Phenotypic Identification and Molecular Characterization of *Citrobacter* sp Isolated from Commercial Fruits and Vegetables in Ile-Ife, Nigeria.

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ABSTRACT

This study investigated antibiotic susceptibility profile, resistance plasmid DNA as well as resistance and virulence genes of *Citrobacter* species isolated from fruits and vegetables. This was with a view to determining the safety of fruits and vegetables sold commercially in Ile-Ife market.

One hundred and fifty (150) fruits and 90 vegetables samples were purchased at Iso-Obi in Sabo, Ile Ife. Each sample was washed with sterile distilled water and serially diluted. The pre-cut of the fruits was homogenized using a sterile mortar and pestle, while fruits with juice were squeezed mechanically to extract the juice. One millilitre of the serially diluted resultant homogenate was plated onto MacConkey, Salmonella-Shigella and Eosin Methylene blue agar plates using spread plate method, and incubated at 37°C for 24 h. Preliminary identification of the isolates was based on their cultural and morphological characteristics. Identities of *Citrobacter* isolates were confirmed using conventional biochemical tests and analytical profile index (API) kits. The antibiotic susceptibility testing of the isolates was carried out using Kirby-Bauer's disk diffusion techniques and the zones of inhibition were interpreted based on the Clinical and Laboratory Standard Institute (CLSI) guidelines. Isolation of plasmid DNA was done using TENS (Tris, EDTA, NaOH and SDS) buffer and plasmid curing was carried out using ethidium bromide. Molecular detection of resistance (*blaCTX, blaSHV and aac(6´)*) and virulence (*eae*) genes was done by Polymerase Chain Reaction (PCR) using appropriate primers.

Forty bacterial isolates were recovered from fruits and vegetables comprising 25 (62.5 %) *Citrobacter*, 7 (17.5 %) *Escherichia coli*, 5 (12.5 %) *Proteus* sp and 3 (7.5 %) *Klebsiella* sp. Antibiotic susceptibility tests showed that all the *Citrobacter* species were resistant to

ceftriazone and amoxicillin, while most were resistant to piperacillin, gentamycin, cefotaxime, tetracycline, cotrimoxazole, nitrofurantoin and augmentin. There was significant difference ($p<0.05$) in the percentage resistance of the *Citrobacter* isolates. Twenty two (88 %) of twenty five *Citrobacter* isolates obtained in this study, *Citrobacter freundii* (60 %), *Citrobacter braakii* (20 %) *and Citrobacter youngae* (8 %) exhibited multiple antibiotic resistances to between two and four different classes. All the twelve selected multiple antibiotic resistant *Citrobacter* isolates harboured single plasmid with molecular weight of 1710 bp. The plasmid curing experiment revealed that ceftriazone resistance was plasmid-borne. Five of the 12 representative isolates namely *Citrobacter freundii* (3), *Citrobacter braakii* (1) *and Citrobacter youngae* (1) harboured *blaCTX* gene with molecular weight of 585 bp. *Citrobacter freundii* (2) and *Citrobacter braakii* (1) had *bla*SHV with molecular weight of 392 bp each. None of the isolates harboured *aac6'* resistant gene. Only *Citrobacter braakii* (1) harboured *eae* (1917 bp) virulence gene.

The study concluded that fruits and vegetables sold in the study area were contaminated with potential Gram negative bacterial pathogens capable of causing food-borne disease in consumers.

Keywords: Phenotypic identification/ Molecular Characterization/ *Citrobacter* sp Isolated/ Commercial Fruits / Vegetables

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CHAPTER ONE

INTRODUCTION

The plant world, particularly the group 'fruits and vegetables', is an enormous store of active chemical compounds and considered as the cheapest and most easily available sources of carbohydrate, fiber, proteins, vitamins, minerals and **amino acids** (Ahmad *et al*., 2008; Gibson *et al*., 2012). They are carotenoid in nature and are resistant to oxidation due to Low Density Lipoprotein (LDL). They lowered the damage of deoxyribonucleic acid (DNA) and voluntarily induced greater activity of repairing in human (Southon, 2000) compared to processed food. They serve as additional source of vitamins A, C and E to supplement the diet of patients suffering from cancer affects negatively their radiotherapy as well as chemotherapy results (Seifried *et al*., 2003). Therefore, globally, there has been an increase in consumption of fresh fruits and vegetables due to their nutritional health benefits to humans and most of which are more affordable to both poor and rich people especially in Nigeria.

Despite their nutritional and health benefits, outbreaks of human infections associated with the consumption of fresh fruits and vegetables have increased (Beuchat, 2002; Eni *et al*., 2010). Fruits and vegetables are widely exposed to microbial contamination through contact with soil, dust, water and by handling at harvest or during postharvest processing. They therefore, harbour a diverse range of microorganisms including plant and human pathogens (Carmo *et al*., 2004). These microbes render fresh fruits and vegetables unfit for human consumption by causing deterioration leading to reduction in quality, texture, off flavour development and loss of nutrients.

The contaminations of these fruits and vegetables have created another burden for consumers. They have been increasingly recognized as significant reservoirs of foodborne

pathogens (Brandl, 2006; Doyle and Erickson, 2008; Berger *et al.*, 2010). In the United State, the proportion of outbreaks associated with fresh fruits and vegetables of all reported food-borne outbreaks with an identified food source has increased from 0.7 % in the 1970s to 6 % in the 1990s (Sivapalasingam *et al.*, 2004) and to 13 % in the 2000s (Doyle and Erickson, 2008). During 1999 and 2000, fruits and vegetables were the incriminated food at 6 %, 10 % and 17 % of all identified cases of food-borne disease outbreaks in Sweden, United Kingdom and in Iceland respectively (Anonymous, 2003). In developing countries such as Nigeria, continued use of untreated waste water and manure as fertilizers for the production of fruits and vegetables is a major contributing factor to contamination (Amoah *et al.*, 2009). Most of the reported outbreaks of gastrointestinal disease linked to the fresh produce have been associated with bacterial contamination, particularly with members of the family Enterobacteriaceae (Tyler and Triplett, 2008). *Citrobacter* species is considered one of the bacterial species that has been isolated from fruits and vegetables (Abdelnoor *et al.*, 2013).

Organisms of the genus *Citrobacter* are Gram-negative bacilli of the family Enterobacteriaceae, tribe Citrobactereae. The genus was originally in the tribe Salmonelleae but, was re-classified as a unique tribe. These bacteria have undergone frequent, sometimes confusing changes in nomenclature that make it difficult to clearly determine whether some reported cases are legitimately attributed to *Citrobacter* or to a particular species within the genus. They are sometimes accepted to be in *Escherichia* or *Salmonella genus* (Kurtoğlu *et al.*, 2011). They have variable culture, biochemical and antigenic characteristics that resemble those of the genus *Salmonella* and *Escherichia coli* (Delgado *et al.*, 2013). Hedegaard *et al.* (1999) also reported that DNA sequence analysis show that *Citrobacter*, *Escherichia* and *Salmonella* form one distinct lineage within the Enterobacteriaceae. This plasticity has prevented a definitive

classification of *C. freundii* and has created a taxonomic problem that has been under discussion for many years (Kurtoğlu *et al.*, 2011). At present and according to Bergey's Manual of Systematic Bacteriology, eleven species are reported in the genus *Citrobacter* and they are localised in the intestinal system (Frederiksen and Genus, 2006).