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Incidence, Characterization and Pathological Features of *Bacillus Cereus* in Soil, Raw Cereals and Meat in Anambra State, Eastern Nigeria

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Abstract
This study was conducted to determine the level of contamination of *Bacillus cereus* in soil, raw cereal and meat in Anambra State Nigeria. Eighty (80) different types of extra human samples made up of 10 each from soil from animal houses, moist soil, soil from plant roots, and dry soil, 10 each from raw cereal- maize, beans, rice and raw meat. Raw cereal and meat were randomly purchased from local food vendors and markets and soil samples were collected from different locations. Eighty human diarrheal stool samples from patients attending clinics and fifty (50) control stool samples from human volunteers were also evaluated for the presence of *Bacillus cereus*. All samples were transported to the laboratory for microbiological analysis. The extra human samples were first homogenized in 0.1% peptone water, incubated at 37^{oc} for 24 hours and then subcultured on to freshly prepared blood, MacConkey and polymyxinB agar plates while a direct stool culture on the above plates were also done. *Bacillus cereus* was identified using cultural characteristics, Gram staining reaction, spore staining for the presence of lipid globules and polymerase chain reaction (PCR). Enterotoxin production by *Bacillus* cereus and its effect on animal models was done. Antibiotic sensitivity tests were carried out. *Bacillus cereus* was isolated in 11(27.5%) of 40 soil samples, 5(16.6%) of 30 cereal crops and raw meat 4(40%). Soil from animal houses and raw meat had the highest contamination of 4(40%) each from 10 samples studied. There was no significant difference ($p>0.05$) in the *Bacillus cereus* isolated from soil, raw cereal and meat samples. *Bacillus cereus* was isolated in 15(18.8%) of 80 diarrheal stool and 4(8%) of 50 control stool samples which difference was not statistically significant. ALL *Bacillus cereus* isolated were positive for the enterotoxin and resistant to ampiclox. The results of this study reveal that *Bacillus cereus* is a common contaminant of soil, raw cereals and meat and is mainly of the diarrheal type.
Keywords: *Bacillus cereus,* incidence, characterization, pathological features

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 (Ryan and Ray, 2004). 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 (Inabo *et* al., 2000). *Bacillus cereus* is an opportunistic human pathogen and is occasionally associated with food poisoning, local and systemic infections (Kotianta 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 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) while in Turkey Wiley and Etats-Unis (2006) reported a prevalence of 22.4% *Bacillus cereus* contamination of meat and meat products . The figures are worrisome given that food borne illnesses associated with *Bacillus cereus* are grossly under-reported, mainly because symptoms such as abdominal cramps, diarrhea, vomiting are mild, mirror those of *Staphylococcus aureus* intoxication and *Clostridium perfringens* and goes undiagnosed (Todar, 2009). Practices identified as contributing to outbreaks of food poisoning include improper refrigeration, prolonged handling and contamination of food by food handlers who worked while ill or poor personal hygiene (Panisello *et* al.,2000,Daniels, 2002; Hedberg *et* al., 2006). Efforts must be made to adhere strictly to hygiene measures by following good hygiene practicesand stringently

implementing hazard analysis critical control point (HACCP) along the whole food chain (Powell *et* al., 2002, Oranusi *et* al 2003).
Epidemiological data on food borne disease outbreaks associated with *Bacillus cereus* contamination with soil, raw cereal and meat is Nigeria is not available but poor storage practices coupled with poor personal hygiene and lack of knowledge in safety practices which is inherent with food handlers in Nigeria are causes of concern. With the above facts in mind the current study aims at evaluating the incidence, characterization and pathological features of *Bacillus cereus* contamination with soil, raw cereal and meat in parts of Anambra State, Nigeria.

MATERIALS AND METHODS

Sampling procedures

Extra human samples
Ten (10) samples each of raw cereal (rice, beans and maize) and raw meat were randomly purchased in disposable sterile containers from different markets and soil samples were collected from different locations in Obosi, Onitsha and Awka areas of Anambra State, Nigeria and transported to the laboratory within 1-2 hours of collection for microbiological studies. Thus a total of eighty (80) extra human samples were analysed for the presence of *Bacillus cereus* see table 1a.

Human samples

One hundred and thirty (130) human stool samples made up of eighty (80) diarrheal stool samples from patients seen in clinics and medical laboratories and fifty (50) control stool samples from apparently healthy human subjects were also submitted to the laboratory for microbiological studies.

Microbiological evaluation

Ten (10g) grams of the different raw cereals samples were rehydrated in 0.8% sodium chloride for one hour at room temperature and 90ml of 0.1% peptone water added and incubated for 24hours at 37^{oc} while 10g of the different types of soil 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 on freshly prepared plates of sheep blood, MacConkey (Oxoid), 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^{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 confirmation of the identity and toxin identification.

Identification of *Bacillus cereus*

Identification methods were in accordance with Oxiod (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 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 ® 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 maize, soil, and raw meat 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, Duncan's multiple comparison test.

Soil and cereal samples tested

Soil		
a. Soil from animal houses	10	Unboiled
b. Moist soil	10	Unboiled
c. Soil from plant roots	10	Unboiled
d. Dry soil	10	Unboiled
Raw cereals		
a. Rice	*Oryza sativa Lin,* 10	Uncooked rice seeds
b. Beans	*Vigna unguiculata*, 10	Uncooked bean seeds
c. Maize	*Zea mays*, 10	Uncooked maize seeds
Raw meat (cow)	10	Uncooked cow meat

RESULTS
Bacillus cereus was isolated 11(27.5%) of 40 different types of soil, 9(22.3%) of 40 different types of cereal and meat.
Table 1 showed the prevalence of *Bacillus cereus* isolated from different types of soil in Nigeria. The results reveal soil from animal houses had the highest contamination. However, dry soil, moist soil had the lowest contamination
Table 2 showed the prevalence of *Bacillus cereus* in raw cereal and meat. Raw meat had the highest contamination of 4(40%) while the least came from rice.
The results of toxin production using Oxoid BCET-RPLA kits showed that 8(88.88%) *Bacillus cereus* isolated were positive for enterotoxin while 1(11.11%) from rice was negative.
Figures 1,2,3,4 also represented the results of liver tests. There was mild to moderate infiltration of the liver cell by inflammatory cells, distension of the central vein and necrosis.

Table1 Prevalence of *Bacillus cereus* in different types of soil in Nigeria

Soil samples	Number tested	Growth	Prevalence
Soil from animal houses	10	4	40%
Moist soil	10	3	30%
Soil from plant roots	10	2	20%
Dry soil	10	2	20%
TOTAL	40	11	27.5%

Table 2 Prevalence of *Bacillus cereus* in Nigeria locally affordable raw cereals and raw meat.

Raw cereals	Number tested	GROWTH	Prevalence
Beans (*Vigna unguiculata*)	10	2	20%
Maize (*Zea mays*)	10	2	20%
Rice	10	1	10%
Raw meat	10	4	40%
TOTAL	40	9	22.3%

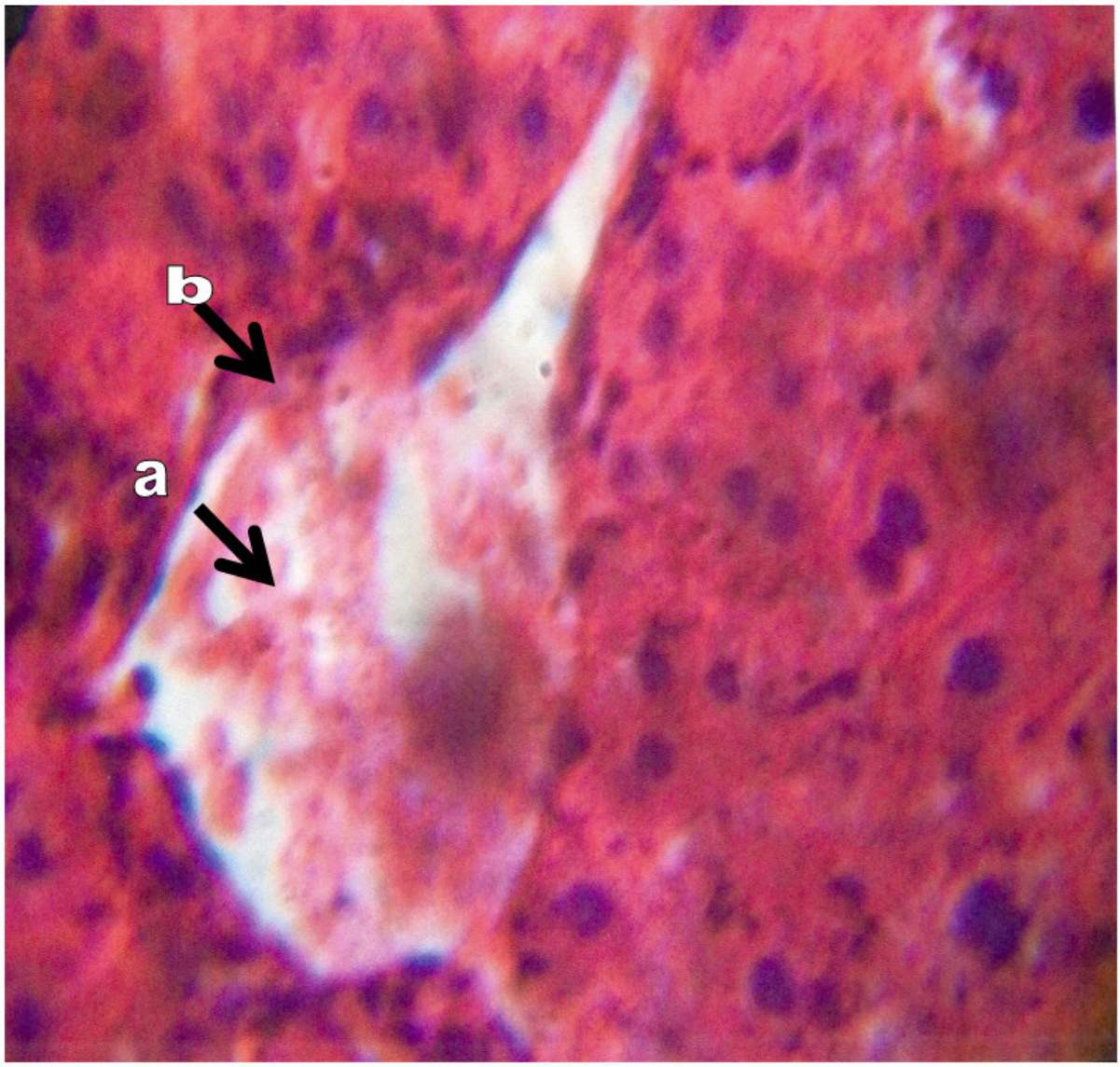

Fig.1 Liver section [a, b] shows distention of the central vein with moderate congestion with eosinophilic materials. *Bacillus cereus* from diarrheal stool sample

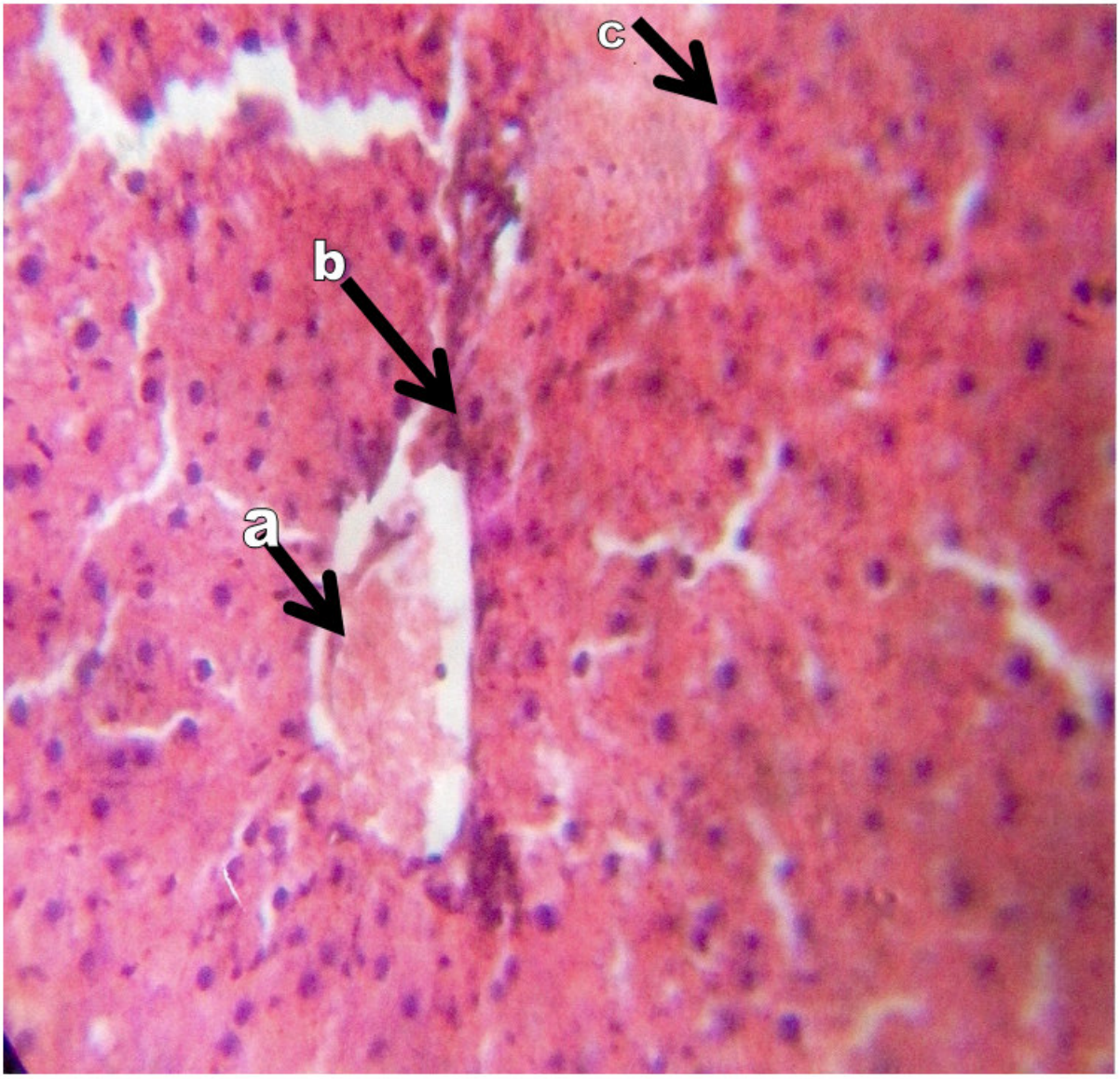

Fig. 2 Liver section shows [a]. Congestion of the central vein. [b]. Dilation of the liver sinusoids. [c].Mild necrosis liver cells. (Source control stool sample.

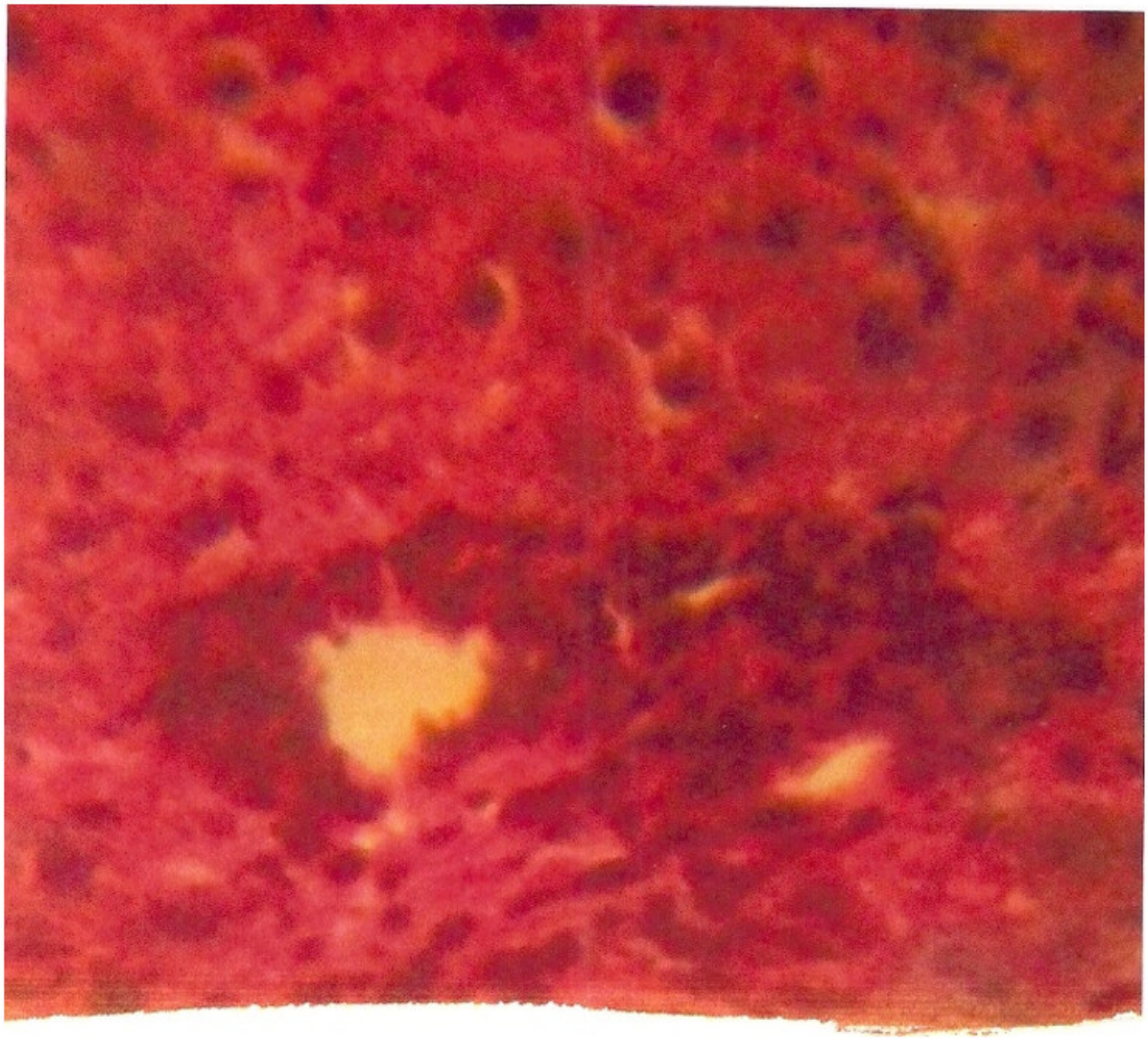

Fig. 3 Liver section shows cluster of mononuclear cells around a central vein with moderate hepatocyte degeneration and necrosis. Source of organism- maize cereal.

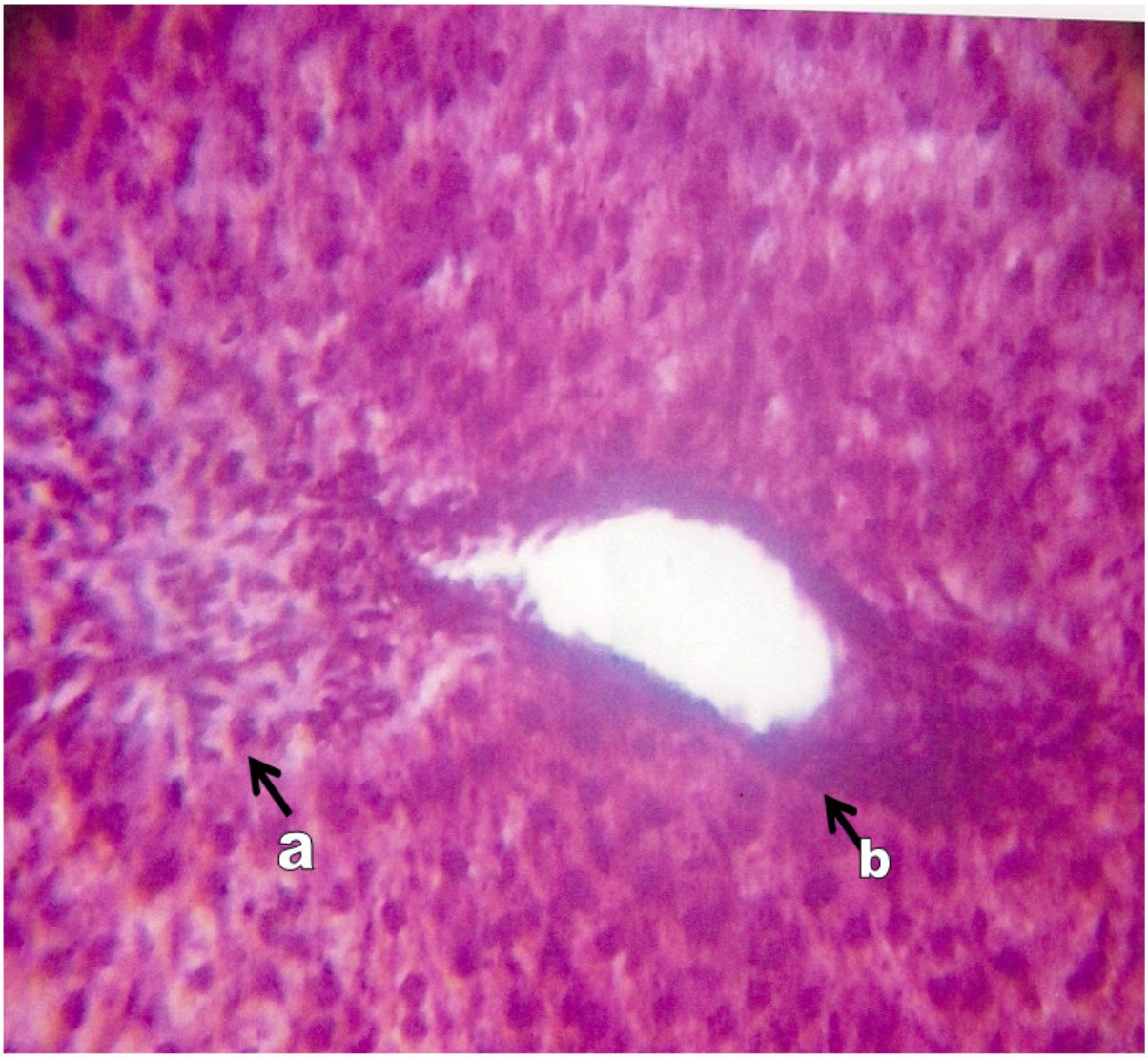

Fig. 4 Liver section showing (a). Hyperplasia of the liver cells at various stages of mitosis. [b]. The epithelial cells around the central vein [endothelial cells] are also hyperplastic [thickened [a]. No evidence of necrosis or infiltration of inflammatory cells. Source of organism peptone control.

DISCUSSION

This study reveals a high prevalence 11(27.5%) of 40 different types of soil samples. This could be explained by the ubiquitous distribution of this organism and its ability to form endospores (Mckillip, 2000). Soil from animal houses and raw meat had the highest prevalence of 4(40%) followed by 3(30%), 2(20%), 2(20%) from moist, dry, and soil from plant roots respectively. *Bacillus cereus* is soil-dwelling and easily found in soil samples (Hobbs and Gilbert, 1994, Vilkan *et* al., 2000). The high contamination in soil from animal houses and raw meat could be because some harmless strains of *Bacillus cereus* are used as probiotic feed additive to reduce *Salmonella* in the cecum (Ehiri et al 2001, Vila *et* al., 2009, Bories *et* al., 2009). Gulam and Nur-Hayati (2000) and Lee (2009) in their respective studies in Malaysia found an alarming rate of 76% contamination of cereal by *Bacillus cereus*. Imwidthaya *et* al., (1987), and Moss, (1989) in their respective studies found *Bacillus cereus* as the only bacteria isolated from cereals and a frequent source contaminant of maize seeds. Wiley and Estats-Unis (2006) in his study in Turkey found a prevalence of 22.4% *Bacillus cereus* contamination of raw meat.

The isolation of *Bacillus cereus* 15(18.75%) from eighty diarrheal stool and 4(8%) from fifty control stool samples is of concern. It could be attributed to contamination/cross contamination of foods and food products by endospores of *Bacillus cereus* from soil and raw cereal and meat. All isolate of *Bacillus cereus* were 100% resistant to ampiclox while one from moist soil was 100% resistant to tested drugs. Lee (2009) reported 100% resistant to ampicillin in his study. There was a significant difference ($p<0.05$) between sensitivity of isolate to antibiotics and their resistant to it and sensitivity of isolate of diarrheal stool to that of control stool to antibiotic tested.

Toxin production using BCET RPLA Oxoid test kit showed that 8(88.8%) of *Bacillus cereus* isolated were positive for enterotoxin and 1(11.11%) negative. Gulam and Nur Hayyati (2000) and Lee (2009) both reported 91.8% and 92% enterotoxin production respectively in their studies.

The results of this study revealed similar effects of diarrhea as in human were observed in the animal models after oral ingestion of *Bacillus cereus.* Histomorphological examination of the liver cells showed evidence of infiltration, distention of the central vein and necrosis. Helmut *et* al., (1997) and Katelijne *et* al., (2005) both in their respective studies found evidence of liver failure and necrosis in a child after eating food contaminated with *Bacillus cereus*

In conclusion, *Bacillus cereus* is a major contaminant of our soil, raw cereal, and meat and could be a probable source of contamination of foods and in food poisoning outbreaks. Efforts must be made to improve hygiene in food processing practices as it is highly recommended for prevention of contamination.

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