

Ecopathological Features of *Bacillus Cereus* Food Poisoning in Anambra State, Nigeria

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Abstract

This study was conducted to determine the level of contamination of *Bacillus cereus* in Nigeria local cooked/boiled foods in Anambra State, Nigeria. One hundred and ninety (190) different types of extra human samples made up of 10 each ready to eat cooked/boiled foods were randomly purchased from local food vendors and restaurants. Eighty (80) human diarrheal stools from patients suspected of food poisoning attending clinics and laboratories and fifty control stool from human volunteers were also examined for the presence of *Bacillus cereus*. The extra human samples were first homogenized in 1% peptone, incubated at 37^oc for 24hours and then sub cultured on to freshly prepared blood, MacConkey, and Polymyxin B agar plates while a direct stool culture on the above agar plates were also done. *Bacillus cereus* was identified using cultural characteristics, Gram staining reaction, spore staining reaction for the presence of lipid globules which is specific for *Bacillus cereus*, some biochemical tests and polymerase chain reaction (PCR). Animal studies results revealed pathological involvement of the liver. Antibiotic sensitivity tests were carried out using agar diffusion methods. *Bacillus cereus* was detected in 44(23.4%) of 190 extra human samples. *Bacillus cereus* was isolated in ready to eat cooked/boiled foods, 20(33.3%) of 90 cooked/boiled foods, soup category 10(20%) of 50 soup samples, 15(30%) of 50 steamed wrapped foods samples. No *Bacillus cereus* was isolated from stew sample while 4(40%) of 10 samples each of abacha (African salad, breadfruit (ukwa), boiled palm nut oil soup (akwu), plantain pudding (ukpogede) were positive for *Bacillus cereus*. *Bacillus cereus* was isolated in 15(18.5%) of 80 diarrheal stool samples and 4(8%) of 50 control stool. 42 (95.45%) of *Bacillus cereus* isolated were positive for the enterotoxins while 2(4.55%) all from cooked rice were negative. The *Bacillus cereus* isolated were 100% resistant to ampiclox. The results of this study showed that *Bacillus cereus* is a common contaminant of Nigerian's local foods and food products and is mainly of the enterotoxin (diarrheal type).

Keywords: Ecopathology, *Bacillus cereus*, Food poisoning, Enterotoxin

INTRODUCTION

Bacillus cereus was first successfully isolated from a case of fatal pneumonia in a male patient (Hoffmaster *et al.*, 2006). It is a Gram positive, rod shaped facultative aerobic spore former (Villia *et al.*, 2006). Spores of *Bacillus cereus* can be found widely in nature including samples of dust, dirt, soils, cereal crops, water among others, so it is a normal contaminant of raw agricultural commodities. Normal contamination levels are generally less than 100organisms/g (Hobbs and Gilbert, 1994). The presence of large numbers of *Bacillus cereus* greater than 10⁵orgs/g in a food is indicative of active growth and proliferation of the organism and is consistent with potential hazards to health (Hobbs and Gilbert, 1994, Inabo, *et al.*, 2000). It is difficult to differentiate superficial contamination with genuine disease caused by *Bacillus cereus*. *Bacillus cereus* is an opportunistic human pathogen and has been reported to cause local and systemic infections (Kotrianta *et al.*, 2000, Wijnards *et al.*, 2006). *Bacillus cereus* is also commonly known to cause food borne intoxications. *Bacillus cereus* food poisoning occurs all the year round and is without any geographic distribution. *Bacillus cereus* causes two types of food poisoning in humans including the diarrheal and emetic syndrome. The diarrheal type is associated with meats, vegetable dishes, soups and occurs 6- 15hrs after ingestion of contaminated foods. Food poisoning results from the production of enterotoxins- haemolytic, non- haemolytic and cytotoxin K. The dosage of ingested *Bacillus cereus* spores leading to the production of enterotoxins is 10⁵- 10⁷ organisms/g of ingested food (Wijnards *et al.*, 2006). The pathogenesis of diarrhea is as a result of the action of enterotoxin on the secretory mechanism of the small intestine leading without invasion. This leads to large watery stool in the absence of blood, pus or severe abdominal pains. It is diagnosed by the isolation of *Bacillus cereus* from stool and food. Isolation from stool alone is not sufficient because 14% of healthy individuals have been reported to have transient gastrointestinal colonization with *Bacillus cereus* (Wijnards *et al.*, 2006). The emetic type is associated with rice dishes and occurs, 30mins -6hours after ingestion of spores containing 10⁵-10⁸organisms/g of ingested food (Wijnards *et al.*, 2006). It is diagnosed by the isolation of *Bacillus cereus* in contaminated food. The virulence factor associated with the emetic syndrome is cereulide while those associated with non-gastrointestinal infections with *Bacillus cereus* includes haemolysins, phospholipase C, haemolysin C (Wijnards *et al.*, 2006). Food poisoning is defined as an illness caused by the consumption of food or water contaminated with bacteria, and or their toxins, or with parasites, viruses or chemicals (Roberts *et al.*, 2009). *Bacillus cereus* causes problems to the food industry because of their proteolytic, lipolytic and saccharolytic activities both by

deteriorating the products (Tegiffel *et al.*, 1996) and endangering people lives upon consuming them (Ghelards *et al.*, 2002, Yint *et al.*, 2004). Between 1973- 1988, Bean and Griffin (1990) reported 53 outbreaks of food borne diseases with *Bacillus cereus* to CDC USA. In 2003 only 2 cases (2-5%) of *Bacillus cereus* food poisoning were reported to CDC, but this is thought to represent only 2% of total cases which have occurred during those periods in USA (Kotrianta *et al.*, 2000). In Britain, Colombia and Canada Lorraine *et al.*, (2000) reported 39 outbreaks of *Bacillus* species food poisoning after eating various foods in the restaurants with *Bacillus cereus* causing 23 outbreaks. Gulam and Nur Hayati Yaacob (2000) reported a prevalence of 42.1% *Bacillus cereus* food poisoning in cooked foods of which 91.8% were enterotoxigenic. The figures are worrisome given that food borne illnesses are grossly under reported, mainly because symptoms such as diarrhea and abdominal cramps and vomiting mirror those of *Staphylococcus* and *Clostridium perfringens* food borne intoxications and common stomach viruses (Maggi, 2007, McCabe-Sellers and Beattie, 2004; Flanigan, 2006). Practices identified as contributing to outbreaks of *Bacillus cereus* include improper refrigeration, prolonged handling and inadequate reheating of cooked food, cross contamination of food and infection from food handlers Parisello *et al.*, 2000, Daniels, 2002, Hedberg *et al.*, 2006, CDC 2009. *Bacillus cereus* is a common food contaminant. Effective control measures depend on destruction by a heat process and temperature control to prevent spore germination and multiplication of vegetative cells in cooked food. Efforts must be made to adhere strictly to good food hygiene practices and stringently implementing hazard analysis critical control points (HACCP) along the whole food chain (Powel *et al.*, 2002, Oranusi *et al.*, 2003). Barriers in implementing good food hygiene practices include lack of funds and or time as well as employee motivation and confidence (Youn and Sneed 2003, Robert *et al.*, 2006). Epidemiological data on foodborne disease outbreaks in Nigeria local food, meat and meat products is lacking but poor storage practices coupled with poor personal hygiene and lack of knowledge in food safety practices which is inherent with food handlers in restaurants and local food vendors were causes for concern. News and information are available on daily bases of people dying after a meal. Many of such deaths are attributed to offended deities in Nigeria. Some of such undisclosed causes may be due to *Bacillus cereus*. With the above facts in mind the current study aims at determining the prevalence, antibiotic sensitivity profile, pathogenic mechanisms and details of the hazardous effects on experimental animals of this usually ignored organism in our environment.

MATERIALS AND METHODS

Extra human samples

One hundred and ninety (190) extra human samples of ready- to- eat cooked food samples made up of 10 each from white rice, yam, beans, pounded cassava (akpu), breadfruit (ukwa), rice and beans, soups- melon (egusi), stew, palm nut oil soup (akwu), draw soup (ogbonu), bitter leaf (onugbu), steamed wrapped- Agidi (white) eku, agidi (jellof), plantain pudding (ukpogede), stone groundnut paste (okpa- cake) and moi moi were randomly purchased from local food vendors and restaurants in Obosi, Onitsha and Awka areas of Anambra State, Nigeria between July 2010 to January 2013 and transferred to the laboratory within 1 to 2 hours of collection for microbiological studies.

Human samples

One hundred and thirty (130) human stool samples made up of eighty diarrheal samples from patients suspected of food poisoning with history of abdominal cramps, diarrhea, nausea and vomiting (these patients were seen at clinics and medical laboratories in the study areas) and fifty stool samples from apparently healthy volunteers were also submitted to the laboratories for microbiological studies. Attempts made to associate the foods with the patients suspected of food poisoning failed as there was no reporting system of food poisoning in the study areas.

Sampling procedures

Samples of foods were collected into sterile specimen containers with metal spoons used by the food handlers for dishing food. Samples were taken to the laboratories.

Microbiological evaluation

Ten (10) grams of the different local food items were homogenized respectively with 90ml of 0.1% peptone water in a screw capped flasks by means of horizontal and vertical agitation for few minutes and incubated for 24 hours at 37°C. After 24 hours clear supernatant of homogenized samples were each subcultured on freshly prepared plates of sheep blood, MacConkey (Difco), and Mannitol Egg Yolk Polymyxin B (MYP) (Oxoid) agar plates. Direct plates culture of loopful of fecal specimens was also done on the above plates. All cultures were incubated for 24 hours at 37°C (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 confirmation of the identity and toxin identification.

Identification of *Bacillus cereus*

Identification methods were in accordance with Oxoid (1988). Blood agar plate was used to observe beta haemolysis. 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. Typical colonies of *Bacillus cereus* on MYP agar plate are crenated about 5mm I diameter and have distinctive turquoise to blue color surrounded by a good egg yolk precipitate of the same color. Rapid confirmatory 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 1988) 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 order 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) liquid broth of peptone water containing *Bacillus cereus* standardized at 10³org/ml from representative colonies of the food samples and peptone water only 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), x² test, Duncan's multiple comparison test, Pie and Bar chart.

RESULTS

Forty four (23.2%) of the 190 different types of cooked foods samples showed growth of *Bacillus cereus*. All *Bacillus cereus* isolated were positive for enterotoxin.

Table 1b showed the prevalence of *Bacillus cereus* in cooked food samples. The result reveal that of the 10 different samples of abacha (Africa salad), breadfruit 4(40%) each were contaminated with *Bacillus cereus* while 3(30%) each of 10 samples of yam, pounded yam, while beans, abacha (white) and rice and beans had the lowest prevalence of 1(10%) of the 10 samples studied.

Table 2 showed the prevalence of *Bacillus cereus* in 50 soup samples. According to the results 10(20%) of the 50 soup samples showed growth of *Bacillus cereus*. The highest contamination of 4(40%) of 10 samples came from boiled palm nut oil (akwu) soup while there was no growth from stew samples.

Table 3 represented the results from 50 samples of different types of steamed wrapped foods. Okpa-cake had the highest contamination of 4(40%) of *Bacillus cereus* while agidi jellof and agidi white had the lowest contamination 1(10%) each of *Bacillus cereus*

Table 4 represented the results of 80 human diarrheal stool samples (40 from male and 40 from female patients) and 50 control (25male, 25 female) 15(18.7%)of 80 diarrheal stool samples showed growth of *Bacillus cereus* out of which 8(53.3%) and 7(46.7%) were from male and female respectively. In the control stool samples 4(8%), two each from male and female subjects showed growth of *Bacillus cereus*

Figures 1,2,3,4,5 showed results of the liver test. Approximately all except that from peptone water

showed evidence of abnormal gross macroscopically and the ileal loop showed evidence of swelling when compared with that of control. Histomorphological results revealed the presence of cellular alterations and infiltration of inflammatory cells, necrosis, and distention of the central vein in various proportions.

Antibiotic sensitivity profile

All isolates of *Bacillus cereus* were 100% resistant to ampiclox and highly sensitive to levofloxacin and gentamycin.

Statistical analysis results

There was no statistical difference in the number of positive samples for *Bacillus cereus* among cooked foods, soups and steamed wrapped foods $p > 0.05$

1. There was no statistical variation in the distribution of *Bacillus cereus* $p > 0.05$ according to groups of food.
2. There was a significant difference between sensitivity of isolates to antibiotics and their resistant to it $p < 0.05$.
3. There was a significant level of difference in the sensitivity of the isolates to the different antibiotics $p < 0.05$

Table1: Prevalence of *Bacillus cereus* isolated from Nigerian's local

Extra Human Samples Food Items	Number tested	Growth	Prevalence
1 Ready-to-eat cooked/boiled foods			
White Rice (<i>Oryza sativa</i>)	10	2	20%
Yam (<i>Discorea rotundata</i> poir)	10	3	30%
Beans (<i>Vigna unguiculata</i>)	10	1	10%
Pounded cassava (<i>Manihot esculentum</i>)	10	3	30%
Milled fried cassava [Gari] (<i>Manihot esculentum</i>)	10	3	30%
Abacha (African salad)	10	4	40%
Abacha white (slice cooked cassava)	10	1	10%
Breadfruit (<i>Treculia africana</i>)	10	4	40%
Rice and beans mixed	10	1	10%

Table 2 Prevalence of *Bacillus cereus* isolated from Nigerian's local soups

Soups	Number tested	Growth	Prevalence
Melon [Egusi] (<i>Cucumis melo</i>)	10	1	10%
Stew	10	0	0%
Boiled palm nut oil (<i>Elias guineensis</i>)	10	4	40%
Draw (<i>Irvingia wombolu</i>)	10	3	30%
Bitterleaf (<i>Vernonia amygdalina</i>)	10	2	20%

Table 3 Prevalence of *Bacillus cereus* in steamed/ boiled wrapped foods

Steamed /boiled Wrapped foods	Number tested	Growth	Prevalence
Agidi [jelof] with oil (<i>Zea mays</i>) [Eko]	10	1	10%
Agidi [white] without oil [Eko]	10	3	30%
plantain pudding [Ukpogede] (<i>Musa sapientum</i>)	10	4	40%
Okpa cake [Stone groundnut] (<i>Vigna subterranean</i>)	10	3	30%
Milled cooked beans [Moim moim] [<i>Vigna unguiculatum</i>]	10	3	30%
TOTAL [cooked/boiled foods]	190	44	23.2%

Table 4 Prevalence of *Bacillus cereus* according to se

Sex	Number tested	Prevalence
Male	40	8 (20%)
Female	40	7 (17.5%)
Control		
Male	25	2 (8%)
Female	25	2 (8%)

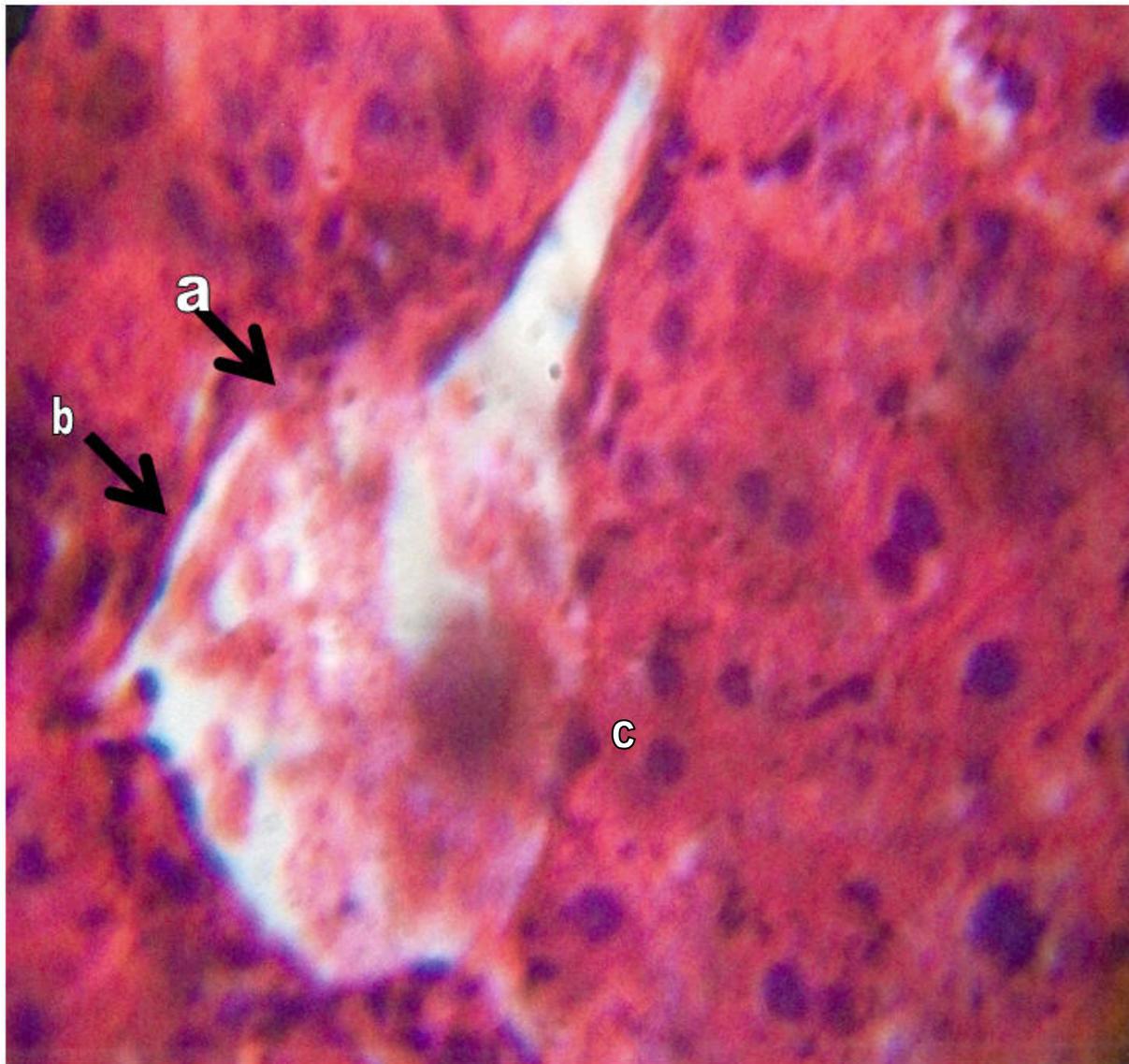


Fig.1 Liver section [a, b] shows distention of the central vein with moderate congestion with eosinophilic materials .(source of organism from diarrheal stool)

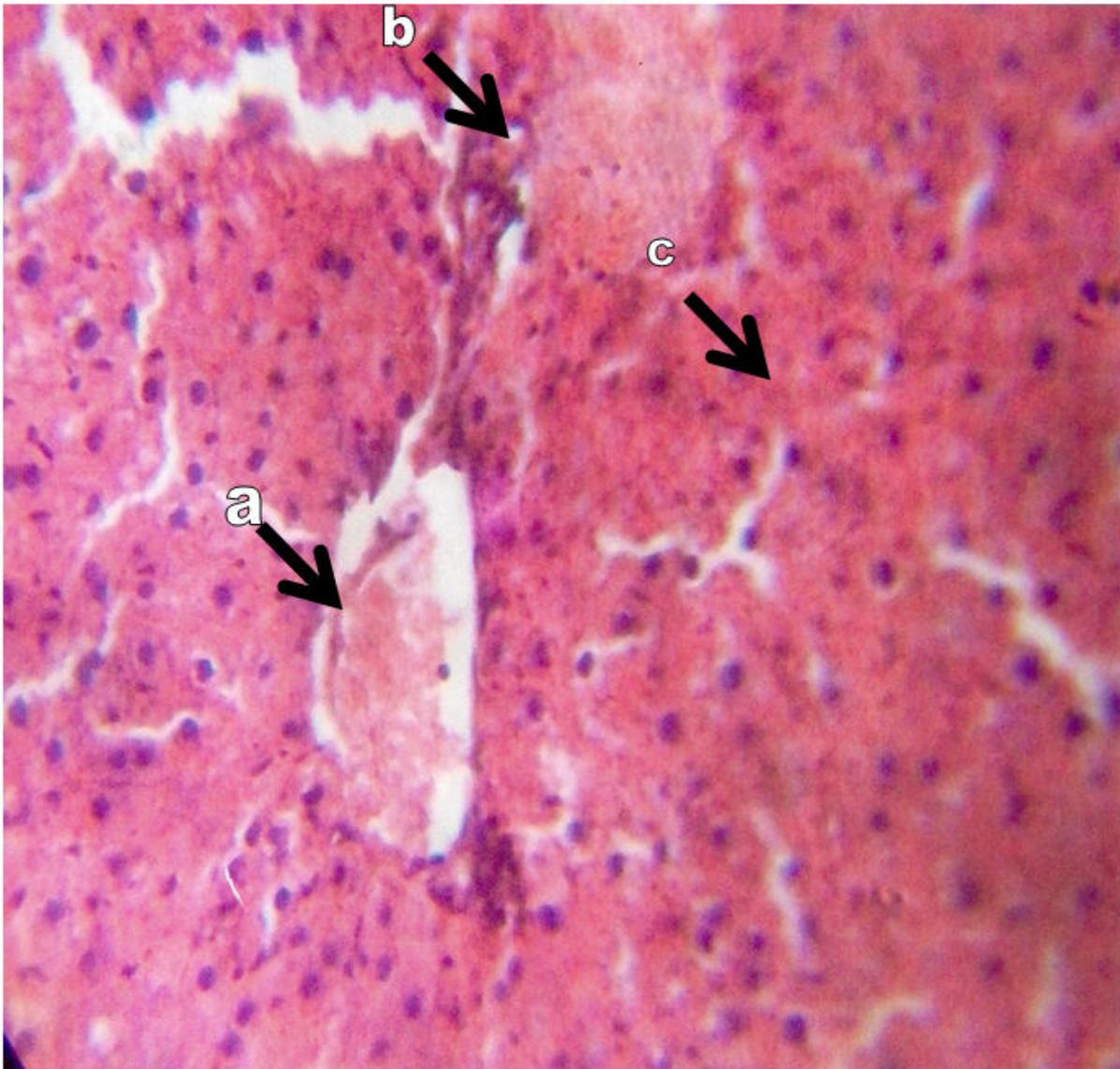


Fig. 2 Liver section shows [a]. Congestion of the central vein. [b]. Dilation of the liver sinusoids. [c]. Mild necrosis liver cells. (Source of organism- control stool sample).

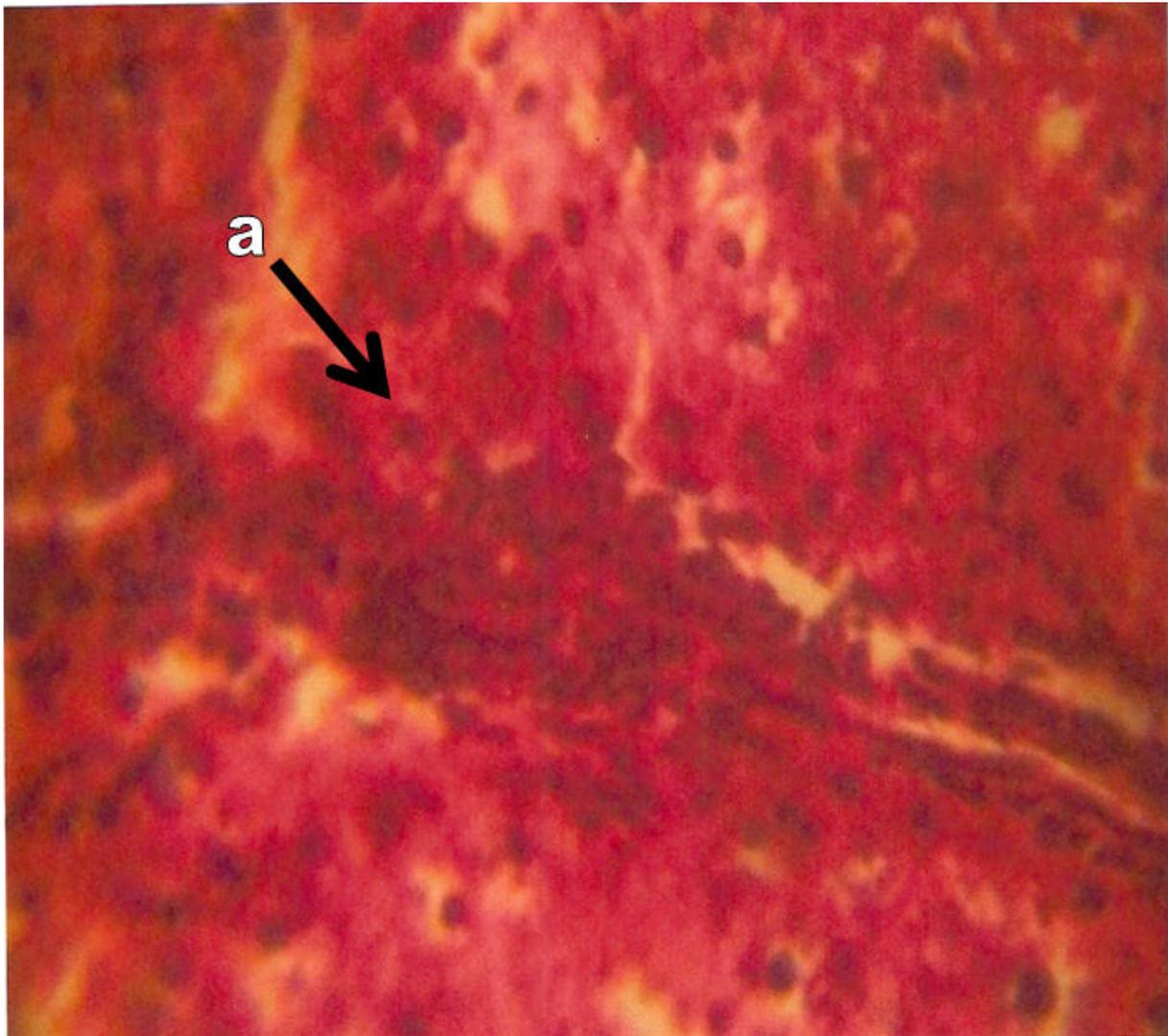


Fig.3 Liver section showing evidence of moderate necrosis and hepatocyte degeneration with concentration of mononuclear cells within and around the portal tract. [b]. There is also moderate distension of the sinusoids. Source of organism-Bean flour (okpa cake).

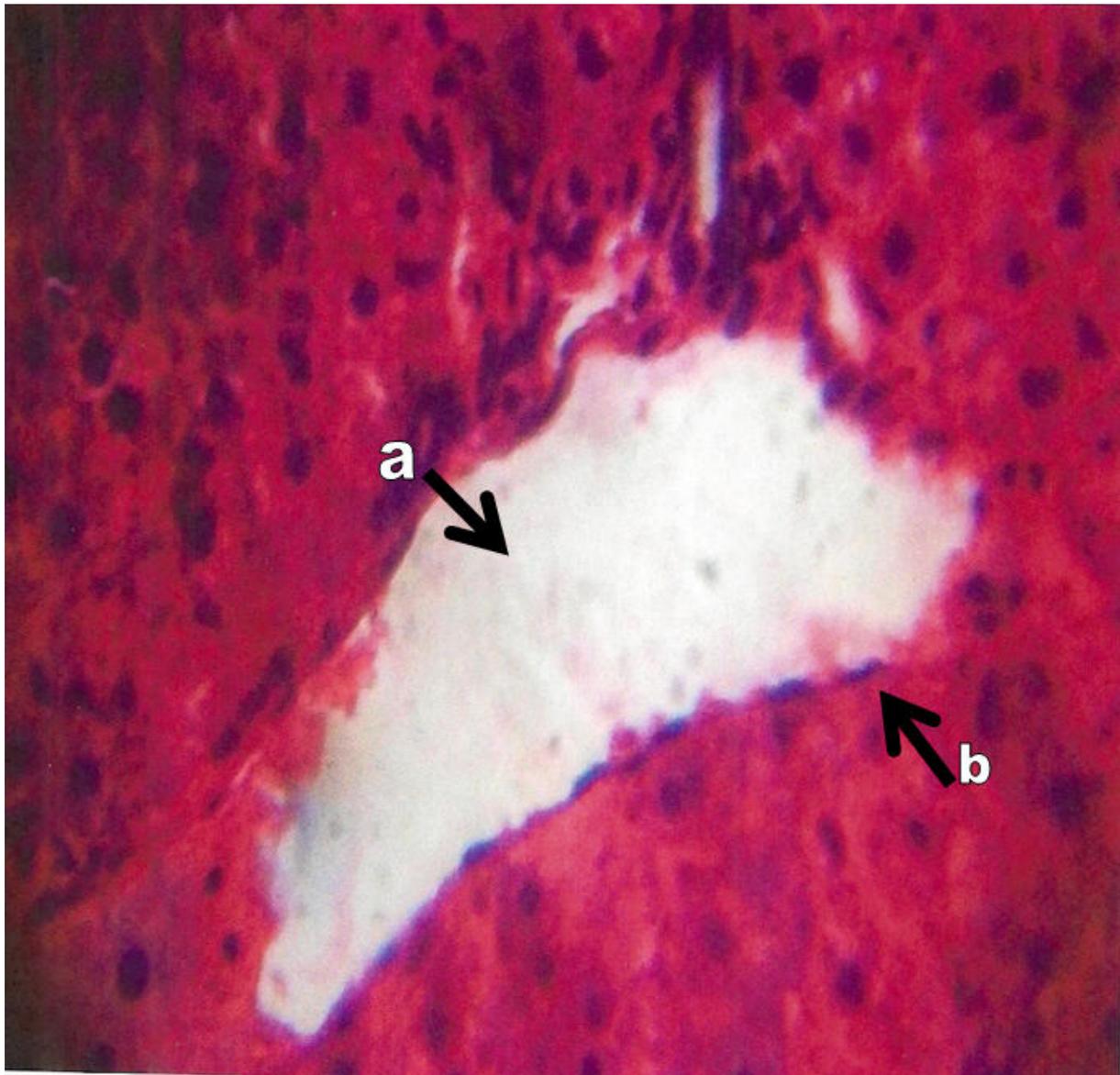


Fig.4 The liver section showing (a) Odematous distention of the central vein with moderate centrilobular necrosis. [b]. There is focal mononuclear cell infiltrates around the periphery of the distended vein. Source of organism- egusi (melon) soup.

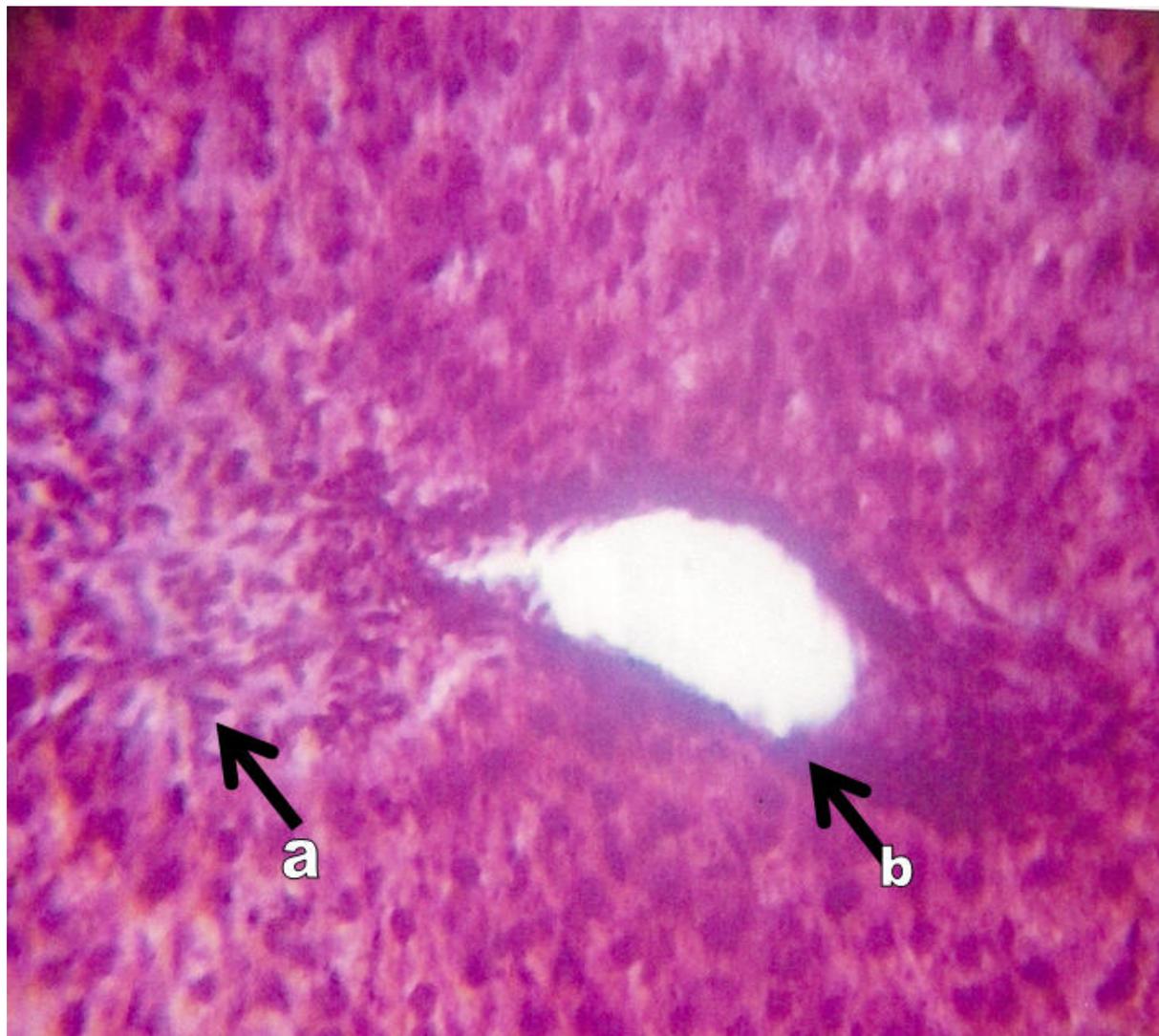


Fig. 5 Liver section showing (a). Hyperplasia of the liver cells at various stages of mitosis. [b]. The epithelial cells around the central vein [endothelial cells] are also hyperplastic [thickened [a]. No evidence of necrosis or infiltration of inflammatory cells. Source of organism peptone control.

Discussion

Bacillus cereus has been reported as an agent of food poisoning since 1955 in developed countries (Todar, 2008). Its effect in Nigeria locally affordable foods is lacking or does not exist. This study revealed a prevalence of 44(23.2%) of 190 cooked foods contamination with *Bacillus cereus* and 15(18.75%) of 80 suspected stool samples from food poisoning outbreaks. Four (8%) of 50 control stool samples also showed growth of *Bacillus cereus*. Breadfruit, African salad (from ready to eat cooked/boiled food group), boiled palm nut oil (akwu) from soup group, plantain pudding (ukpogede) from steam wrapped food category all had 4(40%) of 10 samples contaminated with *Bacillus cereus* while yam, pounded cassava, gari (milled fried cassava) from cooked/boiled group, draw soup from soup group, agidi with oil (Eko), okpa-cake moi moi, all had 3(30%) of 10 samples contamination with *Bacillus cereus*. This was followed by white rice (cooked food), bitterleaf (soup group), all had 2(20%) and finally beans, abacha white, rice and beans(cooked food group), melon (egusi) from soup group, agidi white from wrapped food group revealed 1(10%) of 10 samples contaminated white *Bacillus cereus*. There was no contamination with *Bacillus cereus* in the stew soup. The isolation of *Bacillus cereus* from all cooked food samples in this study is worrisome. This could be explained by the ubiquitous distribution of this organism and its ability to form endospores (Mckillip, 2000), coupled with other factors such as improperly washed utensils and equipment, poor hygiene, dirty environment and presence of animals in the cooking environment (Villa *et al.*, 2009, Bories *et al.*, 2009) contributed to the contamination of Nigeria local cooked foods. This study agrees with the findings of 22.4% of 100 retail food samples by Wiley and Etats-Unis (2006) in Turkey while Oni *et al.*, (2010) found a higher prevalence of 30% in vegetable salad in Nigeria. Also previous studies

done in Britain by Lorraine *et al.*, (2000) and in Malaysia by Gulam and Nur Hayati Yaacob (2000) reported a higher prevalence of 58.97% and 42.11% respectively when compared with this result. Sofroni *et al.*, (2010) in Australia found a lower prevalence of 8(4.7%) of 175 cooked food samples. Histomorphological report of this study revealed the presence of cellular alterations and infiltration of inflammatory cells, necrosis and distention of central veins in various proportions of the liver tissue samples. This liver involvement in animal models agrees with the reports of fatal fulminant liver failure after eating spaghetti (Helmut *et al.*, 1997), and salad (Katelijne *et al.*, 2005) contaminated with *Bacillus cereus* leading to the death of two children. All *Bacillus cereus* isolated in this study is 100% resistant to ampiclox while Lee (2009) reported 100% resistant to ampicillin. There was significant difference ($p < 0.05$) between sensitivity of *Bacillus cereus* to antibiotics and their resistant to it and sensitivity of *Bacillus cereus* isolated from diarrheal stool to that of control stool to antibiotic tested. *Bacillus cereus* was isolated from 8(20%) of 40 male and 7(17.5%) of 40 female stool samples revealing no significant difference $p > 0.05$ in sex distribution of *Bacillus cereus* food poisoning. This agrees with the finding of 58% in male and 42% in female in South Africa by South Africa Department of Health (2002). Forty two (95.45%) of 44 *Bacillus cereus* isolated in the study were positive for the enterotoxins while two (4.55%) were negative. This agrees with the results of Gulam and Nur Hayati Yaacob (2000) 91.8% and 92% by Lee (2009) all in Malaysia. This study could not match particular food item with the patient having food poisoning as a result of eating the food item. This limitation is as a result of many cases of food poisoning being attributed to activities of offended deities, lack of efficient and integrated surveillance and notification system and due to the mild and short duration of the symptoms. The isolation of *Bacillus cereus* 4(8%) of 50 control stool samples in this study is suggestive that some people are carriers of *Bacillus cereus* in the study area. Grosha (2009) found 100(14%) of 711 feces of healthy persons. It is difficult to differentiate superficial contamination by *Bacillus cereus* from genuine disease caused by the organism owing to the mild short duration of the illness.

CONCLUSION

In conclusion, majority of Nigeria locally affordable cooked /wrapped/soups foods are liable to contamination by *Bacillus cereus*. Animal models fed with *Bacillus cereus* presented the symptoms of diarrhea, swelling of ileal loop and infiltration of liver cells by inflammatory cells and necrosis as with human subjects. Food handlers could be important reservoirs for pathogenic bacteria. Increased time/temperature exposure of foods and strict control of mishandling of products during preparation and dispensing is highly recommended and *Bacillus cereus* should be considered in the differential diagnosis of aetiologic agents of food poisoning outbreaks in Nigeria.

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