGCTACAATGGTAGGTACAATAAGACGCA
AGACCGTGAGGTGGGGGCAAAACTTATAAAAC
ATATCTCATTTCGGATTGTATGGCTGCTACTCC
CCTTACATGAAGCTGGAGTTGCTAGTAATCGCG
AATCAAAATGCCCGGTTAAAACTTCCCCCGGC
CCCATAAAAAAAAAA.

4. Discussion

Clostridium botulinum in drinking water and/or water used for food processing poses a potential risk of direct infection of the gut and liberation of botulism toxin. The effect of this is likely to be detrimental in the rural populations where malnutrition and other immunocompromised states predominate. In a study in Enugu and the environs, the organism has been detected in the stool of infants and children [13]. A study found out that the majority of rural dwellers do not have access to potable drinking water. In a water report by the WHO and UNICEF, over 86% of Nigerians lack access to safe drinking water, and many people still rely on surface water for drinking. This is why the burden of water-borne diseases is still a problem [6].

It is wildly known of the association of food containing *C botulinum* spores and gastrointestinal botulism, but few reports have highlighted a similar association in water-containing spores [5,8]. In animals, however, this association exists [8]. Water treatment by boiling will kill the vegetative organism but the spores could be resistant to boiling temperature [10]. Other methods of water treatment are scarce in rural communities [15].

In rural communities, such water is used for processing local food such as Abacha (African salad), vegetables or cooking [16,17]. Many of such food products are consumed directly without further processing. For instance, Iwuoha et, al reported that the rate of consumption of abacha immediately after washing in the stream in villages is high and could lead to food poisoning [17]. Some of the food products are fermented given room for proliferation and liberation of toxins by anaerobic bacteria like *Clostridium botulinum*. Iwuohaet et, al suggested that producers and vendors of such products should be educated on the healthy methods of preparation and handling of such food products. Many workers have recommended that there is a need for regulation of food production and selling at the rural level [16,17,18].

Clinicians should understand that many rare diseases are emerging, and they should broaden their differential diagnosis to accommodate diseases such as botulism, especially in children. Okunromade et, al observed that children with floppy features are neither investigated for botulism [2]. He also highlighted the gross lack of facilities for the diagnosis of anaerobic infections in many tertiary institutions [2]. The study also observed that autopsies are not frequently conducted due to poor implementation of state regulations and cultural beliefs. Currently it is difficult to know the incidence of the disease in Nigeria apart from the scattered case reports.

The public health specialist's role in source tracing should also be broad to accommodate rural sociodemographic peculiarities. This important function is often hindered in Nigeria by poorly funding of the public health institution [19]. Many 'sudden child death syndromes' happen in rural areas without postmortem diagnosis. Many of such deaths are mythically attributed to spiritual causes. Until proper investigation and disease source tracing are routine in Nigeria especially in rural area many rare and emerging diseases will continue to be misdiagnosed, underdiagnosed, and epidemiologically unclear.

5. Conclusion

Clostridium botulinum is the cause of food-borne disease, botulism. This study reported the incidental isolation of *Clostridium botulinum* from the Adada river. Its isolation from drinking and food processing water in a rural area could lead to gastrointestinal botulism in children. There is a need to provide potable water in the rural communities in Enugu. This will help to reduce both common food-borne diseases and rare ones like botulism. The authorities should monitor the water used by local food producers and vendors, especially in the rural area.

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