

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

ABSTRACT

Aim: 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 MALDI-TOF and ribosomal MLST analysis. Antimicrobial susceptibility testing was performed using the Kirby-Bauer method for 15 antibiotics. Isolates were characterized by whole genome sequencing.

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 resistant determinants present.

Conclusion: This study provides genomic characterization of antibiotic resistant isolates from retail leafy and salad vegetables from Nigeria. These results suggest that the vegetables may be a less appreciated source of clinically important resistant organism. 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. 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 [2]. 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. Previous reports have 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 [3, 4, 5]. 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 [6].

Several microorganisms isolated from vegetables have been reported to be resistant to several antibiotics [7, 2, 8, 9, 10, 11] hence have increased the effective prevention and treatment plan of an ever expanding range of infections. This poses 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. In Nigeria, there exists various reports on the susceptibility of isolates from retail vegetables with little/no information about their genetic characterization. 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 obtained 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

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, salmonella shigella agar, 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 [12]. 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 [13] was carried out and results were interpreted using European Committee on Antimicrobial Susceptibility Testing, (EUCAST) criteria [14]. The isolates were tested with 15 antibiotics: meropenem, ertapenem, cefotaxime, amoxicillin/clavulanic acid, ceftazidime, cefpodoxime, piperacillin/tazobactam, cefoxitin, 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 MiSeq V3 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 [15]. 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) [16] 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), *Acinetobacter baumannii* (n=1) and *Klebsiella quasipneumoniae* (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 to ertapenem, ciprofloxacin, cefotaxime and ceftazidime. Another *P. mirabilis* (10%) and two *P. vulgaris* (20%) isolates were resistant to ertapenem and ciprofloxacin respectively. The *Acinetobacter baumannii* isolate was resistant only to meropenem while the *Klebsiella quasipneumoniae* was resistant only to the tetracycline 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* (Table 1). Significantly, one *Proteus mirabilis* isolate was sensitive to the antibiotics in the phenotypic testing but had resistant determinants CRP, *QnrD1* present.

Table 1 Antimicrobial resistance genes of the recovered isolates.

ID No	Isolate	Phenotypic resistance profile	Antimicrobial resistance genes detected
510504	<i>Acinetobacter baumannii</i>	MEM	<i>Acinetobacter baumannii</i> <i>AbaQ</i> , <i>QnrD1</i> , <i>Acinetobacter baumannii</i> <i>AbaF</i> , OXA-106, <i>adeN</i> , ADC-76, <i>adeH</i> , <i>adeF</i> , <i>adeL</i> , <i>abeS</i> , <i>Acinetobacter baumannii</i> <i>AmvA</i> , <i>LpsB</i> , <i>adeJ</i> , <i>Acinetobacter baumannii</i> <i>parC</i> conferring resistance to fluoroquinolones.
510494	<i>Proteus terrae</i>	CIP	CRP
510495	<i>Proteus terrae</i>	CIP	CRP, <i>QnrD1</i> , <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, <i>QnrD1</i>

510496	<i>Proteus mirabilis</i>	-	CRP, <i>QnrD1</i>
510497	<i>Proteus mirabilis</i>	-	CRP, <i>QnrD1</i>
510498	<i>Proteus mirabilis</i>	-	CRP, <i>QnrD1</i>
510500	<i>Proteus mirabilis</i>	-	CRP, <i>QnrD1</i>
510506	<i>Klebsiella quasipneumoniae</i>	TGC	<i>eptB</i> , CRP, <i>Klebsiella pneumoniae KpnG</i> , <i>LptD</i> , <i>OmpA</i> , <i>Klebsiella pneumoniae acrR</i> with mutation conferring multidrug antibiotic resistance

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

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. 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 [17]. In Nigeria, there are several other previous reports on the microbial contamination of vegetables [18, 19, 20, 21, 22, 8]. 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 [23]. 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 [24]. 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 [21]. 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 (25). Antibiotic-resistant

bacteria contamination in the fresh vegetables have been previously reported [7, 9, 26, 27]. 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 suggests that the vegetables may be 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 [9, 10]. 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 resistant 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. 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 in order to eliminate/reduce minimally contaminations and antibiotic resistance gene transfers. 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.

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UNDER PEER REVIEW