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Antibiogram and Multidrug Resistance in Enterobacteriaceae from Peanut Cake in Nigeria

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Abstract

We evaluated the response of enterobacteria species obtained from peanut cake in our previous study to commonly used antibiotics in Nigeria. Consequently, the multidrug resistant (MDR) isolates were tested for presence and functionality of plasmids in MD resistance, and potential virulence using the human blood haemolysis test. All isolates exhibited high resistance (>80%) to amoxicillin, cefuroxime and nitrofurantoin while sensitivity to fluoroquinolones (FQs) was between 70% and 80%. Contrariwise, 67% of Klebsiella showed resistance against the FQs (ciprofloxacin, ofloxacin and norfloxacin) and tetracycline. Salmonella isolates exhibited the highest significant ($P<0.05$) percentage resistance to ≥4 antibiotics (21%) while prevalence of haemolytic MDR strains was highest (100%) in Klebsiella. All MDR isolates exhibited single or double plasmid bands in gel electrophoresis. However on curing the plasmids, sensitivity to FQs and tetracycline increased to 100% while nitrofurantoin and amoxicillin resistance were maintained. The significance of this study lies in the detection of MDR enterobacteria in peanut cake, which are capable of lysing human erythrocytes; a threat to food and public health safety.

Key words: Food safety; Enterobacteriaceae; Multidrug Resistance; Plasmid; Peanut cake

Introduction

Peanut cake in Nigeria has been reported to be contaminated with a wide variety of bacterial species ranging from the simple commensals to the pathogenic types (Adebesin et al. 2001; Ezekiel et al. 2011a). Among these are the enterobacteriaceae, a group of Gram-negative intestinal bacterial that are extremely pathogenic to man and animals (Delost 1997; Da Costa et al. 2007; Cox and Pavic 2010). Also, amongst the notable entero-pathogens such as Escherichia coli, Salmonella, Shigella and Klebsiella, has been the emergence and continuing presence of multidrug resistant (MDR) strains (Garau et al. 1999; Robicsek et al. 2006; Joshi and Amarnath 2007; Esimone et al. 2010; Ezekiel et al. 2011b). This has aroused so much interest in the area of food and clinical microbiology.

It is noteworthy to mention that the fluoroquinolones (FQs), gentamycins and beta-lactams are the commonly used antibiotics in Nigeria against enterobacteria infections (Okeke et al. 2000; Okoli et al. 2002; Okoli 2006). However, several studies have reported emergence of resistance towards those antibiotics (Okeke et al. 2000; Chah et al. 2003; Oyinloye and Ezekiel 2011). Till date, there has been no report on the response of the isolates from this snack and feed ingredient to antibiotics, or even the occurrence of MDR strains amongst the populations. Hence, this study aimed at evaluating the response of previously

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isolated enterobacteria species from peanut cake (Ezekiel et al. 2011a) to commercially available and commonly used antibiotics in Nigeria. The potential virulence of the MDR isolates determined by their ability to lyse human erythrocytes was also evaluated. The data from this study will reveal the level of multidrug (MD) resistance among pathogenic enterobacteria that contaminate peanut cake and the dangers associated with the consumption of this kind of food material.

Material and Methods

Isolates. The enterobacteria used in this study were obtained from our previous work (Ezekiel et al. 2011a). The isolates were E. coli (2), Shigella (23), Salmonella (11), Klebsiella (3), Proteus (3) and Enterobacter (7). The isolates were sub cultured twice from MacConkey agar slants to Eosin-Methylene Blue, Salmonella-Shigella, and MacConkey agars in order to check the consistency of morphological characteristics. Plates were incubated at 37oC for 24–48 hours.

Antimicrobial susceptibility test (AST). The antimicrobial susceptibility test was performed for each isolate on freshly prepared Mueller Hinton agar (Oxoid) using the agar-disk diffusion method described by CLSI, formerly NCCLS (2002). The following antibiotics were tested against each isolate: amoxicillin (25µg), cefuroxime (30µg), ciprofloxacin (10µg), gentamycin (10µg), nitrofurantoin (200µg), norfloxacin (5µg), ofloxacin (5µg) and tetracycline (30µg). E. coli ATCC 25922 was used as quality control for the test. The isolates were grouped as either sensitive (S) or resistant (R) based on zones of inhibition as interpreted using the criteria recommended for enterobacteriaceae (NCCLS, 2002). Isolates that were resistant to four or more antibiotics excluding nitrofurantoin were classified as MDR strains. Nitrofurantoin resistance in enterobacteria has been reported to be greater than 80% in all cases (Okoli 2006; Ezekiel et al. 2011b); therefore, it was not used in this study to determine MD resistance.

Test for virulence. All MDR isolates were tested for the production of haemolysin on blood agar. The blood agar was constituted with agar powder (BDH) and human blood obtained from the Medical Laboratory section of Babcock University Medical Centre, Ilishan Remo, Nigeria.

Molecular studies of MDR strains. The TENS-Mini Prep method was performed for 8 MDR strains in order to determine their plasmid profiles. The obtained plasmids were separated on horizontal gel electrophoresis in 0.8 % agarose in 1X Tris-Borate-EDTA (TBE) buffer according to Lech and Brent (1987) and Kraft et al. (1988). Briefly, each purified bacterial colony was seeded into 10 ml Mueller-Hinton broth (Difco) in a screw cap tube and incubated at 37 oC overnight. The overnight broth (1.5 ml) was centrifuged for 1 min and the pelleted cells were dissolved in 300 µl of TENS solution (Tris 25 mM, EDTA 10 mM, NaOH 0.1 N and SDS 0.5 %) by inverting the tube a few times for thorough mixing. The dissolved cells were iced for 5 min and 150 µl of 3.0 M sodium acetate (pH 5.2) was added. This was followed by vortexing the tube until the content was completely mixed. The solution was microfuged for 5 min at 13,000 rpm to pellet cell debris and chromosomal DNA.

Table 1: Percentage response pattern of each genus of enterobacteriaceae from peanut cake to individual antibiotics

[+]Antibiotics (µg)	*E. coli*			*Enterobacter*			*Klebsiella*			*Proteus*			*Salmonella*			*Shigella*		
	[*]S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
AMX (25)	0	0	100	0	0	100	0	0	100	0	0	100	25	0	75	13.6	9.1	77.3
CEF (30)	0	0	100	0	0	100	0	0	100	0	0	100	25	8.3	66.7	13.6	9.1	77.3
CIP (10)	100	0	0	100	0	0	33.3	0	66.7	100	0	0	75	0	25	91	4.5	4.5
GEN (10)	0	0	100	85.7	14.3	0	100	0	0	0	33.3	66.7	58.4	8.3	33.3	59.1	4.5	36.4
NIT (200)	0	0	100	0	28.6	71.4	0	0	100	0	0	100	0	0	100	0	0	100
NOR (5)	100	0	0	100	0	0	33.3	0	66.7	100	0	0	83.4	8.3	8.3	72.7	18.2	9.1
OFL (5)	100	0	0	100	0	0	33.3	0	66.7	100	0	0	75	16.7	8.3	81.8	9.1	9.1
TET (30)	100	0	0	100	0	0	33.3	0	66.7	100	0	0	58.3	0	41.7	72.7	4.6	22.7

[*]S – sensitive, I – intermediate, R – resistant

[+]Antibiotics used: AMX – Amoxicillin, CEF – Cefuroxime, CIP – Ciprofloxacin, GEN – Gentamycin, NIT – Nitrofurantoin, NOR – Norfloxacin,

OFL – Ofloxacin, TET – Tetracycline

To obtain the plasmid, 400 µl of the supernatant was decanted into an Eppendorf tube, mixed with 800 µl ice-cold absolute ethanol, and centrifuged for 10 min. The supernatant was discarded and the pellet containing the plasmid DNA was rinsed twice in 1 ml of 70 % ice-cold ethanol. The pellet was dried at 45 oC for 15 min, re-suspended in 40 µl TE buffer and stored at 4 oC till further analysis.

The obtained plasmids were separated on a horizontal tank loaded with 5 mm agarose gel stained with 20 µl of 1 mg/ml ethydium bromide. The power supply was fixed at 80 V for 4 h. Exactly 15 µl of plasmid DNA solution was mixed with 2 µl loading dye (bromocresol purple) and carefully loaded onto each well in the gel. This was allowed to run for 2 h. DNA bands were visualized and photographed using Bio-Rad, Mini-Sub Gel GT®. The molecular weight of unknown plasmid DNA was extrapolated using the band mobilities in the gel. To confirm the presence of plasmid and its involvement in antibiotic resistance in the MDRs, 20 µl aliquot of cells from the primary bacterial colony were cured in 0.1 mg/ml acridine orange-supplemented nutrient broth. Antibiotic susceptibility tests were performed on the cured cells.

Data analysis. The SPSS® 14.0 was used for all statistical analysis. The percentage MDRs by genus and across districts were compared by the One-way ANOVA and DMRT at 95% significant level.

Results

Response of enterobacteria from peanut cake to AST. The antibiogram data of all isolates to eight different antibiotics showed that high resistance (>80%) occurred across board to amoxicillin, cefuroxime and nitrofurantoin while the sensitivity of the isolates towards ciprofloxacin was greater than 80%. Ofloxacin, tetracycline and norfloxacin also showed a high potency (70–80% sensitivity) against the isolates (Figure 1). However, of noteworthy is the susceptibility pattern of Klebsiella species to the FQs (Table 1). About 67% of this entero-pathogen showed resistance against the FQs (ciprofloxacin, ofloxacin and norfloxacin) and tetracycline. Contrariwise, all isolates of Proteus, Enterobacter and E. coli were sensitive to the FQs and tetracycline. All Klebsiella isolates showed sensitivity to gentamycin while the isolates of E. coli, Enterobacter and Proteus were all resistant to amoxicillin and cefuroxime. From the nine districts involved, isolates from Sagamu, Ikorodu, Mile 2 and Minna had overall sensitivity of 55% – 63% while the resistance levels were high (>50%) in isolates from Ibadan and Abeokuta (Figure 2).

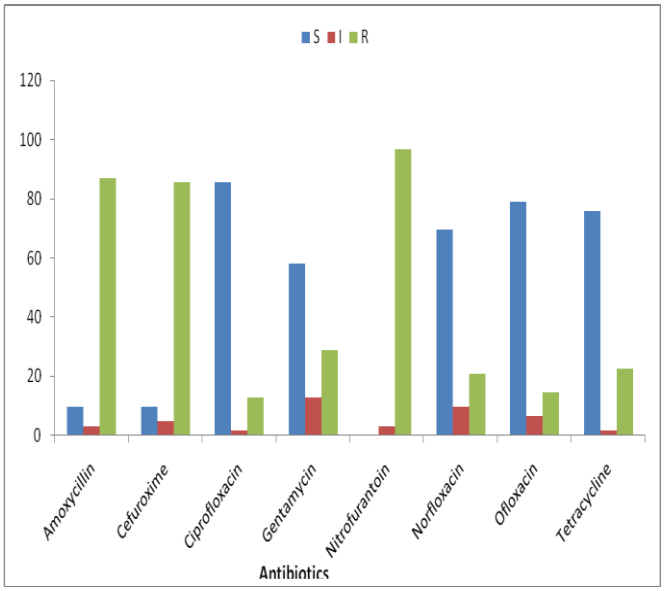

Figure 1: Overall percentage response pattern of entero-pathogens from peanut cake to individual antibiotics.
S – sensitive, I – intermediate, R – resistant

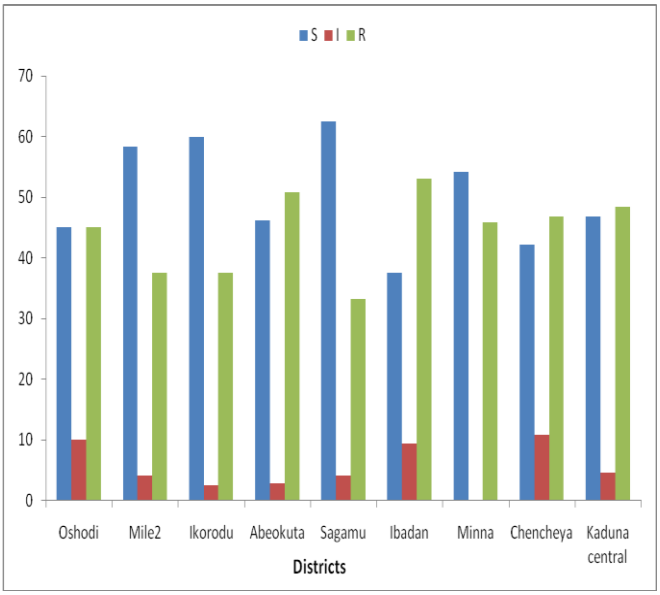

Figure 2: Overall percentage susceptibility pattern of entero-pathogens from peanut cake within nine districts in Nigeria. S – sensitive, I – intermediate, R – resistant

MD resistance and haemolytic virulence in enterobacteria. Of the six genera of enterobacteria considered in this study, three exhibited MD resistance and haemolytic activity on human erythrocyte cells (Table 2). Salmonella isolates exhibited the highest significant ($P<0.05$) percentage resistance to ≥4 antibiotics (21%). Out of the MDR Salmonella, 75% were haemolytic, while of 16% MDR isolates of Shigella species, 67% were haemolytic. About 11% of all Klebsiella isolates were MDR, all being haemolytic.

Of the nine districts sampled, a significant ($P<0.05$) percentage of MDR (21%) in the isolates was observed in Abeokuta, Ibadan, Chencheya and Kaduna central districts over the 10.5% and 5% recorded in Minna and Oshodi districts respectively. All isolates from Abeokuta and Oshodi districts were haemolytic, while a half of all isolates from Chencheya were haemolytic (Table 3). No MDR or haemolytic strains were detected in isolates from Mile 2, Ikorodu and Sagamu.

Table 2: Multi-drug resistance profile and haemolytic reaction of entero-pathogens from peanut cake in Nigeria

Number of multi-resistant antimicrobials	Percentage entero-pathogens showing multi-drug resistance and haemolysis					
	E. coli	*Enterobacter*	*Klebsiella*	*Proteus*	*Salmonella*	*Shigella*
4	---	---	0.0	---	10.5	5.3
5	---	---	5.3	---	5.3	0.0
6	---	---	5.3	---	5.3	10.5
7	---	---	0.0	---	0.0	0.0
[+]% MDR	---	---	10.5^{c}	---	21.1^{a}	15.8^{b}
[*]% Haemolytic MDR	---	---	100.0	---	75.0	66.7

[+]% MDR refers to total percentage of isolates in a genus of enterobacteriaceae showing multi-drug resistance
[*]% Haemolytic MDR refers to percentage of multi-drug resistant isolates in a genus of enterobacteriaceae showing haemolytic potential
Means with different alphabets along a row are significantly different ($P<0.05$).

Table 3: Multi-drug resistance profile and haemolytic reaction of entero-pathogens in peanut cake from nine districts in Nigeria

Number of multi-resistant antimicrobials	Percentage entero-pathogens showing multi-drug resistance in each district								
	Oshodi	Mile 2	Ikorodu	Abeokuta	Sagamu	Ibadan	Minna	Chencheya	Kaduna central
4	0.0	---	---	0.0	---	15.8	0.0	15.8	15.8
5	0.0	---	---	10.5	---	0.0	10.5	0.0	0.0
6	5.3	---	---	10.5	---	0.0	0.0	5.3	0.0
7	0.0	---	---	0.0	---	5.3	0.0	0.0	5.3
[+]% MDR	5.3^{b}	---	---	21.1^{a}	---	21.1^{a}	10.5^{b}	21.1^{a}	21.1^{a}
[*]% Haemolytic	100.0	---	---	100.0	---	0.0	0.0	50.0	0.0

[+]% MDR refers to percentage of isolates in a genus of enterobacteriaceae showing multi-drug resistance
[*]% Haemolytic MDR refers to percentage of multi-drug resistant isolates in a genus of enterobacteriaceae showing haemolytic potential
Means with different alphabets along a row are significantly different ($P<0.05$)

Plasmid profiles of MDR strains. All MDR strains in this study possessed extra-chromosomal DNA (Figure 3). Isolates of Shigella and Klebsiella showed single plasmid bands of 6.9–7.2kb and 8.5–8.7kb weights respectively. On the other hand, single and double band patterns of plasmid DNA were shown in the gel for Salmonella isolates. The isolates with the lighter weighted (4.4–5.6kb) single plasmid bands were identified as S. enteritidis and S. paratyphi while S. typhi showed the double plasmid bands of heavy (23.0kb) and light (6.9–9.4kb) weight bands. However on curing the plasmids, sensitivity to FQs and tetracycline increased to 100% while nitrofurantoin and amoxicillin resistance were maintained.

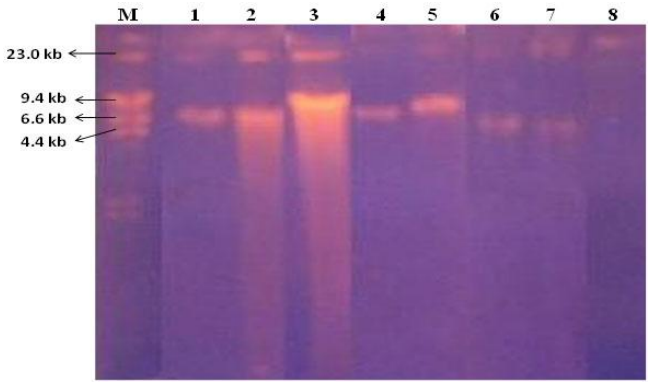

Figure 3: Plasmid bands of MDR strains from peanut cake in Nigeria. M, λ DNA Hind III digested marker;
1 and 4, Shigella; 2-3 and 6-7, Salmonella; 5 and 8, Klebsiella.

Discussion

In food safety analysis, enterobacteriaceae such as Salmonella and Escherichia coli are indicators of faecal contamination. These indicators have been reported to be transmitted from food handlers (potential reservoirs) to the consumers via contaminated foods (Lund et al. 2000; Miranda et al. 2008; Ezekiel et al. 2011a). An increasing number of resistant pathogenic enterobacteria have also been reported recently in diverse food and feed materials (Saenz et al. 2001; Chah et al. 2003; Bouchakour et al. 2010; Ezekiel et al. 2011b). In this study, the six genera of enterobacteriaceae investigated are incriminated in virtually any type of infectious disease and can be recovered from any specimen received in the laboratory (Elmer et al. 1992; Delost 1997; Xilin et al. 2006). Therefore their occurrences in the peanut cake samples and antibiogram profiles are of public health importance.

The prevalence of antibiotic resistant bacteria poses a significant problem today. Globally, FQs have become a vital component of the current antimicrobial arsenal (Hooper 2000; Kerr 2004). In this study, the response pattern of the isolates to the FQs is of great importance especially for Klebsiella species. All Klebsiella isolates showed 67% resistance to each of the FQs tested and also to other four antibiotics tested thereby confirming them as MDR. The resistance percentage reported here is 100% higher than our previous reports for Klebsiella against the FQs (Ezekiel et al. 2011b). This raises serious food safety alarm. A study carried out in England and Wales showed that the prevalence of resistance of Klebsiella species to FQs rose by 171% between 1990 and 1996 (David et al. 2002). This further supports the possibility of a 100% increase in FQ resistance as reported earlier, moreover, since FQ resistance is usually plasmid-mediated (Robicsek et al. 2006; Sjölund-Karlsson et al. 2010). For the other genera, we recorded >75% sensitivity against the FQs. This is in accordance with the reports of Antti et al. (1999), Sharada et al. (2009) and Ezekiel et al. (2011b) but in contradiction to the findings of Joshi and Amarnath (2007) especially for the salmonellae and E. coli.

Recently, significant increases in the prevalence of FQ resistance among gram-negative bacilli (Livermore et al. 2002; Neuhauser et al. 2003; Lautenbach et al. 2001) and its relationship with resistance to multiple other antimicrobial agents have been documented (Paterson et al. 2000; Lautenbach et al. 2001). In this study, all MDR strains of Klebsiella, Salmonella and Shigella had a resistance combination of two or three FQs and AMX-TET/GEN. On the overall, the commonest patterns were NIT-AMX-CEF linked to any other antibiotic; a similar pattern reported by Ezekiel et al. (2011b).

In all MDR enterobacteria, >67% of the isolates had haemolytic potentials. This is a major threat to the consumers of this snack and also to the birds that may consume feed made from such contaminated peanut cake. Haemolysins are important extracellular substances produced by many bacteria to increase pathogenicity. Sekowska et al. (2006) confirmed the ability of these isolates to lyse human red blood cells although at a lower percentage (29.8%) than that in this report. This then implies that our isolates could render the samples unsafe for human and animal consumption. This calls for a high microbiological standard for consumable products in light of the spread of enteropathogens and resistance.

The presence of light and heavy weighted plasmids in the isolates may be a reflection of horizontal extra-chromosomal DNA transfer among isolates, since Esimone et al. (2010) and Ezekiel et al. (2011b) reported them to be mobilizable and conjugative, respectively. The presence of the plasmid bands as MDR determinants in the haemolytic isolates in this study was confirmed with the increase in susceptibility to antimicrobials after curing. Previous studies have reported plasmid-mediated quinolone resistance proteins as being responsible for the conferment of FQ resistance on Shigella, Klebsiella and Salmonella isolates (Munshi et al. 1987; Martinez-Martinez et al. 1998; Sjölund-Karlsson et al. 2010). QnrA determinants have also been identified in C. freundii, E. coli, Enterobacter and K.

pneumoniae in Europe (Nazik et al. 2005). The quinolones resistant proteins (Qnr) protect the DNA-gyrase from quinolones, Aac(6′)-Ib-cr modifies quinolones with a piperazinyl group, and QepA is involved in active efflux (Robicsek et al. 2006). Since curing of plasmids led to increased (100%) sensitivity to FQs and tetracycline but stability in nitrofurantoin and amoxicillin resistance in all isolates, it may be deduced that resistance to the latter antibiotics are chromosome-controlled. In our previous study (Ezekiel et al. 2011b) MDR enterobacteria isolates from poultry feeds had amoxicillin resistance controlled by plasmids, a report negating our present finding.

Conclusively, we have presented for the first time the antibiotic susceptibility and MDR patterns of enterobacteria contaminating peanut cake in Nigeria. A handful of the isolates are MDR while others are virulent. Therefore, with peanut cake being at the centre of the connection between human and animal feeding, it is of importance that contamination of this food and feed ingredient be reduced to the barest minimum. Ezekiel et al. (2011a) recommended some tips for the improvement on the safety of this food. Producers, handlers and consumers of this snack and feed ingredient are therefore obliged to adhere strictly to this to ensure food and public health safety.

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