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Characterization and Phylodiversity of Implicated Enteric Bacteria Strains in Retailed Tomato (*Lycopersicon esculentum* Mill.) Fruits in Southwest Nigeria

Yemisi Dorcas Obafemi[1]*, Paul Akinniyi Akinduti[1], Adesola Adetutu Ajayi[2], Patrick Omoregie Isibor[1], Theophilus Aanuoluwa Adagunodo[3]

[1]Department of Biological Sciences, Covenant University, PMB 1023, Ota, Ogun State, Nigeria; [2]Department of Biological Sciences, Augustine University, PMB 1010, Epe, Lagos State, Nigeria; [3]Department of Physics, Covenant University, PMB 1023, Ota, Ogun State, Nigeria

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***Correspondence:** Yemisi Dorcas Obafemi, Department of Biological Sciences, Covenant University, PMB 1023, Ota, Ogun State, Nigeria. E-mail: yemisi.obafemi@covenantuniversity.edu.ng
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Abstract

BACKGROUND: Tomatoes (*Lycopersicon esculentum Mill.*) have very huge health-promoting benefits due to high nutritional composition; however, these fruits are potential reservoir of enteric food-borne pathogens causing major public health concerns.

AIM: Characterization and phylo-analysis of implicated enteric bacteria strains in retailed Tomato fruits in southwest Nigeria were studied.

METHODS: Ready to be retailed fresh tomato fruits were purchased from common food markets in southwest, Nigeria, which lies between latitudes 6° 21′ to 8° 30′ N and longitudes 2° 30′ to 5° 30′ E. Observation of sample storage potentials at different conditions and bio-typing of associated bacterial strains were carried out for consecutive 14 days. Enteric bacteria strains were genotyped with 16S rRNA assay and further profiled for antibiotic susceptibility to common antibiotics. High population rate frequently consume tomatoes.

RESULTS: Early spoilage characterized with yellow fluid, fungal growth and visible lesions were observed at 25°C storage compare to few patches of lesion at 4°C after 14 days. Higher bacterial count of 4.0–7.18 Log CFU/g was recorded at ambient storage compare to refrigerated samples with more than 10% occurrence rate of *Citrobacter* spp., Klebsiella spp. and Enterobacter spp. Identified *Citrobacter* spp. and Klebsiella spp. showed 100% resistant to beta-lactam antibiotics (ceftazidime, cefuroxime, cefixime, ciprofloxacin, and amoxicillin-clavulanic acid). Two-resistant enteric bacteria strains, *Klebsiella aerogenes B18* and *Citrobacter freundii B27* obtained from Nigerian tomato clustered with *Citrobacter* strains in food (China), water strains (India, Poland, Malaysia), milk (Germany), and human fecal (China).

CONCLUSION: Implicated multidrug-resistant enteric bacilli in retailed tomatoes can cause severe food-borne diseases which public oriented awareness, strategic farm to market surveillance are needed to be intensified.

Introduction

Tomato (*Lycopersicon esculentum* Mill.) belongs to a plant family known as Solanacea [1]. It is one of the most important fruits in the world which can be eaten raw or processed into paste or juice [2]. Tomato supplies about 90% of dietary Vitamin C needed for human nutrition with a high ascorbic acid and lycopene content [3], [4], [5]. In Africa, tomatoes are also used for medicinal purposes for protection against various diseases such as the osteoporosis, cardiovascular disorders, and prostate cancer [6]. Recent reports on the nutritional composition in tomatoes revealed 95% water and 4.5% carbohydrate with other natural nutrients such as vitamins, minerals, dietary fiber, and protein [7], [8]. The rich nutritional components of tomatoes are often exploited by microorganisms which make post-harvest tomato spoilage and contamination to be rampant [9], [10]. Post-harvest spoilage and contamination occur initially on protective outer surface after which movement of water into the fruits occur causing contamination and possible attacks by enteric pathogens [11]. Contamination of tomatoes may result from the treatment of the soil with organic fertilizers, such as sewage sludge and manure, irrigation water, as well as from water used in washing the tomatoes in the market till it gets to the final consumers [12]. In Nigeria, tomatoes are kept in the open markets often displayed in baskets or on benches for the prospective customers, thereby exposing them to opportunistic microbial infections [13]. Post-harvest infections in tomato could occur during storage, transportation, packaging, and distribution (loading and offloading) at various points of sale at which bacteria are present. Therefore, adequate knowledge and careful handling procedure of the tomatoes can reduce wastage of the fruits [14]. However, the incidence of foodborne disease is now becoming a public health issue considering the recent outbreaks of foodborne diseases in various

countries due to consumption of contaminated raw fruits [15], [16], [17], [18]. The previous reports have implicated presence on pathogens on surfaces of fresh fruits and vegetables which includes *Escherichia coli*, *Salmonella paratyphi typhi*, *Klebsiella pneumonia*, *Yersinia pestis*, *Shigella dysenteriae,* and *Listeria monocytogenes*; however, there is still paucity of studies to determine the public health implication of consuming the contaminated tomatoes which are often used by many local food eateries in Nigeria [19], [20], [21], [22], [23]. There is need to determine bacteria contaminants in tomatoes and check their antimicrobial susceptibility to common content that may constitute risk to public health [24]. Reports are suggesting possible contamination of fresh edible tomatoes resulting in high microbial diversity including the family Enterobacteriaceae that invariable signifies poor post-harvest sanitation practices by the farmers and local handlers [25], [26], [27], 28], [29], [30]. Therefore, the prevailing enteric pathotypes and genotypes in retailed tomato fruits in Southwest Nigeria were studied.

Materials and Methods

Sample collection and bacteria growth kinetics

One hundred and twenty fresh tomato fruits were purchased from 18 major food markets in southwest Nigeria between April and June 2017 and transported in the cold chain. Accurate coordinates of the sampling markets were recorded with the Global Positioning System device, as described by Obeng *et al*. [31]. Each tomato sample batch was assayed for spoilage bacteria growth kinetics by 2 × 2 contingency factorial design of each group of 60 samples each at various ambient temperatures (25°C) and refrigerated condition (4°C) experimental design. These selected temperatures are the common condition of storage in many households in Nigeria and all the samples in each conditional group were daily analyzed for bacteria growth kinetics for a period of 14 days while evidence for visible lesion was observed during deterioration stages.

Viable bacteria count and phenotypic characterization

At each successive day of the storage, 1 g of mashed tomato sample from each experimental condition was thoroughly grounded and homogenized in 9 ml sterile water, serially diluted, inoculated into brain heart infusion agar and incubated at 37°C for 24 h. Total bacteria colonies were enumerated and phenotypically biotyped with the taxonomic scheme of Bergey's Manual of Determinative Bacteriology [32].

Antimicrobial susceptibility assay

Characterized bacterial strains were profiled for an antibiogram using the Kirby–Bauer disc diffusion method [33]. Briefly, the broth culture of 0.5 MacFarland turbidity was spread on Muller-Hilton Agar. The antibiotic disc of Ceftazidime (30 μg), Cefuroxime (30 μg), cefixime (30 μg), ofloxacin (5 μg), ciprofloxacin (5 μg), Gentamicin (10 μg), nitrofurantoin (30 μg), and augmentin (30 μg) was added and incubated at 37°C for 24 h. The zone of inhibition obtained was measured and evaluated [34].

Genotyping

The chromosomal DNA of each bacteria isolates was extracted (Jena JmbH, Germany) and amplified for 16S rRNA using previously described polymerase chain reaction (PCR) assay [35]. Amplification was carried out in a Hybaid PCR express unit (Hybaid Ltd., Middlesex, UK) at initial denaturation temperature of 94°C for 5 min for 1 cycle which was followed by 30 cycles of denaturation at 95°C for 1 min. Annealing at 55°C for 45 s and extension was at 72°C for 1 min. Final extension was carried out at 72°C for 7 min (Table 1). Primer pair used for this study was 27F:5′-AGAGTTTGATCCTGGCTCAG-3', and 1492R: 5'-GGTTACCTTGTTACGACTT-3'. Amplicons were separated on a 1.5% Agarose gel and electrophoresis carried out at 120 Volts for 40 min with the DNA ladder (Solis Biodyne), visualized with ultraviolet photo-documentation. Amplicons were purified using the QIAquick PCR purification kit (Qiagen, USA) and quantified with Thermoscientific Nanodrop Spectrophotometer at an absorbance of 260 nm to obtain required dilution of amplicon for sequencing. Purified products were sequenced using ABI Prism Big Dye Terminator version 3.0, and products were subsequently analyzed on ABI PRISM 3700 DNA Analyzer. Obtained nucleotide reads were aligned into Basic Local Alignment Search Tool Web-based tool of the National Institutes of Health (USA) software to search for homologous sequences with not more 99% identity [36].

Table 1: Amplification condition used for PCR carried out in the study

Procedure	Temperature (°C)	Time (seconds)	Number of cycles
Initial denaturation	94	300	1
Denaturation	95	60	
Annealing	55	45	30
Extension	72	60	
Final extension	72	420	1

PCR: Polymerase chain reaction

Phylogenetic analysis

Each bacteria sequence reads were manually aligned to make a dendrogram tree with maximum parsimony regarding the sequences as independent, unordered, and equally weighted according to Fitch parsimony with a calculated bootstrap option for each

node of 1000 replicates of heuristic search using MEGA software version 6 [37].

Data analysis

The significance of the viable count at different kinetic temperature conditions was determined using Chi-square at $p < 0.05$, while categorical data were analyzed with descriptive statistical methods. Bacteria antibiogram profile significance was evaluated with ANOVA ($p < 0.05$).

Results

Sampling of tomato fruits from food markets

The tomato fruits were on display when purchased from 18 major food markets located at *lat*. 7.2571 ± 1.500° and *long*. 5.2058 ± 1.5500° (Figure 1) distributed in six states (Ekiti, Lagos, Ogun, Ondo, Osun, and Oyo) in Southwest, Nigeria, with approximate population of 27,534,432 in 2006 (Table 2). Daily observation shows that early spoilage and rottenness were observed in tomato fruits stored at ambient temperature (25°C) on day 5 while refrigerated samples (4°C) remain fresh throughout the experimental period. After 5 days at both ambient and refrigerated temperatures, no visible lesion was observed in refrigerated samples but only fungal growth at 25°C. Broken cell wall, production of yellow fluid and heavy fungal growth was observed at 25°C after 14 days.

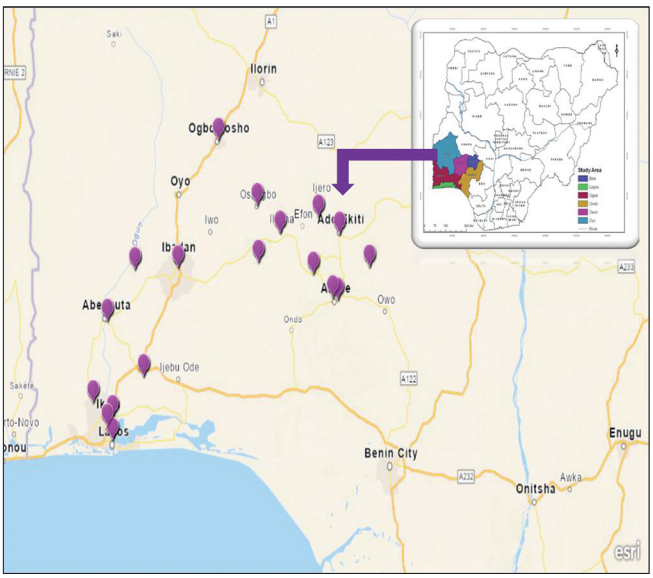

Figure 1: Geo-spatial distribution of sampled food markets in Southwest Nigeria

Table 2: Distribution of potential tomato consumers in various states in Southwest, Nigeria

Surveyed states	Area (km^2)	Population (persons)
Ekiti	6,353.00	2,210,957.00
Lagos	3,577.00	9,113,605.00
Ogun	16,980.55	3,751,140.00
Ondo	15,500.00	3,460,877.00
Osun	9,251.00	3,416,959.00
Oyo	28,454.00	5,580,894.00

Map was designed using ArcGIS software online (https://www.arcgis.com/). Attribution: Esri, USGS | Esri, HERE, Garmin, METI/NASA, USGS.

Viable bacteria count and phenotypic characterization

The total bacterial count for tomato fruits stored at the ambient temperature was within the range of 4.0–7.18 LogCFU/g and higher when compared with the refrigerated samples. However, steady rise in bacteria count was observed on day 8–10 of ambient

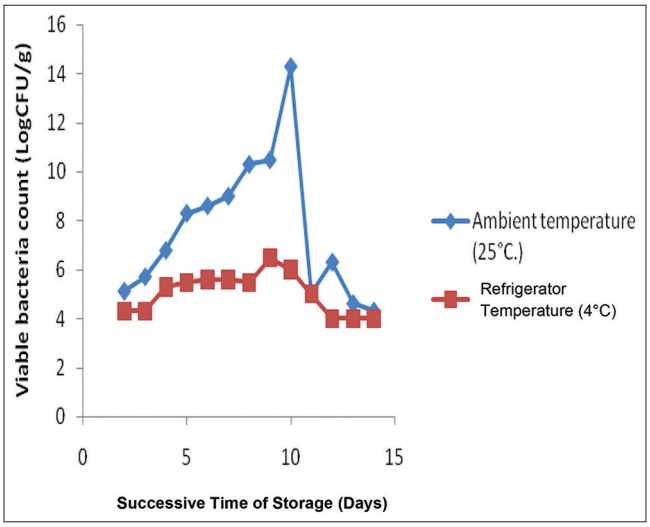

Figure 2: Successive viable bacteria count in different experimental conditions

storage indicating a very high spoilage rate than refrigerated samples (Figure 2). Occurrence of enteric bacteria isolates obtained was identified as *Aeromonas* spp. (2.6%), *Bacillus* spp. (18.2%), *Citrobacter* spp. (25.5%), *Proteus* spp. (7.2%), *Klebsiella* spp. (35.7%), and *Enterobacter* spp. (10.8%) (Figure 3).

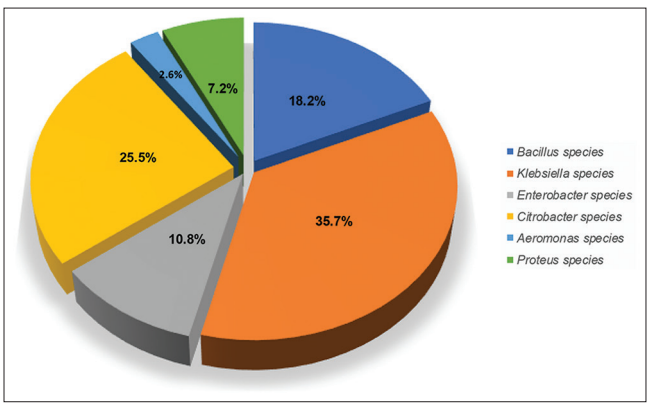

Figure 3: Occurrence of enteric bacilli in tomato fruits purchased from Southwest Nigeria (n = 120)

Antimicrobial susceptibility assay

The antibiotic resistance profile of enteric bacteria strains from tomato fruits showed high multi-antibiotic resistance particularly to commonly used beta-lactamase antibiotics such as ceftazidime, cefuroxime, cefixime, ciprofloxacin, and amoxicillin-clavulanic acid. A significantly low resistance was recorded rate for gentamycin, nitrofurantoin, and ofloxacin (Figure 4). High multidrug resistance rate observed in *Citrobacter* spp. (100%) and *Klebsiella* spp. (100%) to beta-lactam antibiotics.

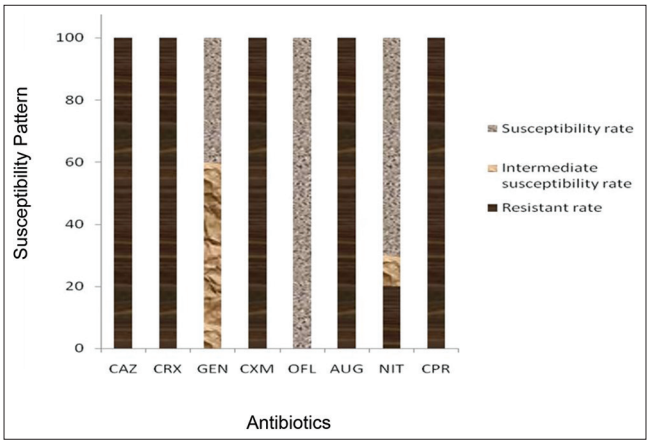

Figure 4: Susceptibility pattern of biotypes analyzed from tomato fruits. R: Resistant, I: Intermediate, S: Susceptible, CAZ: Ceftazidime, CRX: Cefuroxime, GEN: Gentamycin, CXM: Cefuroxime, OFL: Ofloxacin, AUG: Augmentin, NIT: Nitrofurantoin, CPR: Ciprofloxacin

Genotyping and phylogenetic analysis

The extracted and purified DNA yield was 5 ng–25 ng and the absorbance ratio (A_{260}/A_{280}) 1.60–1.80 which informed constitution for PCR analysis using conditions presented in Table 1. Amplicon from the strains expressed high molecular weight band on Agarose gel (Figure 5). Two-resistant enteric bacteria strains were characterized as *Klebsiella aerogenes B18*

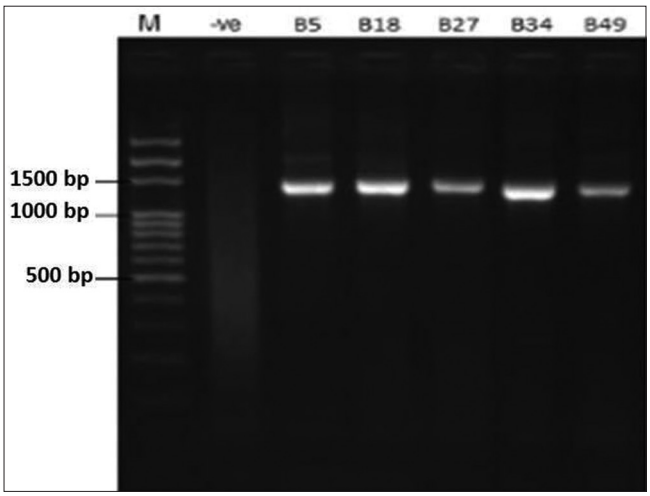

Figure 5: Amplicons of enteric bacterial strains expressed on Agarose gel (M – 500bp DNA Ladder, −ve: Negative Control, B5: Bacillus sp.; B18: Klebsiella sp.; B27: Citrobacter sp.; B34: Enterobacter sp. B49: Aeromonas sp.).

and *Citrobacter freundii B27*. Both *Citrobacter freudii* from Nigerian tomato clustered with *Citrobacter* strains in food (China), water strains (Indian, Poland, Malaysia), milk (Germany), and human (China) while *Klebsiella aerogenes* showed genetical relatedness with other *Citrobacter* strains (red rectangular notch) (Figure 6).

Discussion

The tomato fruits are fresh farm products retailed in major food markets located in many cities in Southwest Nigeria. Residents in these areas are potential consumers who patronize the markets for fresh farm products including fruit retailers, food handlers, vendors, and canteen operators [38], [39], [40]. It is evidenced from the results that tomato fruits are well preserved at lower temperature below 8°C, high humidity and could maintain its integrity as well as the nutritious value when compared to easily spoilt samples stored at ambient condition of 25°C [41], [42], [43]. High spoilage rate is expected considering the ubiquitous nature and optimum growth condition of many mesophilic bacteria which are mostly enteric pathogens transferred to the fruits during harvesting, storage, and transportation from farm to markets. Some of these strains could be pathogenic, causing various intestinal infections and severe enteric diseases particularly among the immune-compromised individuals. The previous studies reported 5–8 days of storage before for spoilage initiation is observed. This depends on environmental factors in preserving the post-harvest shelf life of tomato fruits because it has a tremendous influence on the rate of biological processes [44], [45]. Occurrence of enteric bacteria strains particularly coliforms suggests fecal contamination of tomato retailed in most of these markets, initiating spoilage, reduce post-harvest shelf life and increases gastrointestinal diseases due to poor hygiene and handling from farm to the markets. This agrees with previously published reports indicating six of the Gram-negative bacteria species isolated from the fruit samples could as fecal coliforms [46], [47], [48], [49], [50], [51]. Contamination of fresh tomatoes from the farm and in the market by enteric bacteria species including *Escherichia coli* and other coliforms were similarly reported in post-harvest tomatoes in Nigeria [11]; Ghana [52], India [53], Europe, and USA [54], [55].

High multidrug resistance observed in *Citrobacter* spp. (100%) and *Klebsiella* spp. (100%) to beta-lactam antibiotics suggest an increase in use of antimicrobial agent for preservation and plant treatment resulting to acquisition of resistance in common food associated pathogens could intensify foodborne disease outbreak among the consumers. Occurrence of these resistant strains further suggests imminent danger of gastrointestinal disease outbreak from retailed tomato in most parts of southwest Nigeria. Poor post-harvest

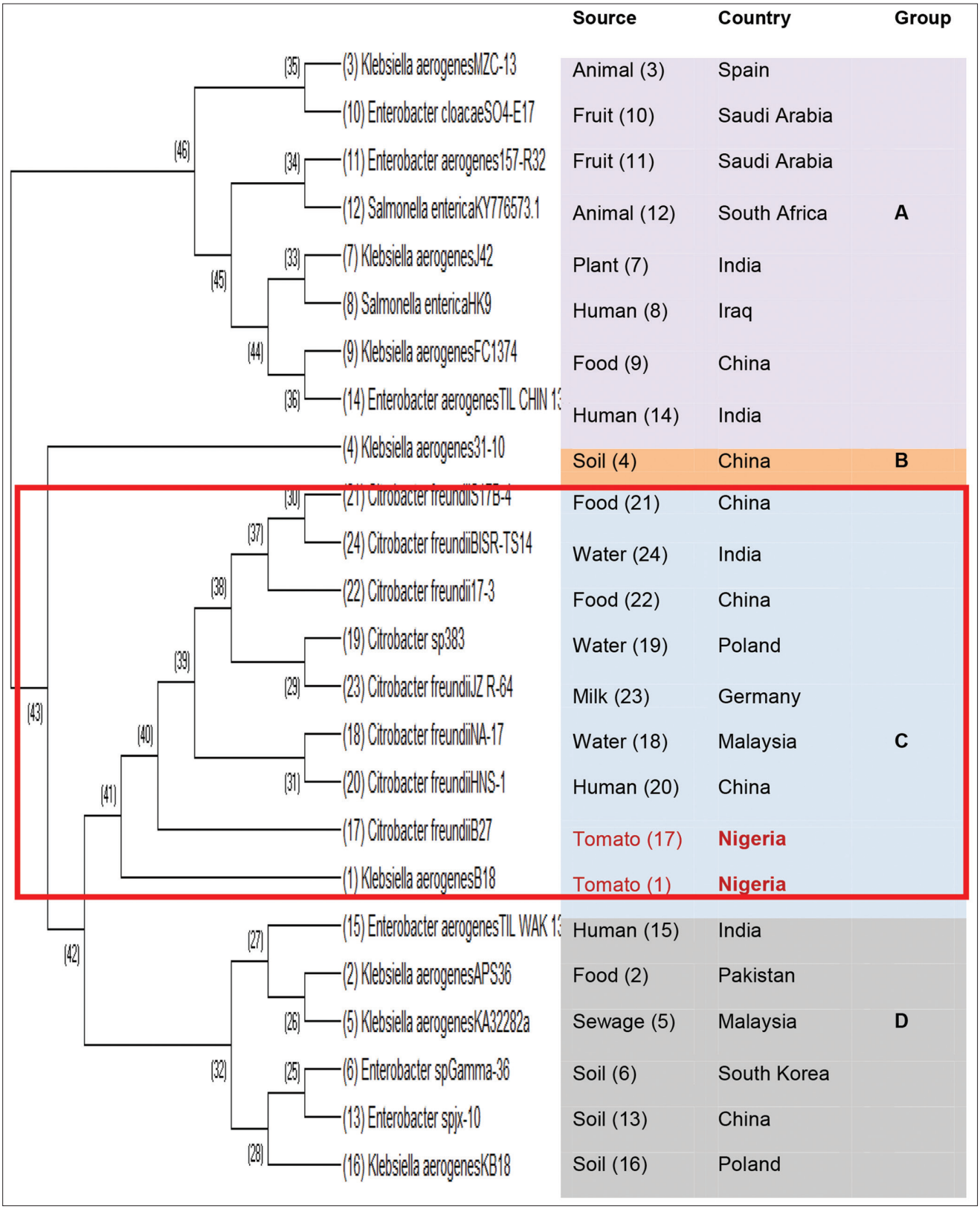

Figure 6: Phylogenetic diversity of 16s rRNA gene sequence of enteric bacteria strains in retailed Tomato fruits in Nigeria analyzed with GenBank reference

storage conditions of food materials, poor food handling practices, and unhygienic cooking habits in common street canteens and restaurants are gradually increasing resistant foodborne pathogens as well as other extended-spectrum beta-lactamases-producing strains in food materials in many communities [21], [56]. Clonal relatedness of highly resistant enteric *Klebsiella aerogenes* and *Citrobacter freundii* from Nigerian tomatoes with human, food, and milk products from other countries further indicate high genetic

mobility and transfer from animal milk products, human contamination probably from fecal that could drive genetic reassortment leading to spread of phylo-diverse pathotypes [20], [57], [58], [59].

Conclusion

This study has established the fact that fresh tomato fruits serves as host to several bacteria pathogens that can cause food-borne diseases. Multidrug-resistant pathogens indicated potential causes of food borne infections and food contamination which is a major cause of public health concern. There is need for public awareness to prevent rising food contamination from tomatoes commonly called “Esa” in Nigeria which are fondly used by most food canteen operators and also to detect the new antimicrobial resistant strains using high throughput genomic techniques.

Authors’ Contributions

YDO and AAA conceived and designed the background experiments in the study and collected the samples. PAA carried out all the isolation of the bacteria strains and antimicrobial sensitivity experiments. YDO carried out the phenotypic characterization of the bacterial strains with POI. The authors PAA and YDO carried out extraction, purification and quantification of DNA amplicon form the bacterial isolates. PAA carried out the PCR assay. TAA helped collection of samples and in drafting the manuscript figures and tables. All authors read and approved the final manuscript.

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