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Evaluation of *Bacillus Cereus* Contamination of Local Vegetables in Obosi, Nigeria.

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Abstract
Seventy Nigerian local vegetables were examined for contamination by *Bacillus cereus*. The vegetables were purchased from different markets in Obosi, Anambra state, Nigeria and transported to the laboratory for microbiological analysis. The samples were first homogenized in 90ml of 0.1% peptone water and incubated at 37^{oc} for 24 hours. The clear supernatant were sub-cultured on to freshly prepared blood, MacConkey, PolymyxinB agar plates and incubated for 24hours at 37^{oc}. *Bacillus cereus* was identified using cultural characteristic, some biochemical tests, spore staining for the presence of lipid globules and polymerase chain reaction (PCR). Those positive were investigated for the presence of enterotoxin and their pathogenic effects using animal models was also done. Antibiotic sensitivity tests were carried out using agar diffusion methods. Thirteen (18.6%) of 70 vegetable samples were contaminated by *Bacillus cereus.* All *Bacillus cereus* were positive for enterotoxin and 100% resistant to ampiclox while one from cabbage was resistant to tested drugs. Histomorphological tests using animal models revealed infiltration of the liver cells by inflammatory cells, necrosis. This study showed that *Bacillus cereus* is a common contaminant of local vegetables in Obosi, Nigeria.
Keywords: Bacillus cereus, contamination, local vegetables, Obosi.

INTRODUCTION

Bacillus cereus is a large 1x3.4um, Gram positive, rod shaped, motile, have beta haemolytic activity facultative aerobic spore former whose spores do not swell the sporangium (Vilian *et* al., 2006). *Bacillus cereus* can be found widely in nature including samples of dust, dirt, cereal crops, rhizospher of some plants and is commonly found in the soil as saprophytic organism (Vilkan *et* al., 2000). As a soil bacterium, it can spread easily to many types of foods such as eggs, plants, meat, cereal crops, vegetables and dairy products and is a common contaminant of raw agricultural products (Lambert and Peferoen 1992, Ryan and Ray, 2004). Normal contamination levels are generally less than 100/g (Hobbs and Gilbert, 1994). The presence of large numbers of *Bacillus cereus* greater than 10^6 organisms/ g in a food is indicative of active growth and proliferation of the organism and is consistent with potential health hazards. *Bacillus cereus* is an opportunistic human pathogen and is occasionally associated with food poisoning, local and systemic infections (Kotrianta et al., 2000, Hoffmaster *et* al., 2006, Wijnards *et* al., 2006). *Bacillus cereus* causes two distinct types of food poisoning. The long incubation (diarrheal) type is associated with meat or vegetable containing foods. The bacterium has been isolated from 50% of dried beans and cereals and from 25% of dried foods –spices and potatos (Todar, 2008) while the short incubation (emetic) is associated with rice dishes. Bean and Griffin (1990) reported 53 outbreaks of food-borne diseases associated with *Bacillus cereus* to CDC. According to Centre for Disease Control (CDC) report (2009) food borne disease outbreaks, there were 1270 outbreaks or 27634 cases reported within 48 states with 11 deaths. The CDC estimates that 97% of all food poisoning result from improper food handling, 79% from food prepared in commercial or institutional establishments and 21% of all cases from food prepared at home (Malek *et* al., 2009). Kristine *et* al., (2006) reported 17.97% *Bacillus cereus* contamination of fresh fruits and vegetables in Denmark while Oni *et* al., (2010) reported 30% contamination of vegetable salad by *Bacillus cereus.* With the facts, the current study aims at evaluation the level of contamination of vegetables in Nigeria by *Bacillus cereus*.

MATERIALS AND METHODS

Sampling procedures

A. **Extra human samples**

Ten (10) samples each of ugu, utazi, okazi, cabbage, onugbu, green leaves, anara were randomly purchased in disposable sterile containers from different markets in Onitsha and Obosi areas of Anambra State, Nigeria and transported to the laboratory within 1-2 hours of collection for microbiological studies. Thus a total of eighty (70) vegetable samples were analysed for the presence of *Bacillus cereus*.

Microbiological evaluation

Ten (10g) grams of the different vegetable samples were added in 90ml of 0.1% peptone and incubated at 37^{oc} for 24hours. After 24 hours clear supernatant of the samples were each subcultured onto freshly prepared plates of sheep blood, MacConkey (Oxoid), and Mannitol Egg Yolk Polymyxin B (MYP) (Oxoid) agar plates. All

cultures were incubated for 24 hours at 37^{oc} (Oxoid 1998). Initial reading of the plates was done. From each positive plate one to three representative colonies of presumptive *Bacillus cereus* were subcultured on nutrient agar (Oxoid) slopes and kept in the refrigerator for further studies.

Nigerian local vegetables

Vegetables		
a. Pumpkin [Ugu]	*Telfairia occidentalis*, 10	Fresh leaves
b. Utazi	*Gongronema latifolia*, 10	Fresh leaves
c. Okazi	*Gnetum africanum*, 10	Fresh leaves
d. Cabbage	10	Fresh leaves
e. Bitter lea (Onugbu)	*Vernonia amygalina*, 10	Fresh leaves
f. Green Leaves	*Amaranthus hybridus,* 10	Fresh leaves
g. Garden egg (Anara)	*Solanum melongena*, 10	Fresh leaves

Identification of *Bacillus cereus*

Identification methods were in accordance with Oxiod (1988). Blood agar plate was used to observe beta haemolysis. In MacConkey agar plate colonies of *Bacillus cereus* are large, irregular and pale. The Mannitol egg yolk polymyxin B developed by Holbrook and Anderson (1980) is a selective and highly specific medium for the isolation and enumeration of *Bacillus cereus* from foods and vegetables. Typical colonies of *Bacillus cereus* on MYP agar plate are crenated about 5mm I diameter and have distinctive turquoise to blue colour surrounded by a good egg yolk precipitate of the same colour. Rapid confirmatory spore staining test according to Oxoid (1988) for the presence of lipid globules which is specific for *Bacillus cereus* among other *Bacillus* species grown on MYP agar plate, Gram staining reaction and some biochemical tests which include IMViC tests, sugar fermentation for the production of acid and gas Frazier and Westerhoff (1991), Polymerase chain reaction (PCR) was done on isolates grown on MYP agar plates. This involves DNA isolation, PCR amplification using random primers- R1 (GAAGCAGCGTGG) and R2 (GTCGTTATGCGGTA). The processes include denaturation, annealing, extension polymerization. PCR products were analysed by agarose gel electrophoresis.

Tests for antimicrobial sensitivity

This was done using the agar diffusion method in accordance with Oxoid (1988). Identified *Bacillus cereus* was subcultured on to nutrient agar (Oxoid) plate and incubated at 37^{oc} for 24 hours. The degree of sensitivity of *Bacillus cereus* to the drugs was determined by measuring using Vernier calipers visible areas of inhibition of growth of *Bacillus cereus*

Tests for detection of *Bacillus cereus* enterotoxin in cultured fluids according to Oxoid 2012 *Bacillus cereus* enterotoxin reversed passive latex agglutination kit.

Identified *Bacillus cereus* from MYP agar plates were used to perform the test according to manufacturers (Oxoid 2012) instruction which include inoculating loopful of confirmed *Bacillus cereus* in to 5ml of brain heart infusion and incubated at 37^{oc} for 18 hours. After growth, the tube was centrifuged for 20 minutes in other to get clear supernatant for the assay of the toxin.

Animal studies.

Eighteen albino wister mice were purchased from the animal house of the college of medicine, university of Nigeria, Enugu campus. The animals were put in triplicate in different cages and kept for 2 days to acclimatize. They were maintained on standard laboratory conditions and fed with animal feed (super starter Guinea feed ^{R} Nigeria PLC and water. The animals were weighed and their weight noted before oral inoculation of 0.5ml or 1ml (depending on weight of mice) of liquid broth of peptone water containing representative colonies of *Bacillus cereus* from ugu, bitterleaf, (onugbu) were standardized at 10^3org/ml and peptone water only (act as control) through oral canula. Within 24 hours of ingestion of *Bacillus cereus* the animals were observed. At the end of the study the animals were sacrificed under chloroform anaesthesia. The liver was harvested, ileal loop observed. The harvested organs were kept in containers containing 10% formal saline and left for 24 hours before being subjected to histological processing, haematoxylin and eosine staining, microscopy and photomicrography.

Statistical analysis

All data generated were subjected to analysis of variance (ANOVA), x^2 test and Duncan's multiple comparison test.

Results

Bacillus cereus was isolated in 13(18.6%) of 70 vegetable samples tested. All isolates tested positive for enterotoxin and 100% resistant to ampicillin. Isolate from cabbage was 100% resistant to tested drugs.

Table 1 showed the level of contamination of *Bacillus cereus* in the different vegetable samples. According to the results bitterleaf (onugbu) had the highest (40%) contamination.

Figure 1 and 2 represented the results of pathogenic effect of the enterotoxin on the liver cells of animal models.

Table 1Prevalence of *Bacillus cereus* in Nigerian's local vegetables

Vegetables	Total	No contaminated	Prevalence
Punkin [Ugu] (*Telfairia occidentalis*)	10	2	20%
Utazi (*Gongronema latifolia*)	10	1	10%
Okazi (*Gnetum africanum*)	10	1	10%
Cabbage	10	2	20%
Bitterleaf (*Vernonia amygalina*)	10	4	40%
Green (*Amaranthus hybridus*)	10	2	20%
Garden egg (*Solanum melongena*)	10	1	10%
TOTAL	70	13	18.6%

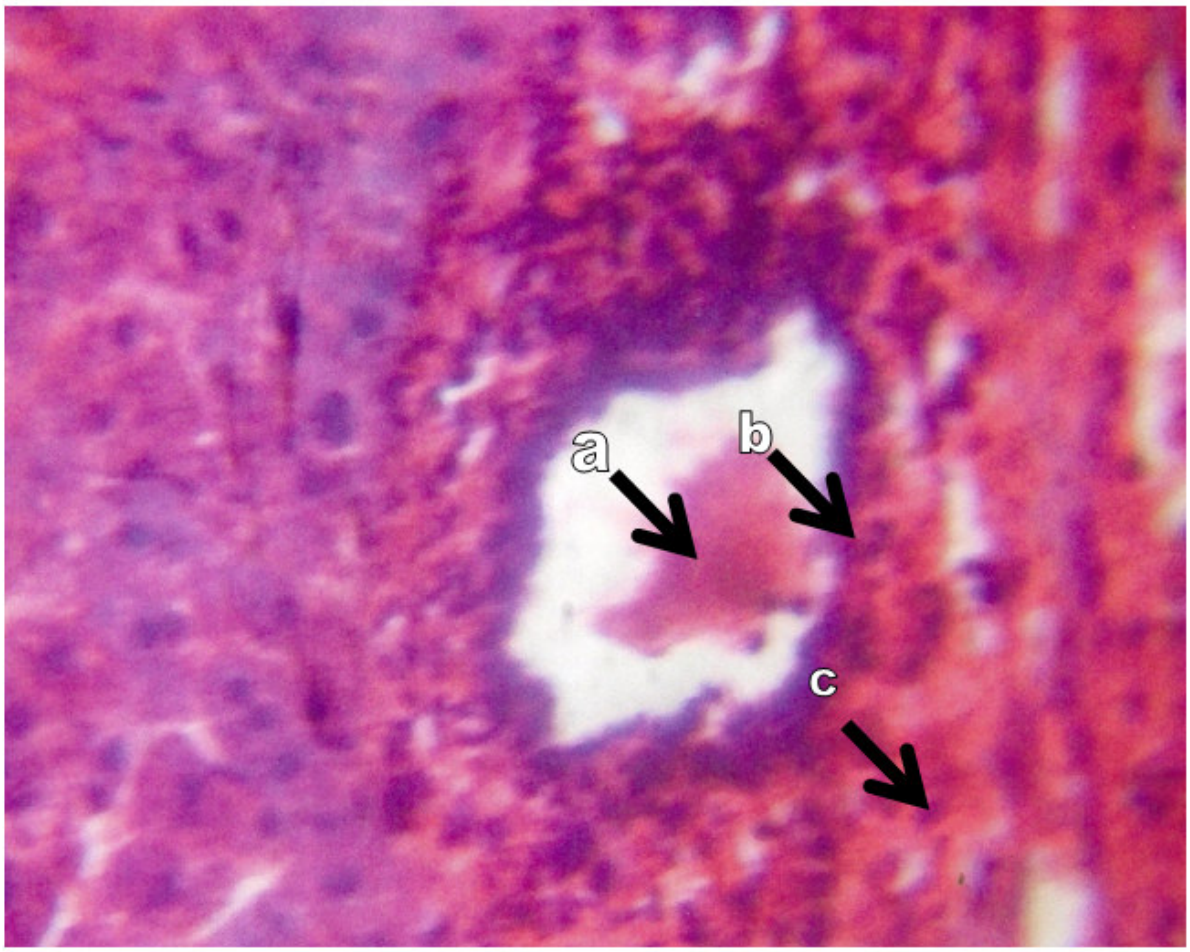

Fig.18: Liver section Showing distended central vein with [a], fibrin plug at the center [b]. There is moderate mononuclear cell infiltrates around the vein, hepatocyte degeneration and necrosis are also evident. Source of organism (bitterleaf vegetable)

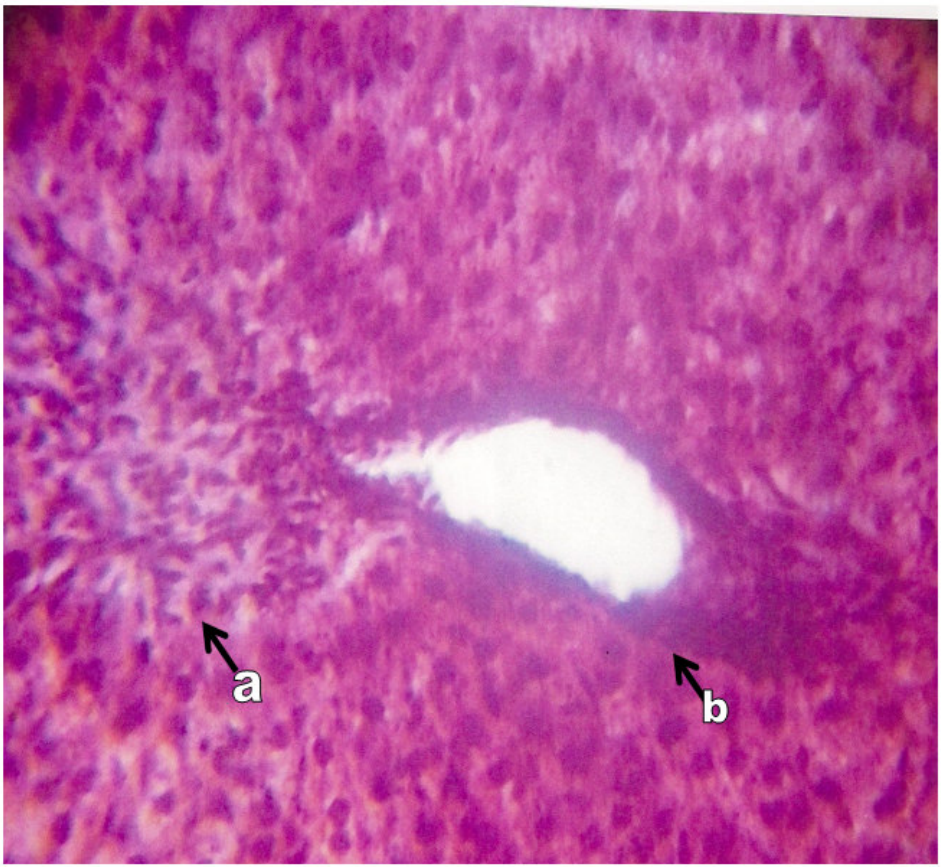

Fig.2 Liver section showed (a). Hyperplasia of the liver cells at various stages of mitosis. [b]. The epithelial cells around the central vein [endothelial cells] are also hyperplastic [thickened. No evidence of necrosis or infiltration of inflammatory cells. Source of organism peptone control.

Discussion

The results of this study indicated that most of Nigeria local vegetables are subject to *Bacillus cereus* contamination with an overall prevalence of 13(18.6%) of 70 samples tested. Kristine *et* al., (2006) reported 17.97% contamination of vegetables by *Bacillus cereus.* There was no statistical difference in the level of contamination of the different vegetables ($p>0.05$). All isolates were 100% resistant to ampiclox while one isolate from cabbage was 100% resistant to tested drugs. Lee (2009) reported 100% resistant to ampicillin and 54% resistant to tested drugs by Wiley and Etats- Unis (2006). The results revealed a significant difference ($p<0.05$) between sensitivity of isolates to antibiotics and their resistance to it. The results of this study showed that similar effects of diarrhea were observed in the animal models after oral ingestion of *Bacillus cereus.* Histomorphological examination of the liver tissues of the animals showed evidence of infiltrations of inflammatory cells, congestion of the central veins, necrosis. This liver involvement in animal models agreed with the works of Hellmut *et* al., (1997) and Katelijne *et* al., (2005) who demonstrated in a post-mortem examination of the liver cells evidence of microvascular and extensive necrosis suggestive of liver failure in a case of fatal family outbreak after eating food contaminated by *Bacillus cereus.*

In conclusion, vegetables could be important reservoir of *Bacillus cereus.* Increased time/temperature exposure of vegetables and strict control of mishandling of product during preparation is highly recommended for prevention of contamination of vegetable by *Bacillus cereus* which could cause food poisoning if consumed by humans.

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