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ISOLATION AND IDENTIFICATION OF *CLOSTRIDIUM BOTULINUM* IN STOOL SAMPLES OF INFANTS IN NURSERY SCHOOLS IN ENUGU, ENUGU STATE.

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Abstract - *Clostridium botulinum*, a ubiquitous organism is one of the most common pathogens known to man. The pathogenicity of this organism is enhanced by the production of highly resistant spores and toxins implicated in tissue necrosis and sclerosis. Foodborne botulism is rare but it may kill rapidly, and contaminated products may expose many persons. The prevalence of food poisoning by this bacterium is not well known and documented in Nigeria. Fifty nine stool samples each were taken from infants in three different nursery schools in Enugu State, Nigeria and examined for the presence of this organism. Isolates were identified by culture and microscopy, after which the isolates were confirmed by their biochemical and molecular characteristics. *Clostridium botulinum* was detected in the stool samples of some of the infants in nursery schools. Foodborne botulism represents a medical and public health emergency that places premium on rapid and effective communication between clinicians and public health officials. Health education is therefore paramount in eradicating this infection, hence the introduction of personal hygiene policy into educational curricula, will ultimately help to reduce the menace of botulism in schools.
Keywords: *Clostridium botulinum,* pathogenic, food poisoning, toxins, spores

INTRODUCTION

Clostridium botulium is a gram-positive anaerobic, rod-shaped bacterium measuring 0.5-2.4µm by 1.6-22.0 µm, with oval sub-terminal spores that usually cause the cells to swell. Global occurrence of this organism has been documented (Koekpe, 2008). The bacterium survives in the soil and food such as honey; hence when the spores are ingested, they begin to flourish and produce a potent neuro-toxin (Aureli *et al*., 2002; Midura *et al*., 2007). In other words, infant botulism is due to the endogenous production of toxin by germinating spores of *Clostridium botulinum* in the intestine of infant (Bianco, 2008). The most unique feature of *C. botulinum* strains is the production of botulinum neurotoxin (BoNT), one of the most potent naturally occurring toxins known (Bakheit *et al.*, 2007). All botulinum neurotoxins (B_0NT_S) are di-chain peptide molecules with molecular mass of $150KD_a$.

The most frequent causes of infant botulism are *Clostridium botulinum* group 1 types A and B (Midura, 2006). Infant botulism is a rare disease that usually occurs in babies between 2 and 4 months; however, the number of cases may be underestimated due to a variety of applicable differential diagnoses such as sepsis, different neurological disorders and sudden infant death syndrome (SIDS). The disease was not

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diagnosed until 1976 (Arnon *et al.,* 2005), the incubation period being less than one month. It is the main form of botulism in Europe today (CDC, 2004). Child or adult botulism from intestinal colonization is represented by those cases in which no food vehicle can be identified, there is no evidence of wound botulism, and there is the possibility of intestinal colonization in a person older than 1 year of age (Shapiro and Hatheway, 2009).

Clostridium botulinum requires strict anaerobic conditions for growth; therefore continous work in an anaerobic workstation is a necessity for successful diagnosis. Conventional method of isolation and detection of *C. botulinum* are based on culturing in a liquid selective medium (Douglas *et al.,* 2006). In other words, cultural characteristics of *C. botulinum* are based on foodborne and infant botulism cases (Dezfulian *et al.,* 2004). The spectrum of clinical symptoms is wide: the main clinical features of infant botulism include constipation, listlessness, difficulty in sucking and swallowing, weak cry, general muscle weakness, gastrointestinal disorder and loss of head control (Arnon, 2004). The cornerstone of management of infant with botulism is meticulous supportive care. Moreover, a trial of human-derived botulism immune globulin is promising (Arnon *et al.,* 2006), There is no current evidence to support the effective use of antibiotics: the use of gentamicin or tobramycin may potentiate neuromuscular blockade, and so and is thus contraindicated (Santos *et al.,* 1981; L'Hommedieu *et al.,* 1979). Adequate pulmonary toilet and monitoring of cardiorespiratory function are essential, whereas intubation may be required for airway protection or respiratory failure. Tube feeding may be necessary if swallowing mechanisms are impaired, although parenteral hyper alimentation may be required if significant (Fenicia *et al.,* 2008). Ingestion of honey and age has also been implicated as significant risk factors of infant botulism. Many cases of infant botulism may go unrecognized due to the often insidious course and variable manifestations. Majority of these cases have been reported in California, Utah, and Pennsylvania. This study therefore was geared towards isolation and identification of *Clostridium botulium* in stool samples of infants in nursery schools in Enugu State, Nigeria, and as well determine the frequency of occurrence of the isolates.

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MATERIALS AND METHODS

Tool for Data Collection

Well-structured questionnaires were administered to the parents/guardians of the children, in order to obtain the socio-economic and bio-demographic data of the children such as age, exposure to dust (environmental factors), intake of honey, weakness, poor cry, lack of appetite, head inbalance, intake of breast milk and other infant foods (Guiteerez *et al.,* 2006).

Collection of Samples

Fifty nine stool samples each were collected from infants below one year of age from 'Santa Rosa school, command day care', and 'All saints nursery school in Enugu State', using dry sterile containers, after which they were transported to the laboratory for microbiological analysis.

Isolation of bacteria

At the laboratory, the stool samples were aseptically inoculated in pre-sterilized enrichment broth, Robertson cooked meat (RCM) broth, for the growth of both spore-forming and non-spore forming obligate anaerobes. The culture tubes were then incubated anaerobically for 24 h at 37^{o}C. Thereafter, aliquots of 100 mL from broth culture were aseptically transferred to sterile blood agar plates, prepared according to the manufacturer's specifications. The inoculated plates were incubated at 37 ^{o}C in an anaerobic atmosphere using anaerobic jar (Merck, Darmstadt-Germany) and Gas pak (AnaeroGen 3.51 Oxoid, Basingstoke UK) for 48 h. After examination, the colonies which displayed typical characteristics such as double zone of beta-haemolysis on blood agar, were purified by sub-culturing and re-incubating anaerobically for 48 before storing the pure culture subsequently for morphological and biochemical characterization using the color of colonies, edges, appearance and elevation, gram reaction, spore staining, and motility test (Solomon and Lilly 2001; Grant, 2008; **(**Logan and De Vos, 2009;).

Multiplex-PCR reaction

The primers used were: Tox-A1 (5'-GGA AAT TTA GCT GCA GCA TCT GAC-3'); Tox-A2 (5'-TCT AGC AAA TTC GCT TGT GTT GAA-3'); Tox-B1 (5'-GGT GAT ATG GAG GCA TCA CCA CTA G-3') and Tox-B2 (5'-TCC AGG ATA AGT CTC CTC TAC GTT G-3') (Gibco BRL Technologies) (Chen *et al*., 2006). Amplification was performed in a DNA thermal cycler (Perkin Elmer, GenAmp

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PCR System 9700), programmed for 94^{o}C (5 min) followed by 37 cycles of 94^{o}C (30 sec), 50^{o}C (30 sec) and 72^{o}C (30 sec), and then 72^{o}C (5 min), in order to allow the completion of DNA extension (Kokeala *et al.,* 2006).

Detection of amplified products

PCR products were detected by electrophoretic separation in 1% agarose gel in 1X TBE (Gibco) stained with ethidium bromide (0.5 µg/ml), at 70 V, for 2 h, using 1 Kb DNA ladder (Gibco) as a reference standard. The separated bands were photographed using ultraviolet (UV) trans-illuminator (Electrophoresis Documentation and Analysis System 120, Kodak Digital Science).

RESULTS AND DISCUSSION

Thirteen isolates were identified as *Clostridium botulinum.* These isolates were obtained from samples collected from infants aged 6 and 12 months respectively. Two of these infants had clinical signs or symptoms of food poisoning such as fever, vomiting or diarrhea. The primers used in this study amplified a characteristic 1,217-bp toxin A (gene *tcdA*) and 1,050-bp toxin B (gene *tcdB*) bands. Table 1 shows that the isolates have different types of edges, texture, shape, sizes, consistency and colour on blood agar. Table 2 shows that the isolates exhibit different biochemical reactions. Table 3 indicates that the highest number of isolate was obtained from the sample in Command day care, followed by Santa Rosa school. However, no isolate was obtained from the sample in All saint's School**.** Table 4 shows that consumption of infant's milk formula is the highest risk factor (seconded by exposure to honey) associated with *Clostridium botulinum C. botulinum* infection.

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Table 1: Morphology of isolates on blood agar

Sample	School	Size	Edge	Elevation	Surface Texture	Colour	Consistency	Shape	Isolate
1	S.R.S	Small	Smooth	Flat	Smooth	Creamy	Opaque	Rod	*C. botulinum*
2	S.R.S	Small	Rough	Flat	Rough	Creamy	Transparent	Round	*C. botulinum*
3	S.R.S	Big	Lobate	Raised	Rough	Yellow	Transparent	Oval	-
4	C.D.C	Small	Lobate	Flat	Smooth	Grey	Transluscent	Round	-
5	C.D.C	Small	Smooth	Flat	Smooth	Yellow	Transparent	Round	-
6	C.D.C	Big	Irregular	Flat	Smooth	Yellow	Transluscent	Oval	-
7	C.D.C	Big	Irregular	Raised	Smooth	Grey	Opaque	Round	-
8	C.D.C	Small	Lobate	Flat	Rough	Creamy	Opaque	Rod	*C. botulinum*
9	A.S.S	Big	Smooth	Raised	Smooth	Yellow	Transluscent	Oval	-
10	A.S.S	Small	Lobate	Flat	Rough	Creamy	Transparent	Round	-
11	A.S.S	Small	Rough	Flat	Rough	Creamy	Opaque	Round	-
12	A.S.S	Big	Irregular	Raised	Smooth	Grey	Opaque	Round	-
13	S.R.S	Small	Smooth	Flat	Smooth	Creamy	Opaque	Rod	*C. botulinum*
14	S.R.S	Small	Rough	Flat	Rough	Creamy	Transparent	Rod	-
15	S.R.S	Big	Lobate	Raised	Rough	Yellow	Transparent	Oval	-
16	C.D.C	Small	Lobate	Flat	Smooth	Grey	Transluscent	Round	-
17	C.D.C	Small	Smooth	Flat	Smooth	Yellow	Transparent	Rod	-
18	C.D.C	Small	Irregular	Flat	Smooth	Yellow	Transluscent	Rod	-
19	C.D.C	Big	Irregular	Raised	Smooth	Grey	Opaque	Round	-
20	C.D.C	Small	Lobate	Flat	Rough	Creamy	Opaque	Rod	*C. botulinum*
21	A.S.S	Big	Smooth	Raised	Smooth	Yellow	Transluscent	Oval	-
22	A.S.S	Small	Lobate	Flat	Rough	Creamy	Transparent	Rod	-
23	A.S.S	Small	Rough	Flat	Rough	Creamy	Opaque	Round	-
24	A.S.S	Big	Irregular	Raised	Smooth	Grey	Opaque	Round	-
25	S.R.S	Small	Smooth	Flat	Smooth	Creamy	Opaque	Rod	-
26	S.R.S	Small	Rough	Flat	Rough	Creamy	Transparent	Rod	*C. botulinum*
27	S.R.S	Big	Lobate	Raised	Rough	Yellow	Transparent	Oval	-
28	C.D.C	Small	Lobate	Flat	Smooth	Grey	Transluscent	Round	-
29	C.D.C	Small	Smooth	Flat	Smooth	Yellow	Transparent	Rod	*C. botulinum*
30	C.D.C	Small	Irregular	Flat	Smooth	Yellow	Transluscent	Oval	-
31	C.D.C	Big	Irregular	Raised	Smooth	Grey	Opaque	Round	-
32	C.D.C	Small	Lobate	Flat	Rough	Creamy	Opaque	Rod	*C. botulinum*
33	A.S.S	Big	Smooth	Raised	Smooth	Yellow	Transluscent	Oval	-
34	A.S.S	Small	Lobate	Flat	Rough	Creamy	Transparent	Rod	-
35	A.S.S	Small	Rough	Flat	Rough	Creamy	Opaque	Round	-
36	A.S.S	Big	Irregular	Raised	Smooth	Grey	Opaque	Round	-
37	S.R.S	Small	Smooth	Flat	Smooth	Creamy	Opaque	Rod	*C. botulinum*
38	S.R.S	Small	Rough	Flat	Rough	Creamy	Transparent	Rod	*C. botulinum*
39	S.R.S	Big	Lobate	Raised	Rough	Yellow	Transparent	Oval	-
40	C.D.C	Small	Lobate	Flat	Smooth	Grey	Transluscent	Round	-
41	C.D.C	Small	Smooth	Flat	Smooth	Yellow	Transparent	Rod	-
42	C.D.C	Big	Irregular	Flat	Smooth	Yellow	Transluscent	Oval	-
43	C.D.C	Big	Irregular	Raised	Smooth	Grey	Opaque	Round	-
44	C.D.C	Small	Lobate	Flat	Rough	Creamy	Opaque	Rod	*C. botulinum*

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45	A.S.S	Big	Smooth	Raised	Smooth	Yellow	Transluscent	Oval	-
46	A.S.S	Small	Lobate	Flat	Rough	Creamy	Transparent	Round	-
47	A.S.S	Small	Rough	Flat	Rough	Creamy	Opaque	Rod	-
48	A.S.S	Big	Irregular	Raised	Smooth	Grey	Opaque	Round	-
49	S.R.S	Small	Smooth	Flat	Smooth	Creamy	Opaque	Rod	-
50	S.R.S	Small	Rough	Flat	Rough	Creamy	Transparent	Round	-
51	S.R.S	Big	Lobate	Raised	Rough	Yellow	Transparent	Oval	-
52	C.D.C	Small	Lobate	Flat	Smooth	Grey	Transluscent	Rod	*C. botulinum*
53	C.D.C	Small	Smooth	Flat	Smooth	Yellow	Transparent	Round	-
54	C.D.C	Big	Irregular	Raised	Smooth	Grey	Opaque	Round	-
55	C.D.C	Small	Lobate	Flat	Rough	Creamy	Opaque	Rod	*C.botulinum*
56	A.S.S	Big	Smooth	Raised	Smooth	Yellow	Transluscent	Oval	-
57	A.S.S	Small	Lobate	Flat	Rough	Creamy	Transparent	Round	-
58	A.S.S	Small	Rough	Flat	Rough	Creamy	Opaque	Round	-
59	A.S.S	Big	Irregular	Raised	Smooth	Grey	Opaque	Rod	-

Key: S.R.S (A) represents 'Santa rosa school' C.D.C represents 'Command day care'
A.S.S represents 'All saints school'

Table 2: Biochemical reaction of the isolates

Isolates	Gram reaction	Spore reaction	Motility test	Catalase reaction	Oxidase reaction	Indole reaction
A.S.S	-ve cocci	None	None	Negative	Positive	Negative
S.R.S	-ve cocci	Central	Motile	Positive	Positive	Positive
C.D.C	-ve cocci	None	None	Positive	Negative	Positive

Key: +ve represents positive; -ve represents negative

Table 3: Carriers of *C. botulinum* among the study subjects

Sample location	Number of samples	Number of positive sample
S.R.S	15	6
C.D.C	24	7
A.S.S	20	None
Total	59	13 (22.033%)

Table 4: Socio-economic and bio-demographic data of children

Sample location	Exposure to honey	Consumption of infant's milk	Weakness/ poor cry	Loss of head	Lack of appetite
S.R.S	12	15	5	4	4
C.D.C	18	22	6	5	5
A.S.S	5	17	1	0	1
Total	35	54	12	9	10

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Children are exposed to infectious diseases on daily bases. One of such is a bacterial diseases known as infant botulism. The causative agent is known as *Clostridium botulinum*, which uses its toxin, called botulinum neurotoxin (BoNT) to cause a deadly neuroparalytic illness. According to the report of Dembek *et al.* (2007) and Peck *et al.* (2011), though of a few nanogram quantities in weight (<50ng), the toxin elaborated by this bacterium is globally considered as the most deadly agent of humans exposed to it via food or air. Hence, it is of great public health importance. Previous studies reported that most dairy products and canned foods, which are poorly processed have been found to the sources of major outbreaks of the illnesses (Lindström *et al*., 2010). In this study, thirteen 13 (22.033%) stool samples collected from infants (between the age of 6 months and 1year) in three different nursery schools in Enugu were positive. In a similar study, Johnson in 2001 isolated eight *Clostridium botulinum* from infants in the United in United States of America. The result of this work showed that almost equal number of isolates of *C. botulinum* were isolated from samples gotten from Command day care (7) and Santa Rosa School (6), while there was no isolate of *C. botulinum* from the sample obtained from All Saints School. This pathogen that usually affects infants is the causative agent of infant botulism, and this therefore indicates danger in feeding infants with honey (which might contain spores of *Clostridium botulinum*) (Fenicia *et al*., 2008). Although environmental microscopic dust seem the most source of *C. botulinum* spores, ingestion of honey remains the only identified avoidable risk factor for acquiring infant botulism, as represented by positive samples in Command day care (CDC) and Santa Rosa school (SRS) (CDC, 2014; Canadian Food Inspection Agency, http://www.inspection). Infant botulism is a rare but under recognized disease; therefore the presence of a single confirmed case is an indication of disease outbreak.

An early clinical suspicion is essential for a prompt diagnosis and rapid treatment in order to avoid un-useful and unadvisable therapies. The diagnosis of botulism should be suspected in young infants with the acute onset of weak suck, loss of head control, poor cry, generalized hypotonia, and constipation. Detection of BoNT and/or identification of *C. botulinum* spores in stool samples, support clinical diagnosis. Also, other BoNT-producing clostridia should be considered as agents of the disease and included in laboratory criteria for diagnosis.

Infant botulism may present with a variety of manifestations and may be difficult to differentiate from other disorders solely on clinical grounds. It should be considered in infants presenting with poor feeding, and constipation. Definite diagnosis requires the identification of the organism or its toxin.

CONCLUSION

In this study, isolation and identification of *Clostridium botulinum* from the stool samples of thirteen infants were indicative of *Clostridium* food poisoning. Therefore, there is need to restrict infants from honey which might be contaminated with *Clostridium botulinum.*

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