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Full Length Research Paper

Analysis of selected Enterobacteriaceae for extended spectrum beta-lactamase (ESBL) production and virulence factors associated with fruit samples

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Virulence factors (such as serum resistance, cell surface hydrophobicity, haemolysin, and gelatinase production) and extended spectrum β lactamase were determined for the Gram negative bacilli isolated from ready-to-eat fruit samples comprising of *Ananas comosus, Carica papaya*, *Citrus sinensis* and *Citrullus lanatus*. The Enterobacteriaceae which include *Klebsiella oxytoca* (31.7%), *Klebsiella pneumonia* (22.0%), *Proteus mirabilis* (14.6%), *Enterobacter gergoviae* (13.4%), *Proteus vulgaris* (3.7%), *Pantoea agglomerans* (3.7%), *Hafnia alvei* (3.7%), *Enterobacter aerogenes* (2.4%), *Citrobacter sakazaki* (1.2%), *Citrobacter freundii* (1.2%), *Salmonella arizonae* (1.2%) and *Serratia marcescens* (1.2%) respectively were characterized using Microgen GNA ID kit. The haemolysin production analysis showed that 96.6% of *Enterobacteriaceae* produced cytolytic protein toxins (α-haemolysin), gelatinase (81.7%), serum resistance (87.9%), cell surface hydrophobicity (92.4%) and 95.8% were resistant to cefotaxime and ceftazidime which implies that such organisms are ESBL producers. The research showed that consumption of ready-to-eat fruits processed and sold by unlicensed vendors with poor education levels and untrained in food hygiene may increase the risk of food-borne diseases.

Key words: Serum resistance, haemolysin, extended spectrum β lactamase, fruit, enterobacteriaceae.

INTRODUCTION

Ready-to-eat fruits are fruits bought directly from street vendors, hawkers or at local markets and consumed immediately that is without necessarily having to cut, peel or rinse before consumption as they have already been prepared by the vendors (Eni et al., 2010). Over the last few years, there has been a significant increase in the consumption of sliced/ ready-to-eat fruits in Nigeria because they are easily accessible, convenient and most importantly cheaper than whole fruits (Oranusi and Olorunfemi, 2011). In the food matrix, microbes grow in a compact ecosystem in which they can exploit metabolites produced by other microbes (metabiosis) (Gram et al., 2002). Growth and metabolism of spoilage and pathogenic bacteria produce compounds such as secondary metabolites that can affect the quality of foods, causing either organoleptical, structural, chemical or microbiological changes in the products (Gram et al., 2002). These compounds include volatile compounds, proteolytic and lipolytic enzymes, toxins and biogenic amines (Rokka et al., 2004).

In addition to enteric pathogens, some food spoilage *Enterobacteriaceae* are opportunistic pathogens capable of causing disease in conditions favourable for their growth. Contamination from process surface biofilms is an effective route for food contamination by pathogens or spoilage microbes. Beta-lactamase is an enzyme located in the periplasm on the outer surface of the inner membrane of the cell envelope (Ghuysen et al., 1991). The production of penicillinases and other β-lactamase is the principal mechanism of resistance to β-lactam antibiotics among members of the Enterobacteriaceae family (Bush et al., 2001). Thus, increased consumption rate of ready-to-eat fruits coupled with the associated risk of disease to which consumers may be exposed, is a matter of great concern because ready-to-eat fruit street vending is done without adequate storage conditions, thereby exposing the fruits to flies and other disease-causing agents. The aim of the research work was to

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screen some selected Enterobacteriaceae for extended spectrum beta-lactamase (ESBL) production, phenotypic confirmation of ESBL production through double disk synergy test and detection of virulence factors such as cell surface hydrophobicity using the salt aggregation test (SAT), bacterial serum resistance, gelatinase and haemolysin production.

MATERIALS AND METHODS

Isolation and characterization of Enterobacteriaceae

A total of fifty ready-to-eat fruit samples comprising of *Ananas comosus* (pineapple), *Carica papaya* (pawpaw), *Citrus sinensis* (orange) and *Citrullus lanatus* (watermelon) obtained from five retail outlets in Osogbo, Osun state were analyzed for presence of pathogenic bacteria. The characterization was based on the biochemical characteristics, colonial and cellular morphologies. Colonial morphology such as shape, size, colour, edge, elevation and surface texture of the colonies were observed macroscopically. The cellular morphology of the isolates was determined through Gram staining. Different biochemical tests such as catalase, indole production, oxidase, citrate utilization etc. were carried out using Microgen GNA ID kit (Microbiology International, Frederick, USA).

Detection of virulence factors

Haemolysin production

Cytolytic protein toxins (alpha haemolysin) produced by most haemolytic bacteria were assayed by culturing on 5% sheep blood agar at 37°C for 24 h. Haemolysin production was detected by the presence of complete heamolytic zone around the colony (Sharma et al., 2007).

Gelatinase production

Gelatinase production was assayed for by inoculating test organisms on Gelatin agar and incubated at 37°C for 24 h; the culture was flooded with mercuric chloride solution, thus development of opacity in the medium and a zone of clearance around the bacterial colonies were considered positive for the presence of gelatinase (Doughari et al., 2010).

Cell surface hydrophobicity test

Salt Aggregation Test (SAT) according to Sharma et al. (2007) was utilized to determine the cell surface hydrophobicity of the bacterial isolates. 10 µl of bacterial suspension was prepared using 1 ml of phosphate buffer (pH 6.8) and a loopful of the suspension was mixed with equal volumes of ammonium sulphate solution of different molarities (1.4, 2.0, and 4.0 M) on a glass slide. The mixture was rotated carefully for 1min. and microscopically observed for agglutination. The highest 4.0 M dilution of ammonium sulphate solution having a visible agglutination of bacteria was categorized as the SAT value. Bacterial suspension agglutinating at the lowest dilution (1.4 M) was considered auto aggregative, while those with SAT values of ≤ 2.0 M were considered hydrophobic.

Bacterial serum resistance assay

Bacterial suspension prepared in Hank's Balanced Salt Solution (HBSS) which composed of sodium chloride (0.89 mg/L), potassium chloride (0.04 mg/L), potassium phosphate monobasic (0.06 mg/L), glucose (0.10 mg/L), phenol red (0.001 mg/L), sodium phosphate dibasic (0.048 mg/L), magnesium sulfate (0.098 mg/L), calcium chloride (0.014 mg/L) and sodium bi-carbonate (0.035 mg/L) was used. Equal volume (0.05ml) of the bacterial suspension and serum were mixed in a test tube and incubated at 37°C for 180 min. Viable count (%) was determined by calculating the difference in absorbance value obtained at 600 nm before and after incubation. Resistance of bacteria to serum bactericidal activity was expressed as the percentage of bacteria survival after 180 min of incubation with serum in relation to the original count (count at 0min). Bacteria were termed serum sensitive if viable count dropped to 1% of initial value and resistant if > 90% of organisms survived after 180 min of incubation (Sharma et al., 2007).

Screening of the isolates for extended spectrum beta lactamase production

Isolates were screened for extended spectrum beta lactamase production using disc dilution method according to the recommendation of CLSI (2005). Ceftazidime (30 µg) and cefotaxime (30 µg) were placed on Muller Hinton agar plates previously inoculated with test organism and incubated at 37°C for 18 h. After incubation, ESBLS production was determined by the appearance of zone of inhibition (≤ 22 mm for ceftazidime and ≤ 27 mm for cefotaxime) against the bacteria (Sharma et al., 2007).

Phenotypic confirmation of ESBL production

Bacterial suspension of 0.1 ml equivalent to 0.5 McFarland turbidity standards was spread on Muller Hinton agar and a combined disc containing amoxicillin (20 µg) and clavulanic acid (10 µg) was placed at the centre of the petri dish while ceftazidime (30 µg) and cefotaxime (30 µg) were placed 15 mm to the centre, incubated at 37°C for 18 to 24 h. An enhanced zone of inhibition (synergy, regardless of size) between any one of the beta-lactam disc compared to the combined amoxicillin - clavulanic acid disc was considered to be

Table 1. Biochemical Characterization of Enterobacteriaceae isolated using Microgen GN A ID kit.

Ox	Nt	Ls	Ot	H_2S	Gl	Mt	O.N.P.G	In	Ur	VP	Ct	Xl	T.D.A	Species
-	+	+	+/-	-	+	-	+	+	+	+	+	+/-	+	*Klebsiella oxycota*
-	+	+	-	-	+	+	-	-	+	+	+	+	+/-	*Klebsiella pneumonia*
-	+/-	+	+	+	+	-	-	-	+	+/-	+	+/-	+/-	*Proteus mirabilis*
-	+/-	+	-	+	-	-	-	+	+	-	+/-	+/-	+	*Proteus vulgaris*
+/-	+	-	-	+	-	-	-	+	+	-	+/-	+/-	+	*Pantoea agglomerans*
-	+	+	+	-	+	+	+	-	-	+	+	+	+/-	*Enterobacter gergoviae*
+	+	+	+	-	+	+	+	-	-	+	+	+	+/-	*Enterobacter aerogenes*
-	+	-	-	+	+	+	+	-	+	-	+	+	+	*Citrobacter freundii*
+	+	-	+	+	+	+	+	-	-	+	-	+	-	*Citrobacter sakazaki*
-	+	+	+/-	+	+/-	+/-	+	-	-	+	-	+	+/-	*Hafnia alvei*
-	+	+	+	+	+	+	+	+	+	+	-	+	+	*Salmonella arizonae*
-	+	+	+	+	+	+	+	+	+	+	+	-	+	*Serratia marcescens*

Legend: Ox = Oxidase, VP = Voges-Proskauer, Ls = Lysine, Ct = Citrate utilization, H_2S = Hydrogen sulphide, Nt = Nitrate, Gl = Glucose, Ot = Ornithine decarboxylase**,** Mt = Mannitol, T.D.A= Tryptophan deaminase, O.N.P.G. = *beta*-galactosidase, Nt = Nitrate**,** Id = Indole, Ur = Urease, + = Reactive**,** - = Non-reactive.

Table 2. Incidence rate of *Enterobacteriaceae* isolated from ready-to-eat fruits.

Isolates	Number of strains	Percentage (%)
Citrobacter fruendii	01	1.2
Citrobacter sakazaki	01	1.2
Enterobacter aerogenes	02	2.4
Enterobacter gergoviae	11	13.4
Hafnia alvei	03	3.7
Klebsiella pneumonia	18	22.0
Klebsiella oxytoca	26	31.7
Pantoea agglomerans	03	3.7
Proteus mirabilis	12	14.6
Proteus vulgaris	03	3.7
Salmonella arizonae	01	1.2
Serratia marcescence	01	1.2
Total	82	100

positive for ESBL enzyme production (Doughari et al., 2010).

RESULTS

Morphological characteristics such as colonial morphology (colony shape, colour and surface appearance) and microscopic morphology (cell shape and arrangement) were done according to the Bergeys Manual of Determinative Bacteriology (1994). A total of 82 *Enterobacter*iaceae isolates were obtained based on their biochemical characteristics and identified to specie level using Microgen GN A ID kit (Microgen bioproducts), these include *Klebsiella oxytoca* (31.7%)*, Klebsiella pneumonia* (22.0%)*, Proteus mirabilis* (14.6%)*, Enterobacter gergoviae* (13.4%)*, Proteus vulgaris* (3.7%)*, Pantoea agglomerans* (3.7%)*, Hafnia alvei* (3.7%)*, Enterobacter aerogenes* (2.4%)*, Citrobacter sakazaki*(1.2%)*, Citrobacter freundii* (1.2%)*, Salmonella arizonae* (1.2%) and *Serratia marcescens* (1.2%) (Tables 1 and 2).

Virulence factors such as serum resistance, cell surface hydrophobicity, haemolysin and gelatinase production were determined for the isolates (Table 3). The haemolysin production analysis showed that 96.6% of *Enterobacteriaceae* produced cytolytic protein toxins (α-haemolysin), gelatinase (81.7%), serum resistance (87.9%), cell surface hydrophobicity (92.4%) and 95.8% of the isolates were resistant to cefotaxime and ceftazidime which implies that such organisms are ESBL producers. The ESBL producers obtained were resistant to augumentin (amoxicillin and clavulanic acid), ceftazidime and cefotaxime which implies that the isolates were positive for phenotypic confirmatory test.

DISCUSSION

Ready –to -eat fruits are extraordinary dietary sources of

Table 3. Virulence factors and ESBL analysis of the Enterobacteriaceae.

Parameters analyzed	Percentage of Enterobacteriaceae (%)
Haemolysin production	96.6
Serum resistance	87.9
Cell surface hydrophobicity	92.4
Gelatinase production	81.7
ESBL production	95.8
ESBL Phenotypic confirmatory test	+

Legend: + = All ESBL producers were resistant to augumentin (amoxicillin and clavulanic acid), ceftazidime and cefotaxime, thus there was growth.

nutrients, micronutrients, and vitamins for humans, thus vital for health and well being. Presence of *Klebsiella* species*, Proteus* spp*., Enterobacter* spp*.,* and *Salmonella* spp*.* in the samples analyzed are direct reflection of the sanitary quality of the fruit storage and processing according to the report of Chukwu et al. (2009) and Vantarakis et al. (2011) respectively. Most of these organisms are of intestinal origin which showed inadequate sanitary practices by the fruit vendors. Gram-negative bacteria pathogens isolated also expressed some virulence factors to promote motility, attachment to epithelial or endothelial cell surfaces, avoid host immune responses, activate or inactivate host cellular pathways and ultimately cause clinical disease (Drudy et al., 2006).

Cell surface hydrophobicity enhances the adherence of bacterial cells to host cell surface including mucosal epithelia cells and resistance to phagocytosis by host cells (Costa et al., 2006). It is also a significant determinant of adhesion and biofilm formation on polystyrene surfaces (Pompilio et al., 2008). Expression of cell surface hydrophobic determinant by 92.4% of the isolates shows that effect of antibiotics on the target organisms would not be significant which may eventually lead to tissue destruction by causing soft tissue infection when such fruits are consumed. Production of gelatinase which is an extracellular metalloendopeptidase by 81.7% of the Enterobacteriaceae indicated the ability of the isolates to hydrolyze gelatin and other compounds such as pheromone, collagen, casein and fibrinogen (Makinen and Makinen, 1994). The enzyme plays a very significant role in bacteria pathogenesis by causing direct or indirect damage to host tissue, thus consumption of fruits harboring such microbes will facilitate microbial invasion and survival in the host cell (Alebougeh et al., 2005)

Haemolysin production by 96.6% of pathogens obtained would lead to lysis of the red blood cells, leading to anemia and may result to death of the host. Haemolysis also contribute to tissue injury, survival of renal parenchyma, and entry into the blood stream thus increasing the possibility of establishing acute pyelonephritis (Raksha et al., 2003) because the mode of action of haemolysis involves pore formation on the colonized host cell (Wiles et al., 2008). Serum resistance is the property by which the bacteria resist killing by normal human serum due to the lytic action of complement system thus, gram-negative bacilli (87.9%) that showed serum resistance demonstrated high degree of survival in the blood during bacteraemia (Raksha et al., 2003).

The production of β-lactamase remains the major mechanism of resistance in gram-negative bacilli to β-lactam antibiotics. In recent years, extended-spectrum β-lactamases (ESBLs) have become progressively widespread due to extensive use of third generation cephalosporins in hospital settings (Bradford, 2001). The ESBL producers obtained in the research possessed the ability to hydrolyze β- lactam antibiotics containing an oxyimino group namely augumentin (amoxicillin and clavulanic acid), ceftazidime and cefotaxime which are usually inactivated by β-lactamase inhibitors. Thus, the bactericidal effect of the antibiotics on organisms would not be significant because *Enterobacteriaceae* especially those producing extended spectrum β - lactamases (ESBLs) normally cause various nosocomial infections (Quale et al., 2002).

Summarily, the results obtained in the present study indicate the poor hygienic condition of the fruit vending which implies that the consumers are at risk of contacting food borne infections when ready-to-eat fruits are consumed. Fruit hawkers/vendors in Nigeria are generally unaware of food regulations and have no training in food-related matters thus; there should be awareness amongst the vendors about the safe and hygienic practices; Government agencies and Non Governmental Organizations should establish guidelines for selling of ready-to-eat or precut fruits.

REFERENCES

Alebougeh M, Amirmozafari N, Forohesh H (2005). Evaluation of virulence factors and plasmid related transmissibility among different isolates of *Enterococci*. Iran. Biomed. J., 9(2): 51-55.

Bergeys Manual of Determinative Bacteriology (1994). Retrieved from http://archive.org/stream/bergeysmanualofd1957amer/bergeysmanualofd1957amer_djvu.txt.

Bradford PA (2001). Extended-spectrum beta-lactamases

in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev., 14: 933–951.

Bush K (2001). New ß-lactamases in gram-negative bacteria: diversity and impact on the selection of antimicrobial therapy. Clin. Infect Dis., 32: 1085-1089.

Chukwu OO, Olabode OA, Chukwuedo AA, Umoh EG, Esiekpe MK (2009). Bacteriological evaluation of pre-cut fruits sold in Kano metropolis, Kano State, Nigeria. East Afr. J. Pub. Health, 6(1): 51-54.

CLSI (2005). Clinical and Laboratory Standard Institute. Retrieved from http://isoforlab.com/phocadownload/csli/M45-P.pdf.

Costa GFM, Tognim MCB (2006). Preliminary evaluation of adherence on abiotic and cellular surfaces of *Acinetobacter baumannii* strains isolated from catheter tips. Braz. J. Infect Dis., 10: 346-351.

Doughari JH, Ndakidemi PA, Human IS, Bennade S (2010). Verocytotoxic diarrhogenic bacteria and food and water contamination in developing countries: a challenge to the scientific and health community. Rev. Infect., 1(4): 202-210.

Drudy DO, Rourke M, Murphy M, Mullane NRO, Mahony R, Kelly L, Fischer M, Sanjag S, Shannon P, Wall PO, Mahony M, Whyte P, Fanning S (2006). Characterization of a collection of *Enterobacter sakazakii* isolates from environmental and food sources. Int. J. Food Microbiol., 110: 127-134.

Eni AO, Oluwawemitan IA, Oranusi US (2010). Microbial quality of fruits and vegetables sold in Sango Ota, Nigeria. Afr. J. Food Sci., 4(5): 291-296

Ghuysen JM (1991). Serine beta-lactamases and penicillin-binding proteins. Ann. Rev. Microb, 45: 37-67.

Gram L, Ravn L, Rasch M, Bartholin Bruhn J, Christensen AB, Givskov, M (2002). Food spoilage interactions between food spoilage bacteria. Int. J. Food Microbol., 78: 79- 97.

Makinen P, Makinen KK (1994). The *Enterococcus faecalis* extracellular metalloendopeptidase (EC 3.4.24.30; coccolysin) inactivates human endothelin at bonds involving hydrophobic amino acid residues. Biochem. Biophy. Res. Commun., 200: 981-985.

Oranusi SU, Olorunfemi OJ (2011). Microbiological safety evaluation of street vended ready-to-eat fruits sold in Ota, Ogun state, Nigeria. Int. J. Res. Biol. Sci., 1(3): 22-26.

Pompilio A, Piccolomini R, Picciani C, D'Antonio D, Savini V, Bonaventura GDI (2008). Factors associated with adherence to and biofilm formation on polystyrene by *Stenotrophomonas maltophilia*: the role of cell surface hydrophobicity and motility. FEMS. Microbiol. Lett., 287: 41-47.

Quale JM, Landman D, Bradford PA, Visalli M, Ravishankar J, Flores C, Mayorga D, Vangala K, Adedeji A **(**2002). Molecular epidemiology of a citywide outbreak of extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* infection. Clin. Infect. Dis., 35: 834–841.

Raksha R, Srinivasa H, Macaden RS (2003). Occurrence and characterization of uropathogenic *Escherichia coli* in urinary tract infections. Indian J. Med. Microbiol., 21: 107-120.

Sharma S, Bhat GK, Shenoy S (2007). Virulence factors and drug resistance in *Escherichia coli* isolated from extraintestinal infections. Indian J. Med. Microbiol., 25(4): 369-373.

Vantarakis A, Affifi M, Kokkinos P, Tsibouxi M, Papapetropoulou M (2011). Occurrence of microorganisms of public health and spoilage significance in fruit juices sold in retail markets in Greece. J Anaerobe, 17(6): 288-291.

Wiles TJ, Kulessus RR, Mulevy MA (2008). Origins and virulence mechanisms of uropathogenic *Escherichia coli.* Exp . Mol . Pathol., 85(1).