Research Article

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Fingerprints of resistant *Escherichia coli* O157:H7 from vegetables and environmental samples

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Abstract

BACKGROUND: Some routes of transmission of *Escherichia coli* O157:H7 to fresh produce include contaminated irrigation water and manure polluted soils. The aim of the present study was to determine the genetic relationships of *E. coli* O157:H7 isolated from some produce growing region in Nigeria using enterobacterial repetitive intergenic consensus (ERIC) DNA fingerprinting analysis. A total of 440 samples comprising leafy greens, irrigation water, manure and soil were obtained from vegetable producing regions in Kano and Plateau States, Nigeria. Genes coding for the quinolone resistance-determinant (gyrA) and plasmid (pCT) coding for multidrug resistance (MDR) were determined using polymerase chain reaction (PCR) in 16 isolates that showed MDR.

RESULTS: Cluster analysis of the ERIC-PCR profiles based on band sizes revealed six main clusters from the sixteen isolates analysed. The largest cluster (cluster 3) grouped isolates from vegetables and manure at a similarity coefficient of 0.72.

CONCLUSION: The present study provides data that support the potential transmission of resistant strains of *E. coli* O157:H7 from vegetables and environmental sources to humans with potential public health implications, especially in developing countries.

Keywords: *Escherichia coli* O157:H7; ERIC- PCR; vegetables; irrigation water; manure

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC), in particular serotype O157:H7, is a highly pathogenic subset of shiga toxin-producing *E. coli* (STEC) that causes gastrointestinal illnesses ranging from aqueous and bloody diarrhoea to hemorrhagic colitis in humans.[1] *Escherichia coli* O157:H7 infections are acquired naturally from a wide spectrum of animal species (cattle, sheep, goat, deer, swine, horse, dog, cat, pigeon, chicken, turkey and gull) and, in particular, cattle have been identified as major reservoirs of this pathogen,[1] with the consumption of food contaminated with animal/manure feces being reported to result in STEC infections. Fresh greens, fruits and vegetables are also important sources of this infection.[1] In the USA, reported cases of *E. coli* O157:H7 infections outbreaks from contaminated fruits and vegetables increased from 11% to 41% from 1998 to 2007.[2] Contamination of surface waters from waste disposal can impact food safety through irrigation.[3] Contamination of fresh produce associated with fecal contamination in agricultural irrigation water has been reported.[1] There was an *E. coli* O157:H7 outbreak linked to lettuce from Taco Bell restaurant in the northern USA.[4] The presence of *E. coli* O157:H7 in aquatic environments is the common denominator linking diverse transient habitats and transmission to animals and humans.[1] *Escherichia coli* O157:H7 has been detected in soil, manure, irrigation water and contaminated seeds.[1] It has also been reported to colonize the interior of plants such as radish, lettuce and internal plant compartments;[5] hence, it is difficult to remove or kill by washing and/or disinfection.[1] Irrigation water is the leading pre-harvest source of contamination of fresh produce in the world.[6] The surface water used for irrigation constitutes public health risk because settlements around the dams and rivers pollute them with waste and sewage.[6] The occurrence of *E. coli* O157:H7 in environmental samples poses a potential threat to human health.[7]

On the other hand, antibiotic resistance in *E. coli* O157:H7 is increasing.[8] Antibiotics are extensively used in agriculture to prevent and treat disease as well as promote growth.[9] The abuse of antibiotics in foods of animal origin, especially in livestock production, poses a significant threat to public health because it contributes to the development of antibiotic-resistant bacteria that can be passed on to humans.[10] Consequently, animal waste produced (commonly used as manure) in agricultural settings

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contains resistant bacteria, resistant determinants present in gene transfer units, or combinations of both.[9] Generally, bacteria have a mechanism of rapid evolution towards a resistant phenotype through the sharing of genes that encode resistance.[11]

Resistance can be propagated within and among bacterial strains, species and genera, including commensals and pathogens, by mobile genetic elements such as plasmids, transposons, integrons, gene cassettes and bacteriophages,[12] accounting for more than 95% antibiotic resistance. Quinolones are also considered to exert antibacterial activity by inhibiting DNA gyrase, which catalyses topological changes of DNA.[13] DNA gyrase of *E. coli* consists of subunits A and B, which are the products of the gyr*A* and gyr*B* genes, respectively.[14] Mutations in either gene can cause quinolone resistance.[14] Sequencing of the 0.6 kb Sac1- Sma1 fragments of the mutant gyr*A* genes revealed that these mutations were located within a relatively small region of the A subunit, called the quinolone resistance-determining region.[15]

Pui *et al.*[7] reported that the polymerase chain reaction (PCR) protocol is suitable for rapid and specific analysis of pathogenic *E. coli* O157:H7 in environmental samples for the assessment of microbiological risks and detection of genes. Historically, barcodes (also referred to as fingerprints or profiles) normally assigned to microorganisms based on phenotypic or molecular analyses have been used to identify, compare and track microorganisms.[16] Enterobacterial repetitive intergenic consensus (ERIC) sequences are 127-bp imperfect palindromes that occur in multiple copies in the genomes of enteric bacteria.[17] Previously, Warriner *et al.*[18] used ERIC-PCR to trace the fate of *E. coli* isolates within a pork slaughter line.

The present study assessed cross-contamination based similarities between fingerprints of strains of *E. coli* O157:H7 from different sources (vegetables and environmental; irrigation water and manure treated soils) by ERIC-PCR. The PCR also detected the presence of plasmids (pCT) and quinolone resistance-determining region (QRDR) in strains.

MATERIALS AND METHODS

Study area

Kano is one of the States in Nigeria with extensive irrigation farming. The State has more than 3 million hectares of cultivable land all year round. Plateau State has soil and climatic conditions that favor the production of vegetables and other exotic crops. Sampling sites were selected after a survey of some irrigation sites in the two States. Five sites from each location were selected based on the availability of vegetables on the farms, the cooperation of the farmers, the source of irrigation water and its point source of contamination.

In total, 440 samples comprising salad vegetables (238) and some environmental (84 irrigation water and 118 soil/ manure) samples were collected and analyzed. Samples were collected for two seasons: the wet (May to October 2010) and dry (November to March 2011) seasons.

Water samples were collected in accordance with the procedure recommended by the American Public Health Association[19] in sterile wide mouth, screw capped 250-mL bottles. Vegetables were collected in factory sterile polythene bags, whereas composite soil/manure samples were collected aseptically using ethanol-sterilized spatula. All samples were packed on ice during transportation to the laboratory and were analyzed within 6 h of collection.

Isolation and characterization of isolates

Escherichia coli O157 was isolated from the samples using enrichment in *E. coli* medium, streaked on sorbitol MacConkey (SMAC) agar (Oxoid; Thermo Fisher, Waltham, MA, USA) plates containing cefixime (0.5 L^{-1}) and potassium tellurite (2.5 mg L^{-1}) and incubated at 37 °C for 24 h.[20] Three to four sorbitol negative colonies exhibiting typical *E. coli* O157 colony phenotype were selected, purified on freshly prepared SMAC media and stored on slants at 4 °C. Characterization of the isolates was conducted by biochemical tests as described previously[21] and also by the Microbact computer aided identification kit (Oxoid). Serological identification of *E. coli* O157 isolates was conducted using the *E. coli* O157 latex agglutination test kit (Oxoid) and slide agglutination method to detect antigen O and H flagella serogroups with specific antisera (Denken, Seiken Japan). Confirmed isolates were assayed for verocytotoxin (VT1 and VT2) production by reverse passive latex agglutination using the toxin detection kits (Oxoid).

All procedures for identification and characterisation of *E. coli* O157:H7 are as reported previously.[22]

Antimicrobial susceptibility

Antimicrobial susceptibility of confirmed isolates was performed by the disc diffusion method using eight antibiotics: amoxicillin/clavulanate (30 µg), sulfamethoxazole/trimethoprim (25 µg), ciprofloxacin (5 µg), ceftriaxone (30 µg), gentamicin (30 µg), tetracycline (30 µg), kanamycin (30 µg) and cephalothin (30 µg). A standard strain of *E. coli* ATTCC 25922 was used as a quality control. Isolates were termed resistant or susceptible using the Clinical and Laboratory Standards Institute guidelines.[23]

Template DNA preparation using extraction kit

A single colony of pure *E. coli* O157:H7 was inoculated into 10 mL of Luria-Bertani broth medium and incubated at 37 °C overnight. A sample (1 mL) of the overnight culture was centrifuged for 2 min at 14 000 x *g*. Pelleted bacterial cells were resuspended thoroughly in 480 µL of 50 mmol L^{-1} ethylenediaminetetraacetic acid, digested in 120 µL of 10 mg mL^{-1} lysozyme and incubated at 37 °C for 60 min before being subjected to DNA extraction using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions.

DNA amplification and detection of housekeeping, resistant and multidrug resistant genes in strains

A total of 16 *E. coli* O157:H7 isolated, identified and characterized in the study were further confirmed by detecting the presence of the *uid*A (187 bp) gene as described previously.[24] The presence of the O157:H7 gene was also further confirmed by detecting the *rfb*O_{157} (420 bp) gene as described previously[25] but with slight modification (a different annealing temperature of 60 °C). The presence of the QRDR of gyrA (448 bp) according to Gomez *et al.*[26] and plasmids conferring multidrug and resistance to aminoglycosides (pCT) (428bp) as described by Cottell *et al.*[27] were assayed in the study. The primers, PCR preparations and conditions used are shown in Table 1. Amplification was performed using thermal cycler (Bio-Rad, Hercules, CA, USA). In all of the PCR assays, a negative control (i.e. a reaction tube with nuclease-free water only) was included. The distribution of strains from the different samples assayed is shown in Table 2.

Table 1. Primer sequences, PCR preparations and conditions used in molecular characterization and genotyping of *E. coli* O157:H7 obtained in the present study

Target	Primer sequence	PCR preparation (25 µL)	PCR conditions	Reference (year)
QRDR of *gyr*A	gyrAF 5′-GCGCGTGAGATGACCCGCCGT-3′ gyrAR 5′-CTGGCGGTAGAAGAAGGTCAG-3′	12.5 µL of Dream Taq mastermix, 8.5 µL of nuclease free water, 3 µL of template DNA and 0.5 µL of each primer mix	5 min at 94 °C prior to 35 cycles of 1 min at 94 °C, annealing temperature at 54 °C for 1 min, extension at 72 °C for 1 min and a final extension of 10 min at 72 °C	Gomez *et al.*, (2004)[26]
pCT	pCT(008)F 5′-CATTGTATCTATCTTGTGGG-3′ Pct(009)R 5′-GCATTCCAGAAGATGACGTT-3′	12.5 µL of Dream Taq mastermix, 8.5 µL of nuclease free water, 3 µL of template DNA and 0.5 µL of each primer mix	5 min at 94 °C prior to 35 cycles of 1 min at 94 °C, annealing temperature at 60 °C for 1 min, extension at 72 °C for 1 min and a final extension of 10 min at 72 °C	Cottel *et al.*, (2011) [27]
ERIC	$ERIC_1$ 5′-ATGTAAGCTCCTGGGGATTCAC-3′ $ERIC_2$ 5′-AAGTAAGTGACTGGGGTGAGCG-3′	12.5 µL of Dream Taq mastermix, 8.5 µL of nuclease free water, 3 µL of template DNA and 0.5 µL of each primer mix	10 min at 95 °C prior to 34 cycles of 30 s at 94 °C, Annealing temperature at 50 °C for 1 min, extension at 65 °C for 1 min and a final extension of 8 min at 65 °C	Versalovic *et al.*, (1994) [28]

Table 2. Distribution of *E. coli* O157:H7 strains from the different samples that were used for genotypic typing

	Samples				
State	Vegetables	Irrigation water	Soil	Manure	Total
Kano	4	2	1	1	8
Plateau	3	2	1	2	8
Total	7	4	2	3	16

Genotypic determination of diversity among *E. coli* O157:H7 isolates using a DNA fingerprinting assay

From the characterized *E. coli* O157:H7 isolates, 16 representative isolates were analysed using ERIC-PCR to determine their genetic relatedness.

Electrophoresis of PCR amplicons

The PCR products (amplicons) were separated by electrophoresis on a 2% (w/v) agarose gel containing 2 µL of florosafe. Electrophoresis was run at 100 V for 30 min for gene detection, whereas, for DNA fingerprinting, electrophoresis was run at 120 V for 45 min and visualized on an ultraviolet transilluminator gel imaging system (Bio-Rad Gel Imaging system; Bio-Rad). Bands were photographed and band positions were determined and compared to molecular weight markers (1-kb and 100-bp DNA ladders; First BASE, Seri Kembangan, Malaysia).

Cluster analysis and calculation of discriminatory index

DNA fingerprint profiles were defined as presence of a DNA band (a score '1') and the absence of a DNA band (a score '0'). The scores were entered into NTedit (Exeter software, Serauket, NY, USA) to obtain a data matrix and then inserted into NTSYSpc, version 2.2 (http://www.exetersoftware.com/cat/ntsyspc/ntsyspc.html) for the construction of a dendrogram based on the simple matching coefficient and unweighted pair-group arithemetic average clustering.

RESULTS

An antibiogram of identified *E. coli* O157:H7 isolates to eight commonly used antibiotics revealed seven resistance patterns. All isolates exhibited multidrug resistance, with each isolate being resistant to three or more antibiotics (Table 3). Resistance to kanamycin was the most common resistance phenotype observed among all isolates, whereas isolates were most susceptible to the antimicrobial effects of ceftriaxone. Two isolates JMA3 and KMA3 obtained from manure exhibited complete resistance to six and all eight antibiotics tested, respectively.

Genetic diversity of *E. coli* O157:H7 isolates

To further characterize isolates for the genetic basis of resistance, fragments of QRDR and pCT conferring multidrug resistance to aminoglycosides were amplified in all isolates by PCR. Multidrug resistance in isolates was confirmed by primer amplification of the QRDR and pCT with band sizes of 428 and 484 bp, respectively (data not shown). Figure 1 shows fingerprints of *E. coli* O157:H7 strains, demonstrating three to nine bands with a molecular weight of 150 bp to 1 kb. A common intensive band of approximately 7000 bp was observed in most of the strains. Cluster analysis at a coefficient of similarity of 0.53 grouped the sixteen into five different groups, designated 1–5 (four clusters and one singleton) (Fig. 2). Table 4 shows the diversity and relatedness of the *E. coli* O157:H7 strains obtained from the different sources grouped in clusters and singletons as represented on the dendrogram.

Cluster 1 comprises isolates (Jva1, Jva2 and Jsa6) from vegetables and soil with a similarity coefficient of over 0.58; cluster 2 comprises strains (Jwa4, Jwa5 and Jma7) obtained from irrigation water and manure with a similarity coefficient of 0.67; cluster 3 comprises strains (Jma3, Kva7, Kva2 and Kva40) with a similarity coefficient of 0.72; and cluster 4 comprises strains (Jva3, Ksa5, Kwa29, Kwa7 and Kma3) with a similarity coefficient of 0.40. However, isolate Kva31 from vegetable exists as a singleton.

DISCUSSION

Antibiotics are distributed in the environment via agricultural run-off from precipitation, animal and poultry faeces used as fertilizer and wastewater effluent.[29] None of the antibiotics used recorded 100% sensitivity from *E. coli* O157:H7 in the present study; hence, strains were multidrug resistant. This could be as a result of increased use of antibiotics in poultry and animal breeding and its resultant residue in the manure used by farmers in vegetable production in the study area.

Table 3. Distribution, phenotypic resistance and multidrug resistance gene in *E. coli* O157:H7 strains

E. coli O157:H7 Isolate code	Source	Site of origin/point source of contamination	Isolate resistance pattern								Multidrug resistance genes	
			KF	SXT	AMC	K	CIP	CN	TE	CRO	pCT	QRDR
JVA1	Vegetables	Domestic waste	R	R	S	S	S	S	R	S	+	+
JVA2	Vegetables	Domestic waste	R	R	S	S	S	R	S	S	+	+
JMA3	Manure	Abattoir waste	R	R	R	R	S	R	R	S	+	+
JVA3	Vegetables	Domestic waste	R	R	S	R	S	S	R	S	+	+
JWA4	Irrigation water	Abattoir waste	R	R	R	S	S	S	R	S	+	+
JWA5	Irrigation water	Abattoir waste	R	S	S	R	R	S	R	S	+	+
JSA6	Soil	Abattoir waste	R	R	S	R	S	S	R	S	+	+
JMA7	Manure	Abattoir waste	R	R	S	R	S	R	R	S	+	+
KVA2	Vegetables	Abattoir waste	R	S	R	R	R	S	R	S	+	+
KMA3	Manure	Domestic waste	R	R	R	R	R	R	R	R	+	+
KSA5	Soil	Domestic waste	R	S	S	R	R	R	R	S	+	+
KVA7	Vegetables	Abattoir waste	R	S	S	R	R	R	R	S	+	+
KWA7	Irrigation water	Domestic waste	R	R	R	R	S	R	R	S	+	+
KWA29	Irrigation water	Industrial waste	R	S	R	R	R	S	R	R	+	+
KVA31	Vegetables	Abattoir waste	R	R	R	S	S	R	R	S	+	+
KVA40	Vegetables	Domestic waste	R	R	R	S	R	S	R	S	+	+

K, Kano; J, plateau; V, vegetables; A, *E. coli* O157:H7; R, resistant; S, susceptible; KF, cephalothin (30 μg); SXT, sulfamethoxazole (25 μg); AMC, amoxicillin/clavulanic acid (10 μg); CN, gentamicin (10 μg); CIP, ciprofloxacin (5 μg), ceftriaxone (30 μg); TET, tetracycline (30 μg); K, kanamycin (30 μg).
Abattoir, domestic and industrial = wastewater sources used for irrigating vegetables in the study area and considered point sources of contamination to sites in the study apart from manure.
M = 1000-kb DNA ladder; 1–16 = *E. coli* O157:H7 strains used for genotyping.

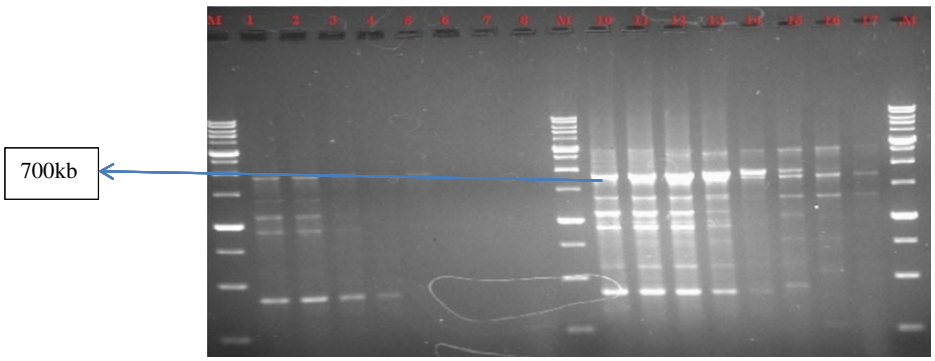

Figure 1. DNA fingerprint of *E. coli* O157:H7 isolates in the present study.

This supports the idea that the use of antibacterials to treat *E. coli* O157:H7 is a contentious area in the management of infection and is currently contra-indicated because it may exarcerbate the disease.[30] Hence, the use of these or any antibacterial for therapeutic and non-therapeutic indications may exert selective pressure and result in the development and maintenance of serotype O157:H7 strains of *E. coli* that are resistant to antibacterials.[31] *Escherichia coli* carrying several antibiotic resistance genes may act as a donor of those resistance genes to other pathogenic *E. coli* and even other species.[32] Hence, the resistance observed in this organism in the present study could be transferred to other pathogens and represents a significant threat to health. Antibacterial therapy may be of value for some forms of diarrhoeagenic *E. coli*,[33] although this resistance pattern is an emerging problem for diarrhoeagenic *E. coli* strains isolated in developing countries[34] and for other enteric bacteria worldwide.[34] *Escherichia coli* O157:H7 has been reported to be a refectory to developing resistance among pathogenic bacteria;[35] hence, the antibacterial resistance being transferred is of great relevance in pathogen emergence and re-emergence.[5]

The pathogens in the present study were resistant to one to several antibiotics tested against them. Bacterial plasmids are key vectors of horizontal gene transfer, mediating the mobilization of genetic material from bacteria to bacteria.[28] The detection of multidrug resistance plasmids and the QRDR in these isolates is in agreement with the findings of a previous study.[28] This supports the suggestion that pCT persistence and dissemination have been driven by constant β-lactam exposure and that pCT can remain stable within a population. The ability and frequency with which antimicrobial resistance genes disseminate between bacteria in humans, the environment and in animals is still debated. The role of plasmids in the movement between ecosystems, including the food chain, is also still contested, despite mounting evidence that it occurs.[36] Interestingly, isolates in the present study were obtained from vegetables, animal manure and irrigation water. Representative isolates from these sources demonstrated the

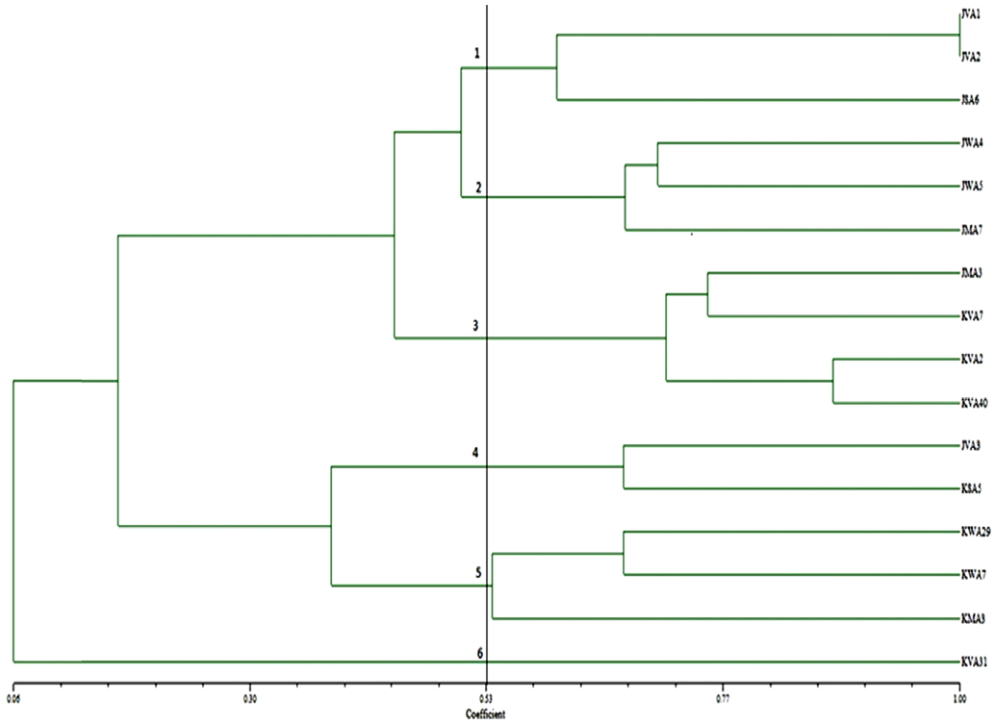

Figure 2. Dendrogram showing relatedness of *E. coli* O157:H7 from environmental isolates in the study.

Table 4. Diversity and relatedness of strains as shown by dendrogram

Clusters	Isolate codes	Source	Coefficient of similarity
Cluster 1			
	JVA1	Vegetables	
	JVA2	Vegetables	0.58
	JSA6	Soil	
Cluster 2			
	JWA4	Irrigation water	
	JWA5	Irrigation water	0.67
	JMA7	Manure	
Cluster 3			
	JMA3	Manure	
	KVA2	Vegetables	0.72
	KVA7	Vegetables	
	KVA40	Vegetables	
Cluster 4			
	JVA3	Vegetables	
	KSA5	Soil	
	KWA29	Irrigation water	0.42
	KWA7	Irrigation water	
	KMA3	Manure	
Singleton 5	KVA31	Vegetables	

K, Kano; J, plateau; V, vegetables; M/Soil , manure treated soil; W, irrigation water.

presence of antibiotic resistance plasmids. Hence, the results of the present study support the hypothesis that pCT is disseminated broadly between bacteria in animal and human ecosystems.[28] Antimicrobial resistance remains a serious global health concern.[37]

Molecular typing of bacteria has been widely used in epidemiological studies with respect to tracing the transmission of food-borne pathogenic bacteria[38] and the determination of relatedness among strains. In the present study, *E. coli* O157:H7 isolates obtained from vegetables were found in the same cluster as isolates obtained from environmental sources: irrigation water and soil/manure. Earlier studies have reported that isolates in the same cluster are genetically related and this indicates the possible contamination of pathogens.[38] In cluster 1, strains obtained from two different vegetable samples and soil samples were found, hence indicating their genetic relatedness. Isolates in clusters 2, 3 and 4 also comprised strains obtained from different samples; hence, these were genetically related at respective coefficients of similarity. These genetic similarities possibly indicate that the isolates obtained from vegetables could have resulted from the water used for irrigating the vegetables or the untreated manure used to supply nutrients to the vegetables for maximum yield. Isolate Kva31 clustered as a singleton, indicating that this isolate is genetically diverse[38] from other *E. coli* O157:H7 isolates obtained in the present study. The diversity observed could be a result of other sources of contamination to produce, such as wildlife. Based on the results of the present study, some isolates from different sources/samples may have originated from a single source and been transmitted through different routes to vegetables or from diverse sources, as shown by similar and differing banding patterns. Our results are in agreement with previous studies[39] reporting the similarity of fingerprints strains of *E. coli* O157:H7 obtained from different sources.

CONCLUSIONS

Antimicrobial resistance was observed in isolated organisms, indicating the role of the environment/irrigation water in emerging antibacterial resistance levels. The high level of antibiotic

resistance observed in the present study was most probably a result of the widespread and heavy use of numerous antimicrobial agents in human therapy, poultry and generally in livestock production. However, despite the ability of typing methods to discriminate and reveal close similarities between isolates from different sources in properly managed and poorly designed public health systems, technology cannot prevent cross-contamination. It is therefore suggested that the implementation of proper farm management techniques, routine surveillance of pathogens and proactive measures be undertaken to prevent cross-contamination of farm-to-fork infection.

Limitations of study

A limited number of representative isolates was used for the molecular aspect of the present study because it was undertaken outside the country of study at the Universiti Putra, Malaysia, which entailed a restricted research isolate size.

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