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BACTERIOLOGICAL ASSESSMENT OF FRIED-READY-TO EAT (RTE) VENDED FOODS SOLD IN OSOGBO, OSUN STATE, NIGERIA

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Abstract: *The purpose of this paper is to investigate the bacteriological profile of Enterobacteriaceae from ready-to-eat (RTE) fried foods and to also screen for the presence of shiga-toxins, intimin and haemolysin genes which are indicator genes for food-borne infections and diseases. The morphological and biochemical characterizations were carried out using standard isolation techniques while enumeration was done using a colony counter. To study the resistance and susceptibility pattern of the isolated Enterobacteriaceae, the characterized isolates were subjected to antibiotics susceptibility test using the Kirby-Bauer disk diffusion method. The presence of stx 1 and 2 (shiga toxin genes), eaeA (intimin gene) and hylA (haemolysin gene) were screened for in the multi-drug resistant (MDR) isolates. Six (6) species of bacteria were isolated from the RTE food samples namely: Proteus spp (20%), Escherichia coli (60%), Salmonella spp (30%), Klebsiella spp (30%), Staphylococcus spp (20%) and Shigella spp (50%) while the colonial count ranged from 60×10^5-299×10^3. The phenotypic antibiotics profile result showed that 60% of the multi-drug resistant organisms harboured stx1 and 2 while 40% harboured hylA. However, none of the MDR isolates were positive for eaeA. In conclusion, commercially vended RTE food handlers should be continuously educated on the importance of basic food hygiene and sanitation.*

Key words: *Food-borne infections, Escherichia coli, Salmonella spp., haemolysin, hygiene*

1. Introduction

Food can simply be defined as any substance (s) that is usually composed of the six classes of food such as: carbohydrate, water, proteins, mineral salt, vitamins and fat that can be ingested by an animal or human for pleasure or nutrition [1, 2]. It is a requisite need of all animals and human beings to sustain a reproductive, productive and healthy life style. The reports of [3] also documented that food can be defined as any nutritious material that provides energy, strength, power when consumed by all living things. Since the existence of mankind, food-borne diseases have been known to be a critical challenge globally. According to the [4], Hippocrates (460 B.C) was one of the early scientists to document that there is a very strong connection between food ingestion/consumption and food borne illnesses. Several authors have reported that food-borne illnesses are posing significant health-related problems in both developed and developing countries with severity varying from one country to another. There are quite a large number of pathogenic microbes causing food borne diseases such as: *Staphylococcus aureus,*

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Salmonella sp, *Escherichia coli* 0157:H7, *Shigella* sp, *Clostridium* sp, *Bacillus cereus*, etc. that have been isolated and characterized from ready-to-eat fried foods (such as cheese, fish, puff-puff, mosa, akara, yam, etc.) sold in public areas such as: restaurants, markets, schools and street vendors [5 - 9].

Ready-to-eat foods (RTE) are foods meant for immediate consumption at the point of purchase without the need for further processing [10]. This implies that such foods do not require any other processing other than reheating/rewarming [11]. Most of these RTEs are usually prepared in advance before sale and consumption. However, due to the minimal cooking procedures, there is the likelihood of cross-contamination, inappropriate holding temperatures and time, leading to the production of inconsistent food products with deteriorating microbiological quality [12, 13]. Knowing the bacteriological load of RTE food components is very essential because predisposing factors such as: processing, display, storage, handling, and processing may increase their bacteriological load at the point of sale [14]. Other factors such as: improper holding temperatures, dirty environment, inappropriate selling points like congested train motor parks and train stations with polluted air aerosols, use of contaminated crude utensils and water are also among the predisposing factors to RTE contaminations [15 - 17]. In addition, food sellers and handlers can also contribute to the transmission of pathogenic food borne microorganisms either during or after convalescence when they no longer have symptoms [18].

The reports of [7] had earlier documented that *E. coli* and *Shigella* spp. can survive on hands and surfaces (such as cutting slabs and knives) for hours or even days after initial contact with the infectious pathogens. It is needful to ascertain the capacity of development and the progression of bacteria existence on the foods with the aim of monitoring not only the bacteriological quality but also to assess and determine the consumer wellbeing [19].

According to the reports of [20], bacterial food borne pathogens are among the major microbes affecting the quality and safety of RTE foods globally. Fried sweet potatoes, cheese, bean cake, mosa, puff-puff, etc are among the RTEs sold within Osogbo metropolis.

The shiga toxins (Stxs) were named in the early 1900s after a novel cytotoxin was detected in *Shigella dysentriae* type 1. Like the other bacterial toxins such as: the heat-labile enterotoxin of *Escherichia coli*, (cholera toxin (CT) of *Vibrio cholera*, the shiga toxins belong to the heteromeric protein toxins that consists of one active (Stx A, 32kDa) and five receptor-binding (StxB, 7.7 kDa) subunits. The two (2) types of shiga toxins namely: Sxt1 (shiga toxin 1) and Stx2 (shiga toxin 2) [21, 22]. The study of [23] also documented that some *E. coli* strains harbouring Stx 2 have the same mode of action with Stx,1 but are antigenically different. However, humans get infected with shiga toxins as a result of ingesting *S. dysenteriae* or *E. coli* (such as 0157:H7) contaminated foods and water which results into bloody diarrhoea, haemolytic uremic syndrome (HUS); a clinical condition characterized by thrombocytopenia, anaemia and kidney damage [24]. Other virulence factors associated with shiga toxins include: effacing (eae) and enterohaemolysin (ehxA) genes [21].

2. Materials and methods

Collection of RTE food samples

Ten (10) samples each of ready-to-eat fried foods: sweet potato, beans cake, fried yam and fried fish were purchased from different locations in Osogbo, Osun State

inside sterile zip lock bags on ice packs. After purchase, the samples were transported immediately to the Microbiology laboratory of Osun State University.

Morphological, colonial, and biochemical characterizations of isolated bacteria

Using the methods of [25] with slight modifications, ten (10) g of each ready-to-eat food sample was weighed and added into 90ml of 0.1% sterile peptone water to make a 100ml sample suspension. The suspension was homogenised for 20 min. Tenfold serial dilutions of each sample were prepared and 0.1 ml of appropriate aliquots (10^3 and 10^5) were spread plated in duplicates on nutrient agar plates (NA) and incubated at 37^0C for 24 hr. After 24-hr incubation, the numbers of distinct colonies were enumerated on the Petri dish using a colony counter. After counting, the distinct colonies were picked from the NA plates and aseptically streaked on *Salmonella-Shigella* agar (SSA) and Eosin methylene blue agar (EMB) for the isolations of *Samonella* sp, *Shigella* sp *E. coli*, and other enteric. The streaked plates were incubated at 37^0C for 24 hr and pure cultures were picked from the EMB and SSA plates and transferred aseptically into freshly prepared NA slants, incubated for 24 hr at 37^0C and kept inside the refrigerator at 4^0C as stock cultures. All the isolates were Gram stained. All isolates were phenotypically identified based on cultural characteristics such as: shape, colour, elevation, edge, size opacity and biochemical tests such as: catalase, oxidase, urease, MRVP (Methyl-red/Voges-Proskauer), starch hydrolysis, triple sugar iron, sugar fermentation,

citrate, indole and motility according to the Bergey's Manual of Determinative Bacteriology, 9th edition protocols. *E. coli* isolates were confirmed by re-streaking them on Eosine methylene blue (EMB) agar. Furthermore, all isolates were also subjected to haemolysis test on blood agar to identify organisms that have the potential to break down red blood cells by the production of haemolysin toxin using the method of [26].

Antibiotic Susceptibility Testing of Isolated Bacteria from RTEs

The phenotypic antibiotics profile of the isolates was carried out using Kirby-Bauer disc diffusion method. Their multi-drug antibiotics resistance (MAR) index was calculated by the ratio of number of antibiotics ineffective. The antimicrobial agents used includes Clindamycin (10 µg), Erythromycin (15 µg), Gentamycin (10 µg), Doxycline (30 µg), Oxacillin (10 µg), Linezolid (30 µg), Nitrofurantoin (30 µg) and Vancomycin (30µg) which were all obtained from Oxoid UK. Using a flame sterilized inoculating loop, a colony of each isolate was taken and inoculated inside nutrient broth. The cultures were standardized to obtain turbidity that is optically comparable to 0.5 McFarland standards as documented by [27]. Sterile swab stick was dipped into the standardized broth cultures and pressed gently on the wall of the tube to remove excess inoculums from the swab. Sterile and solidified Mueller Hinton agar plates were inoculated by streaking the swab after which antibiotic discs were fixed on the media using disc dispenser and incubated at 37^oC for 24 h. Zones of inhibition were measured in mm and analysed using EUCAST break point version 11. The MDR indicator implies resistance of each isolate to ≥1 antibacterial agent in ≥3 classes of antibiotics.

MAR index = No. of antibiotic against which isolate is resistant /Total no. of antibiotic used for testing.

Molecular Analysis (Detection of Shiga and Shiga-like Toxins)

DNA Extraction

The chromosomal DNA was extracted from all hemolysis positive isolates using Zymo Research Genomic DNA^{TM}- Tissue MiniPrep Kit following the manufacturer's instructions. A UV-Vis ThermoScientific^{TM}Nanodrop Lite Spectrophometer (model S-22, Boeco, Germany) was used to qualify and quantify the extracted chromosomal DNA and stored at -80^{o} C for further analysis.

16SrRNA gene detection and Gel Electrophoresis of toxigenic isolates:

Polymerase chain reaction (PCR) was used to detect the presence of shiga toxins (stx1 and stx2), Intimin (eae) and hemolysin (hylA). Amplifications of the genes were achieved by employing the specific oligonucleotide primers as shown presented in Table 1. Duplex PCR was used to detect *stx1* and *stx2* genes [28] which is a 25µL reaction consisting of: master mix (12.5µL), primer (4µL), DNA template (5µL) and water (3.5µL). The PCR cycling started with an initial denaturation at 94 °C for 7 min; 35 cycles of 94 °C for 1 min, annealing at 60 °C for 1 min, elongation at 72 °C for 2 min; and a final extension at 72 °C for 10 min and allowed to hold at 4°C. 10µL of amplicons were electrophoresed in a 2% (w/v) agarose gel for 1 hr at 5 V/cm with a standard molecular weight marker and visualized using a gel documentation unit (Vilber ^{TM}).

Table 1:

Lists of primers used for PCR

Primers	Specificity	No of nucleotides	Primer sequence (5'-3' orientation)	Expected band size (bp)	References
stx1	Shiga toxin 1	20 20	F: ACACTGGATGATCTCAGTGG R: CTGAATCCCCCTCCATTATG	614	[28]
Stx2	Shiga toxin 2	20 21	F:CCATGACAACGGACAGCAGT R:CCTGTCAACTGAGCAGCACTT	799	[28]
eaeA	Intimin	20 21	F:CCCGAATTCGGCACAAGCAT R:CCCGGATCCGTCTCGCCAGTA	881	[29]
hylA	Hemolysin	20 20	F: ACGATGTGGTTTATTCTGGA R: CTTCACGTGACCATACATAT	165	[29]

3. Results and discussion

Potentially pathogenic *Enterobacteriaceae* are among the major cause of many food/water borne infections and diseases such as: diarrhoea, shigellosis, cholera, haemolytic uremic syndrome (HUS), haemorrhagic colitis, etc. [29]. Therefore, this study was designed to determine the bacteriological load of fried RTEs and also to detect the presence of shiga-toxin, intimin and haemolysin genes which are indicator genes for the onset of food borne infections and diseases. Four (4) different ready-to-eat fried foods

namely: potato, fish, bean cake and yam. The six (6) bacterial species (Tables 2 and 3) isolated from this study correlates to the reports of [30] that isolated similar organisms from fried yam, potato, bean cake and plantain sold in Enugu metropolis namely: *E. coli, S. aureus, Salmonella* sp, *Shigella* sp, *Proteus* sp and *Klebsiella* sp and *Bacillus cereus*. The studies of [31] documented the isolation of shiga-toxin producing *E. coli* from ready-to-drink milk products namely: cheese (wara) and fermented cow milk (nunu) sold in Abeokuta, Ogun State. In addition, [28] also documented similar results of bacteria isolated from domesticated buffaloes. [29, 32] also documented similar reports from RTEs. The colonial morphology of the organisms includes size (1-4mm), shape (circular, irregular), surface (smooth), elevation (raised), edge (entire, lobate, crenate), opacity (translucent and opaque), colour (bluish-purple, pinkish, black, amber and light blue). From the findings of this study, the highest bacterial count was observed in Oke-fia market while the lowest was recorded from Sasa market. This observation could be traceable to the unsatisfactory environmental conditions of these markets.

Table 2:

Morphological characterization of isolated bacteria

Isolate code	Size (mm)	Shape	Surface	Elevation	Edge	Opacity	Agar/color
PT1B	2	circular	smooth	raised	entire	translucent	EMB/bluish purple
PT3B	2	circular	smooth	raised	entire	translucent	EMB/bluish purple
PK04	2	circular	smooth	raised	entire	translucent	EMB/bluish purple
PTIA	1	irregular	rough	raised	crenate	opaque	SSA/blackish
PT3A	2	circular	smooth	raised	entire	translucent	EMB/bluish purple
PT1A	2	circular	smooth	raised	entire	translucent	EMB/bluish purple
PK01	2	irregular	rough	raised	lobate	opaque	SSA/blackish
PT3A0	2	circular	smooth	raised	entire	translucent	EMB/ light blue
	1	circular	smooth	raised	entire	translucent	EMB/bluish purple
FK04	1	circular	smooth	raised	entire	translucent	EMB/bluish purple
FS01	2	circular	smooth	raised	entire	translucent	SSA/ amber
FO01	4	circular	smooth	raised	entire	translucent	EMB/bluish purple
PT3A	2	circular	smooth	raised	entire	translucent	SSA/pinkish
	1	circular	smooth	raised	entire	translucent	SSA/pinkish
FKB05	2	circular	smooth	raised	entire	translucent	SSA/blackish
FIG05	2	circular	smooth	raised	entire	translucent	SSA/pinkish
FIG03	1	circular	smooth	raised	entire	translucent	SSA/blackish
FOJ01	2	circular	smooth	raised	entire	translucent	SSA/ amber
FOJ02	2	circular	smooth	raised	entire	translucent	SSA/ amber
BOJ01	2	circular	smooth	raised	entire	translucent	EMB/bluish purple
BK02	2	irregular	Rough	raised	crenate	Opaque	SSA/blackish
BS05	2	circular	smooth	raised	entire	translucent	EMB/bluish purple
YIG5	2	circular	smooth	raised	entire	translucent	EMB/bluish purple

Key: *P= Fried sweet potato; F= fried fish; B= fried bean cake; Y= fried yam*
EMB= Eosine methylene blue agar; SSA= Salmonella-Shigella agar
Table 1 above shows the phenotypic characteristics of bacteria isolated from the RTE food samples which includes: size, shape, surface, elevation, edge, opacity and colour

Table 3:

Gram staining and biochemical characterizations

Isolate code	Gram's reaction	Oxidase	Catalase	Methyl red	Voges-Proskauer	Citrate	Starch hydrolysis	Urease	Mannitol	Indole	TSI slant/butt	TSI H_2S	TSI gas production	Hemolysis test	Presumptive %	Presumptive organism
PT1B	-	-	+	-	+	-	-	+	+	-	Y/Y	+	-	α	83.1	*P. vulgaris*
PT3B	-	-	+	-	+	-	-	+	+	+	Y/Y	+	-	α	88.1	*E. coli*
PK04	-	-	+	-	-	-	+	-	+	-	R/R	+	-	β	88.4	*Salmonella* spp.
PTIA	-	-	+	-	-	-	-	+	+	+	Y/Y	+	-	β	82.4	*Klebsiella* spp.
PT3A	-	-	+	-	-	-	-	-	+	-	Y/Y	+	-	α	86.4	*E. coli*
PT1A	+	-	+	-	-	+	-	+	+	-	Y/Y	+	-	β	89.9	*Staphylococcus* spp.
PT3A	-	-	+	-	+	-	-	+	+	+	Y/Y	+	-	α	89.4	*E coli*
PK01	+	-	+	-	+	+	+	-	+	-	R/R	+	-	α	85.5	*S. hemolyticus*
PT3A0	-	-	+		-	-	-	+	+	+	Y/Y	+	-	α	88.2	*Shigella* spp.
FK04	-	-	+	-	+	+	+	-	+	-	Y/Y	+	-	α	98	*Klebsiella aerogenes*
FS01	-	-	-	-	+	+	+	-	+	+	Y/Y	+	-	β	97	*E. coli*
FO01	-	-	+	-	-	+	+	-	-	-	R/Y	+	-	β	97	*Proteus* spp.
FKB05	-	-	+	-	-	+	+	-	+	-	Y/R	+	-	β	86	*Salmonella* spp.
FIG05	-	-	+	-	-	+	+	-	+	-	Y/R	+	-	α	98	*Shigella* spp.
FIG03	-	-	+	-	+	+	+	-	+	+	Y/R	+	-	α	98	*Shigella* spp.
FOJ01	-	-	+	-	-	+	+	-	-	-	Y/R	+	-	β	96	*Salmonella* spp.
FOJ02	-	-	+	-	-	+	+	-	+	-	Y/R	+	-	α	99	*Klebsiella* spp.
FSA01	+	-	+	-	-	+	+	-	+	-	Y/R	+	-	α	98	*Staphylococcus* spp.
FAL03	-	-	+	-	+	+	+	-	+	+	Y/R	+	-	α	92	*E. coli*
BOJ01	-	-	+	-	+	+	-	-	+	-	Y/R	-	-	β	98	*E. coli*
BK02	-	-	+	-	-	+	-	-	+	-	Y/Y	-	-	α	98	*Shigella* spp.
BS05	-	-	+	-	-	+	-	-	+	-	Y/Y	-	-	β	97	*Salmonella* spp.
YIG5	-	-	+	-	-	+	-	-	+	-	Y/R	-	-	α	98	*Salmonella* spp.

Key: *S. hemolyticus* = *Staphylococcus hemolyticus*; α= alpha hemolysis; β= beta hemolysis; Y= yellow, R= red, - = negative; += Positive. The Gram staining, biochemical and haemolysis characterizations of isolated bacteria are represented in Table 3 above including the presumptive percentage of the probable organisms.

Figure 1 represents the colonial count of bacteria isolated from the RTE food samples. The bacterial load depicts the hygiene of the production environment as well as that of the food handlers. The lowest and highest bacterial load observed in the sampled RTEs were obtained from Sasa and Oke-fia markets respectively. The high bacterial count observed in RTEs sold in some of the sampled markets might be due to the busyness and unsanitary conditions of these markets as the surroundings are usually unkept. This, however, calls for a public health concern.

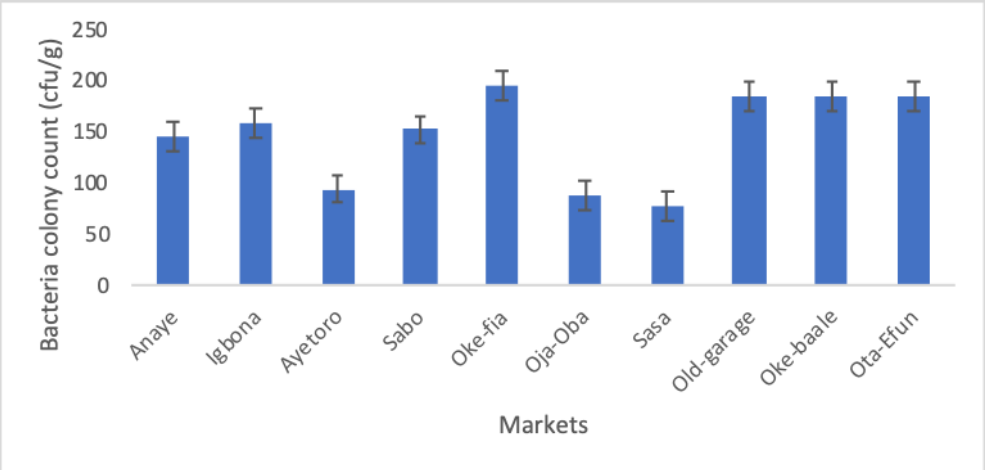

Fig. 1. Bacterial counts from RTEs

The phenotypic antibiotics profile (Table 4) shows that all the isolates were 100% resistant to clindamycin and erythromycin, and 100% susceptible to nitrofurantoin which is similar to the reports of [30, 29, 32, 33] while the multiple antibiotic resistant (MAR) index which signifies the ratio between the number of antibiotics an organism is resistant to and the total number of antibiotics it is been exposed to is presented in figure 2. It was observed that *Shigella* spp. had the highest % MAR index (80%) while *Klebsiella* spp. had the least (21%).

Table 4:

Phenotypic antibiotics profile of isolated bacteria from RTE food samples

Antibiotics	*Proteus* spp. (n=2)	*Escherichia* spp. (n=6)	*Staphylococcus* spp. (n=3)	*Klebsiella* spp. (n=3)	*Shigella* spp. (n=4)	*Salmonella* spp. (n=5)
Clindamycin (DA/2µg)	R (2), I (0), S (0)	R (5), I (0), S (1)	R (3), I (0), S (0)	R (2), I (0), (1)	R (4), I (0), S (0)	R (5), I (0), S (0)
Erythromycin (E/15µg)	R (2), I (0), S (0)	R (3), I (1), S (2)	R (3), I (0), S (0)	R (3), I (0), S (0)	R (4), I (0), S (0)	R (2), I (0), S (3)
Gentamycin (GN/10µg)	R (0), I (0), S (2)	R (3), I (0), S (3)	R (2), I (0), S (1)	R (3), I (0), S (0)	R (4), I (0), S (0)	R (5), I (0), S (0)
Doxycycline (DO/30µg)	R (0), I (0), S (2)	R (2), I (0), S (2)	R (3), I (0), S (0)	R (0), I (0), S (3)	R (4), I (0), S (0)	R (2), I (1), S (2)
Oxacillin (OX/1µg)	R (2), I (0), S (0)	R (6), I (0), S (0)	R (3), I (0), S (0)	R (0), I (0), S (0)	R (4), I (0), S (0)	R (5), I (0), S (0)
Linezolid (LZD/30µg)	R (2), I (0), S (0)	R (3), I (0), S (3)	R (1), I (1), S (1)	R (0), I (0), S (3)	R (2), I (0), S (2)	R (2), I (0), S (3)
Nitrofurantoin (F/300 µg)	R (0), I (0), S (2)	R (0), I (0), S (6)	R (0), I (0), S (3)	R (0), I (0), S (3)	R (1), I (0), S (3)	R (0), I (0), S (5)
Vancomycin (VA/30µg)	R (2), I (0), S (0)	R (1), I (1), S (4)	R (1), I (1), S (1)	R (0), I (0), S (2)	R (1), I (1), S (2)	R (0), I (0), S (4)

Key: I= intermediate R= Resistant S= susceptible n= number of identified isolates

Several studies had earlier documented that *Enterobacteriaceae* especially *E. coli* is used as important indicator organism for faecal contamination which connotes the unsatisfactory and deteriorating qualities of

some vended ready-to-eat food products [32, 34 - 37].

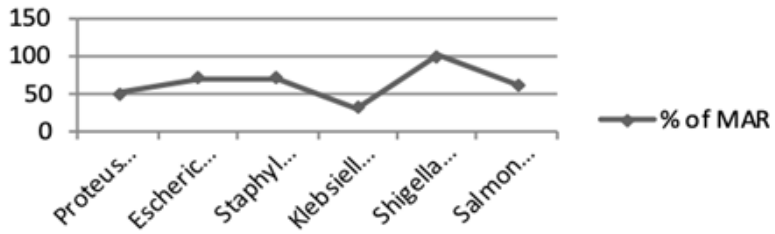

Fig. 2. Percentage (%) of multiple antibiotic resistant (MAR) indexes of recovered bacterial isolates from commercially sold RTE food

About 80% of the isolated bacteria harboured hylA genes, while 40 % harboured stx1 genes (Figures 3 and 4). However, none of the isolates were positive for stx 2 and eaeA in this study. Therefore, all the isolates that were positive for the presence of stx1 can be regarded as "shiga-toxin" producing *Enterobacteriaceae*.

The study [29] had previously reported the presence of stx1 and 2 in *E. coli* O157:H7 isolates recovered from raw/uncooked beef sold in Abeokuta, Ogun state, Nigeria. Organisms producing these toxins are the leading course of food and water borne bacterial infections which menace needs to be curbed.

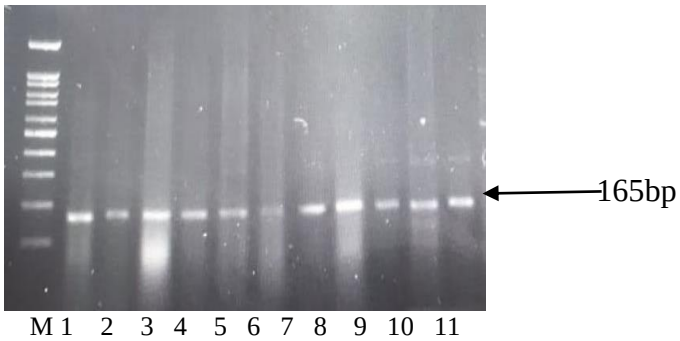

Fig. 3. Agarose gel electrophoresis of amplified products of hylA genes in the bacteria isolates
M: maker, Lane 1-11: Resistant bacteria isolated from RTEs

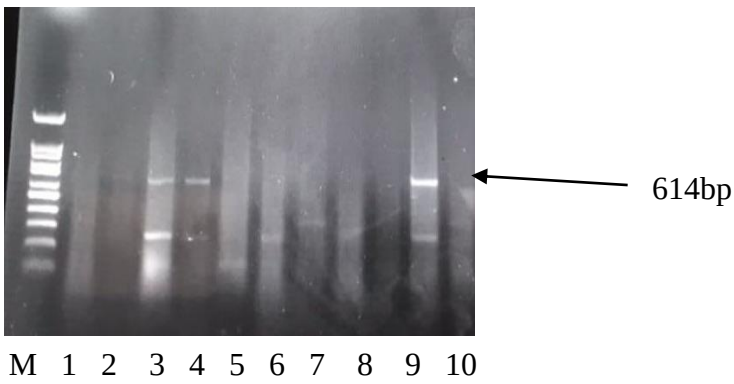

Fig. 4. Agarose gel electrophoresis of amplified products of Stx 1 genes in the bacteria isolates.

4. Conclusion

It can be concluded that the occurrence of hylA and stx 1 genes present in bacteria isolated from the food samples calls for public health attention. This signifies the state of the hygiene involved during the preparations of these RTEs. Hence, basic trainings on food hygiene towards the prevention of cross contamination during food processing and sale of RTEs is necessary and needful. In addition, the misuse of antibiotics should be curbed in order to prevent the spread of antibiotic resistance as observed in this study. More so, since RTEs are basically sold in commercial places, adequate sanitary measure should always be made available.

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