

Isolation and Genetic characterization of Gram-negative bacteria from different fresh retail vegetables in Nigeria

ABSTRACT

Aim: The proportion of foodborne disease outbreaks because of contaminated produce has increased over the years. In this study, the genetic characteristics of antibiotic resistant Gram-negative bacteria from different fresh retail vegetables in Okada, Edo state Nigeria was investigated.

Place and Duration of Study: In April-May 2021, the study was carried out in the Department of Pharmaceutical Microbiology, Igbinedion University Okada Edo state Nigeria.

Methodology: One hundred and eight isolates were isolated from sixteen different retail leafy and salad vegetable samples. Recovered isolates from samples were identified using standard microbiological techniques. Species identification for ten randomly selected isolates was performed by Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and ribosomal multilocus sequence typing (rMLST). Antimicrobial susceptibility testing was performed using the Kirby-Bauer method for 15 antibiotics. Isolates were characterized by whole genome sequencing (WGS).

Results: Species identification using MALDI-TOF-MS and ribosomal MLST assigned the 10 randomly selected isolates to four different species. Identified isolates include *Proteus mirabilis*, *Proteus vulgaris*, *Acinetobacter baumannii* and *Klebsiella quasipneumoniae*. Out of the 10 randomly selected isolates, 60% (6/10) were antibiotic resistant in the antibiotic susceptibility test. WGS data confirmed the identities of the isolates except *Proteus vulgaris* identified as *P. terrae*. More than one resistant determinant was detected on the draft genome sequence of 80% (8/10) of the randomly selected isolates especially the regulatory system modulating antibiotic efflux CRP and the plasmid mediated quinolone resistant determinant *qnrD1*. Significantly, one *Proteus mirabilis* isolate was sensitive to the antibiotics in the phenotypic testing but had resistance determinants present.

Conclusion: This study provides genomic characterization of antibiotic resistant isolates from retail leafy and salad vegetables from Nigeria. Further study is important to understand the public health importance of such resistance and the amount of risk posed to human health by these resistant organisms.

Keywords: whole genome sequencing, Gram-negative bacteria, antibiotic resistance, retail vegetables

1. INTRODUCTION

“Vegetables include the leafy, fruit, seed or root vegetables that are edible for human consumption. These parts are edible as completely or in part, raw or cooked as a supplement to other food crops” [1]. “Vegetables are highly beneficial food that protect the body and are important for the maintenance of good body health. In contrast to their health benefits, the

consumption of fresh vegetables has also been associated to risk for consumers” [2]. “Outbreaks of food infections associated with consumption of ready-to-eat vegetables have been on the increase, which has been linked epidemiologically to the consumption of a wide range of these vegetables. In Nigeria, vegetables are consumed raw or blanched to retain its organoleptic properties that may result in food– borne infections. In the process of planting and harvesting or storage, disease-causing microorganisms may contaminate these vegetables through contact with natural wastes such as fecal matter, sewage and other organic wastes that are used as humus to aid the growth process of the vegetables” [3]. “Water has been reported as one of the possible principal agents for the spread of various enteric diseases because of consuming contaminated vegetables” [1]. “The process of transporting these food products from the farm to households also contributes to the contamination of these vegetables, thus posing a serious problem in food safety” [2]. “Previous reports has shown various pathogens that include; *Escherichia coli*, *Salmonella spp.*, *Listeria monocytogenes*, *Aeromonas spp.*, *Staphylococcus spp.*, *Streptococcus spp.*, *Vibrio spp.* and *Pseudomonas spp* to be associated with the contamination of vegetables causing different outbreaks of gastroenteritis” [4, 5, 6]. “In developing countries, food borne illness caused by contaminated vegetables are frequent but rarely properly documented and reported due to many reasons such as poor diagnostic facilities and lack of food borne disease investigation and surveillance”[7]

“Several microorganisms isolated from vegetables have been reported to be resistant to several antibiotics” [8,3, 9, 10,11,12]“hence have increased the effective prevention and treatment plan of an ever expanding range of infections. A previous study isolated *Shigella spp.* from vegetables which was susceptible to ofloxacin and amoxicillin, intermediate resistant with nalidixic acid and resistant to tetracycline, nitrofurantoin, gentamycin and augmentin” [1]. “*E. coli* isolated from samples was resistant to all test antibiotics except amoxicillin. *Salmonella* showed intermediate reaction to gentamycin, was susceptible to tetracycline and resistant to cotrimoxazole, cloxacillin, erythromycin, augmentin, streptomycin and chloramphenicol” [1]. “Another study reported most of the *Citrobacterspp* isolated from fruits and vegetables sold in Ile-Ife, Nigeria were resistant to cefotaxime and ceftriaxone” [10]. “This pose a threat to safety of foods and the consumers resulting in a global threat as new resistant mechanisms emerge and rapidly spread across the globe” [10]. In Nigeria, there exist various reports on the susceptibility of isolates from retail vegetables with little/no information about their genetic characterization. Genetically characterizing microorganisms using whole genome sequencing creates a platform to gain knowledge and understanding of infectious diseases and clinical microbiology. It provides a detailed knowledge for strain characterization and epidemiological analyses [13]. This study was designed to evaluate the microbial quality, the antibiotic resistance pattern of gram-negative bacteria isolates from retail leaf and salad vegetables found in Okada market Edo state Nigeria and to characterize the isolates using whole genome sequencing.

2. MATERIALS AND METHODS

2.1 Study site and sample collection

In April-May 2021, one hundred and eight isolates were isolated from sixteen different retail leaf and salad vegetable samples. Okada, a rural community in the headquarters of Ovia North East Local Government area of Edo State, Nigeria was the study site where samples were obtained. The estimated population is 155,344 people comprising of farmers, lecturers, civil servants, students and children. The main market was the selected choice of study area. The vegetables were randomly obtained from retail sellers and include fluted pumpkin leaf

(*Telfairia occidentalis*), waterleaf (*Talinum triangulare*), scent leaf (*Ocimum gratissimum*), uziza leaf (*Piper guineense*), green leaf (*Desmodium intortum*), jute leaf (*Corchorus olitorius*), carrots (*Daucus carota*), green beans (*Phaseolus vulgaris*), cabbage (*Brassica oleracea var. capitata*) and green peas (*Pisum sativum*). All samples were collected in a sterile ziploc bag and transported immediately to the laboratory for microbiological analysis.

2.2 Isolation of Bacteria

The method of Halablab et al., 2011 was used with slight modifications [14]. Fifty gram (50g) of each vegetable sample was weighed and dissolved in 150ml of normal saline (0.9% NaCl) for about 10 minutes. A serial dilution of five different strengths ranging from 10^{-1} to 10^{-5} was prepared using sterile distilled water. Dilution 10^{-2} and 10^{-5} were used for the study. 1ml aliquot from each corresponding samples was inoculated into different agar bottles; Macconkey agar (Oxoid, Hampshire, United Kingdom), Salmonella Shigella agar (Oxoid, Hampshire, United Kingdom), Thiosulfate-citrate-bile-salts-sucrose media (Oxoid, Hampshire, United Kingdom) and poured on different plates using the seeded plate technique. "The inoculated plates were incubated at 37°C for 24 hours. Distinct colonies were randomly obtained from the selective agar plates and sub cultured on nutrient agar plates to obtain pure colonies. Isolates were identified using standard microbiological techniques" [15]. Biochemical tests carried out included Indole, Urease, VP, Citrate and Methyl red. Identities of twelve randomly obtained isolates were further confirmed by MALDI-TOF mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany) analysis.

2.3 Antibiotic Susceptibility Testing

"The Kirby-Bauer susceptibility testing technique was carried out and results were interpreted using European Committee on Antimicrobial Susceptibility Testing, (EUCAST) criteria" [16, 17]. Antibiotics used in testing were clinically relevant and obtained based on selection criteria. The isolates were tested with 15 antibiotics: meropenem, ertapenem, cefotaxime, amoxicillin/clavulanic acid, ceftazidime, cefpodoxime, piperacillin/tazobactam, ceftiofuran, cefepime, tigecycline, ciprofloxacin, amikacin, ampicillin, cefuroxime, gentamicin (Oxoid, Basingstoke Hampshire, UK). The *Acinetobacter baumannii* isolate was tested with 5 relevant antibiotics: meropenem, tobramycin, amikacin, ciprofloxacin and trimethoprim/sulfamethoxazole. The selected resistant isolates were subjected to whole genome sequencing for characterization of resistant isolates.

2.4 Whole genome sequencing

"High-molecular-weight DNA from the 10 bacterial overnight cultures was isolated using a MagAttract HMW DNA kit (Qiagen, Hilden, Germany). DNA was quantified using DropSense 16 (Trinean NV/SA, Gentbrugge, Belgium). Library for WGS was prepared with a NexteraXT kit (Illumina, Inc., San Diego, CA, USA) according to manufacturer's instructions and a 300-bp paired-end sequencing run was performed on an Illumina MiSeq instrument using the MiSeqV3 reagent kit (Illumina Inc., San Diego, CA, USA) for the 10 bacterial isolates. Raw reads were de novo assembled into draft genomes using SPAdes version 3.15.2" [18]. "Species identification via MALDI-TOF MS was confirmed using ribosomal multilocus sequence typing (rMLST) (<https://pubmlst.org/species-id>). Assembled genomes were uploaded to the Comprehensive Antibiotic Resistance Database-Resistance Gene Identifier (CARD-RGI)" [19] to identify antimicrobial resistance genes. ARGs were identified based on a minimum cutoff of 98% nucleotide identity for perfect or strict hits predicted by RGI.

2.4.1 Nucleotide Sequence Accession Numbers

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession numbers JANFNI000000000-JANFNR000000000. The version described in this paper is version JANFNI010000000-JANFNR010000000.

3. RESULTS AND DISCUSSION

3.1 Phenotypic characteristics of the isolates

A total of one hundred and eight isolates were isolated from sixteen different retail leafy and salad vegetable samples obtained from retail sites in Okada market, Edo state Nigeria. Identities of ten randomly obtained isolates on MacConkey agar were pink for the lactose fermenters and colourless for the non-lactose fermenters. Species identification using MALDI-TOF-MS and ribosomal MLST assigned the 10 randomly selected isolates to four different species. Identified isolates include *Proteus mirabilis* (n=6), *Proteus vulgaris* (n=2), *Acinetobacterbaumannii* (n=1) and *Klebsiellaquasipneumoniae* (n=1).

3.2 Antibiotic Susceptibility testing of the isolates

Out of the 10 randomly selected isolates, 60% (6/10) were antibiotic resistant in the antibiotic susceptibility test. Supplementary table 1 represents the antibiotic susceptibility pattern of the isolates. One of the *P. mirabilis* (10%) isolates was resistant toertapenem, ciprofloxacin, cefotaxime and ceftazidime. Another *P.mirabilis*(10%)and two *P. vulgaris* (20%) isolates were resistant to ertapenem and ciprofloxacin respectively.The *Acinetobacterbaumannii* isolate was resistant only to meropenem while the *Klebsiellaquasipneumoniae* was resistant only to the tigecycline antibiotic. Intrinsic resistance of the isolates to antibiotics were not considered.

3.3 Antimicrobial resistance genes of the recovered isolates

WGS data identified *P.vulgaris* as *P.terrae*. More than one resistant determinant was detected on the draft genome sequence of 80% (8/10) of the randomly selected isolates especially the regulatory system modulating antibiotic efflux CRP and the plasmid mediated quinolone resistant determinant *qnrD1* among the *Proteus spp* (Table 1). Other resistance genes detected in the recovered isolates include beta-lactamase genes *bla*_{OXA-106}, *bla*_{ADC-76}. The regulatory system modulating antibiotic efflux (Resistance-Nodulation-Division multidrug efflux pumps), small multidrug resistance (SMR) antibiotic efflux and resistance determinants that encode multidrug efflux pumps of the major facilitator superfamily were also detected. Other resistance determinants in the *Acinetobacterbaumannii* isolate include an efflux pump mediating quinolone resistance *AbaQ*, an efflux pump of the small multidrug resistance family of transporters *AbeS*, fosfomycin efflux major facilitator superfamily transporter *AbaF*, *Acinetobacterbaumannii* *parC* conferring resistance to fluoroquinolones and the *Acinetobacterbaumannii* disinfectant resistance protein *AmvA*. The regulatory systems, *adeN* and *adeL*, modulating antibiotic efflux (Resistance-Nodulation-Division multidrug efflux pumps) was also detected in the *Acinetobacterbaumannii* isolate. Resistance determinants in *Klebsiellaquasipneumoniae* isolate include the major facilitator superfamily antibiotic efflux pump, *KlebsiellapneumoniaeKpnG*, *KlebsiellapneumoniaeacrR* with mutation conferring multidrug antibiotic resistance and the phosphoethanolamine transferase *epfB* that confers resistance to peptide antibiotics. Significantly, one *Proteus mirabilis* isolate was sensitive to the antibiotics in the phenotypic testing but had resistance determinants CRP, *QnrD1* present.

Table 1 Antimicrobial resistance genes of the recovered isolates.

ID No	Isolate	Phenotypic resistance profile	Antimicrobial resistance determinants detected
510504	<i>Acinetobacterbaumannii</i>	MEM	<i>Acinetobacterbaumannii</i> AbaQ, QnrD1, <i>Acinetobacterbaumannii</i> AbaF, OXA-106, <i>adeN</i> , ADC-76, <i>adeH</i> , <i>adeF</i> , <i>adeL</i> , <i>abeS</i> , <i>Acinetobacterbaumannii</i> AmvA, <i>LpsB</i> , <i>adeJ</i> , <i>AcinetobacterbaumanniiparC</i> conferring resistance to fluoroquinolones.
510494	<i>Proteus terrae</i>	CIP	CRP
510495	<i>Proteus terrae</i>	CIP	CRP, QnrD1, <i>Mycobacterium tuberculosis</i> intrinsic <i>murA</i> conferring resistance to fosfomycin
510490	<i>Proteus mirabilis</i>	CTX,CAZ,ETP,CIP	CRP
510493	<i>Proteus mirabilis</i>	ETP	CRP, QnrD1
510496	<i>Proteus mirabilis</i>	-	CRP, QnrD1
510497	<i>Proteus mirabilis</i>	-	CRP, QnrD1
510498	<i>Proteus mirabilis</i>	-	CRP, QnrD1
510500	<i>Proteus mirabilis</i>	-	CRP, QnrD1
510506	<i>Klebsiellaquasipneumoniae</i>	TGC	<i>epfB</i> , CRP, <i>Klebsiellapneumoniae</i> KpnG, <i>LptD</i> , <i>OmpA</i> , <i>KlebsiellapneumoniaeacrR</i> with mutation conferring multidrug antibiotic resistance

Key:MEM-Meropenem,CIP-Ciprofloxacin, CTX-Cefotaxime, CAZ-Ceftazidime, ETP-Ertapenem, TGC-Tigecycline

“This study showed the presence of microbial contamination present in retail leaf and salad vegetables obtained and identified isolates are pathogenic and antimicrobial resistant.Fruits and

vegetables cultivated and harvested on the surface or in the soil are usually contaminated because of contact with soil, manure, irrigation water, waste, and animal discharges" [20]. There are several other previous reports on the microbial contamination of vegetables [21,22,23, 24,25, 10]. "A previous report from the region of North Africa reported Gram-negative bacteria resistant to third generation Cephalosporins in Fruits and Vegetables"[20]. The study showed fruits and vegetables to constitute a reservoir of third generation cephalosporin resistant Gram-negative bacteria and multi-drug resistant bacteria that can be transferred to humans through food. This correlates with results obtained from this study as a multidrug resistant *Proteus mirabilis* isolate was detected which had ceftazidime and cefotaxime as part of the drugs it was resistant to. "Another previous Nigerian study that isolated and molecularly characterized *Citrobacter* species in fruits and vegetables reported the samples to be contaminated with multiple antibiotic resistant potential *Citrobacter* species capable of causing food-borne disease in consumers" [10]. In this study the resistance of the isolates to broad spectrum antibiotics like ertapenem, meropenem, ceftazidime, cefotaxime and other clinically relevant antibiotics like ciprofloxacin and tigecycline is of great concern. "These antibiotics for example ertapenem, meropenem and tigecycline are useful for managing multidrug resistant bacterial infections. They are used alone or in combinations as reserve antibiotics to treat fatal bacterial infections [26]. However, reports of resistance to these last resort antibiotics have been on the increase which correlates with the result obtained from this study" [27]. "The high occurrence of *Proteus spp* among the isolates analyzed in this study also correlates with a previous study on the occurrence of human pathogens in spinach and tomato that detected the presence of *Proteus mirabilis*. Analyses of resistance of the isolates to antibiotics revealed resistance to penicillins G, ampicillin, cephalosporin (cefoxitin), amoxiclav, and Sulfametoxazole-trimethoprim"[22] Multiple strains of the bacteria isolates recovered from samples might be a reflection of poor handling during the period of harvest, transportation, unhygienic handlers and storage [28]. Prolong exposure in the market place, use of organic manure as fertilizers, improper washing/rinsing of the vegetables and the use of contaminated water for irrigation are also possible sources of contamination of the vegetables [29]. The high rate of contamination of the vegetable samples with microbes is a call for concern as in most cases these vegetables are consumed raw or slightly cooked to preserve the taste and their nutritional contents. The implication of this is that consumptions of unhygienic raw vegetables could be a source of resistance and virulence genes from antimicrobial resistant isolates for both humans and other flora. "Also, consumers of unhygienic raw vegetables could get infected with diseases that are difficult to treat. A previous report show that more than 90% of food poisoning cases each year are bacteria which include *Staphylococcus aureus*, *Salmonella*, *Clostridium perfringes*, *Clostridium botulinum*, *Campylobacter*, *Vibrio parahaemolyticus*, *Bacillus cereus* and enteropathogenic *Escherichia coli* commonly found in many raw foods" [24]. Significantly, isolates identified in this study, which include *Proteus spp*, *Acinetobacter baumannii* and *Klebsiella quasipneumoniae* are organisms that have are scarcely reported previously to be isolated from retail vegetables. "This confirms a previous report that pathogenic bacteria species, which include but not limited to species of *Salmonella*, *Shigella*, *Escherichia coli*, *Escherichia coli* O157:H7, *Clostridium botulinum*, *Campylobacter*, *Listeria monocytogenes* and *Bacillus cereus* can contaminate vegetables" (30). "The presence of resistance genes like beta-lactamase genes *bla*_{OXA-106}, quinolone resistance determinants *qnrD1*, the regulatory system modulating antibiotic efflux CRP, regulatory system modulating antibiotic efflux (Resistance-Nodulation-Division multidrug efflux pumps), and resistance determinants that encode multidrug efflux pumps of the major facilitator superfamily in the resistant isolates is of great concern.. This slightly correlates with some previously reported studies"[8, 10, 31, 32]. One of the resistance determinants that occurred

frequently in this study are the active efflux systems. Active efflux systems in bacteria have been reported to carry out an important role in acquired multidrug resistance (MDR) [33]. Resistance-nodulation-cell division (RND) pumps for example among the efflux systems are the most prevalent systems in Gram-negative bacteria [33]. Another mechanism of resistance that occurred frequently is the plasmid mediated quinolone resistance mechanism, It constitute the second most important group of quinolone resistance mechanisms. Plasmid mediated quinolone resistance are based on: Qnr proteins, which protect the quinolone targets; the enzyme Aac(6')-Ib-cr, which acetylates aminoglycosides, ciprofloxacin, norfloxacin; and efflux pumps, QepA or OqxAB which decrease the intracellular concentration of quinolones.[34] Phenotypic characterization of bacteria like antimicrobial studies is very important for therapeutic purposes, but further genetic characterization may a times seems appropriate. Whole-genome sequencing is important in enhancing our understanding of how bacteria evolve, are transferred, and monitoring of antimicrobial resistance [35]. This study suggests that the vegetables are a less appreciated source of clinically important resistant organism. Significantly, one *Proteus mirabilis* isolate was sensitive to the antibiotics in the phenotypic testing but had resistant determinant *qnrD1* and the regulatory system modulating antibiotic efflux CRP present. The importance of genotypic characterization of isolates cannot be overemphasized. The presence of these resistance determinants in the isolates is a real threat to public health as they are significant determinants of resistance that are transmissible especially when they are located on mobile elements like plasmids, integrons, transposons etc [10, 11]. This will certainly pose a significant challenge in man or animal, thus causing a wide range of infectious diseases that are difficult to treat.

4. CONCLUSION

This study provides genomic characterization of isolates from retail leafy and salad vegetables from Nigeria. The isolation of these bacteria species with the various resistance determinants indicates the need for continuous surveillance of antibiotic resistance from vegetables/food samples in the developing world with the view of effective rationalization of antibiotics in clinical systems. Further study is important to understand the public health importance of such resistance and the amount of risk posed to human health by these resistant organisms. In addition, farmers and vendors of these vegetables should implement proper environmental hygiene until they reach the final consumers. Vegetables should be properly washed before consumption especially where they are not going to be cooked before consumption. Safe and clean wash water for the vegetables is advocated among the rural areas rather than the use of untreated and unsafe water that will negatively affect the community health. Irrigation water supplies should also be properly screened before use. Application of good cooking practices and adequate food hygiene measures is also important for the prevention of food-borne pathogens in cooked vegetables hence the regulatory authorities should educate the vendors and consumers on good sanitary practices during processing, display and retail sale of vegetables. These hygienic practices could possibly eliminate/reduce minimally contaminations and antibiotic resistance gene hosting and transfers.

COMPETING INTERESTS

Authors declare that no competing interests exists

AUTHORS' CONTRIBUTIONS

C. Jesumirhewe designed the study, wrote the protocol, and wrote the first draft of the manuscript. All Authors were involved in the project and managed the analyses of the study. C. Jesumirhewe, A.A Dauda and S.I Akhimien managed the literature searches. All authors read and approved the final manuscript.

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