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Research Paper

Characterization of Multidrug-Resistant *Escherichia coli* Isolated from Two Commercial Lettuce and Spinach Supply Chains

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ABSTRACT

Leafy green vegetables have increasingly been reported as a reservoir of multidrug-resistant pathogenic *Enterobacteriaceae,* with Shiga toxin–producing *Escherichia coli* frequently implicated in disease outbreaks worldwide. This study examined the presence and characteristics of antibiotic resistance, diarrheagenic virulence genes, and phylogenetic groupings of *E. coli* isolates ($n = 51$) from commercially produced lettuce and spinach from farms, through processing, and at the point of sale. Multidrug resistance was observed in 33 (64.7%) of the 51 *E. coli* isolates, with 35.7% (10 of 28) being generic and 100% (23 of 23) being extended-spectrum β-lactamase/AmpC producing. Resistance of *E. coli* isolates was observed against neomycin (51 of 51, 100%), ampicillin (36 of 51, 70.6%), amoxicillin (35 of 51, 68.6%), tetracycline (23 of 51, 45%), trimethoprim-sulfamethoxazole (22 of 51, 43%), chloramphenicol (13 of 51, 25.5%), Augmentin (6 of 51, 11.8%), and gentamicin (4 of 51, 7.8%), with 100% (51 of 51) susceptibility to imipenem. Virulence gene *eae* was detected in two *E. coli* isolates from irrigation water sources only, whereas none of the other virulence genes for which we tested were detected. Most of the *E. coli* strains belonged to phylogenetic group B2 (25.5%; $n = 13$), B1 (19.6%; $n = 10$), and A (17.6%; $n = 9$), with D (5.9%; $n = 3$) less distributed. Although diarrheagenic *E. coli* was not detected, antibiotic resistance in *E. coli* prevalent in the supply chain was evident. In addition, a clear link between *E. coli* isolates from irrigation water sources and leafy green vegetables through DNA fingerprinting was established, indicating the potential transfer of *E. coli* from irrigation water to minimally processed leafy green vegetables.

HIGHLIGHTS

- Multidrug resistance was observed in 64.7% of all *E. coli* isolates.
- *E. coli* isolates predominantly belonged to phylogenetic group B2.
- There was a clear link between irrigation water and leafy green vegetable *E. coli* isolates.

Key words: *Escherichia coli*; Extended-spectrum β-lactamase/AmpC producing; Farm to fork; Food safety; Leafy green vegetables

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Leafy green vegetables are generally considered healthy; however, the increase in consumption of minimally processed, including ready-to-eat, leafy green vegetables increases the potential for human exposure to foodborne pathogenic bacteria through the food chain *(17, 38, 47)*. The increased demand for ready-to-eat leafy green vegetables has led to an increase in the production and expansion of the market for minimally processed vegetables *(10, 39)*. However, harvesting, preparation, processing, and distribution of leafy green vegetables have resulted in increased complexity in the supply chains and potential contamination with foodborne pathogenic bacteria *(3, 6, 18, 34, 36)*. Consequently, leafy green vegetables were ranked as the highest priority in terms of their microbiological hazard *(22)*.

Escherichia coli is a gram-negative bacterium belonging to the *Enterobacteriaceae* family *(23)* and has frequently been isolated from vegetables, including lettuce and spinach *(1, 41)*. Although most *E. coli* strains are commensal, some strains are pathogenic, containing virulence factors, and one diarrheagenic group called enterohemorrhagic *E. coli* is known as the main cause of foodborne illnesses *(32)*. Other subgroups include enterotoxigenic *E. coli,* enteroaggregative *E. coli,* enteroinvasive *E. coli,* and enteropathogenic *E. coli (39)*. The enterohemorrhagic *E. coli* serotype O157:H7 is a bacterial pathogen of concern linked to fresh produce contamination; it is found to be a highly pathogenic subset of Shiga toxin–producing *E. coli* and implicated in various gastrointestinal

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illnesses including bloody diarrhea in humans *(1, 27, 38, 42, 46)* and several highly publicized foodborne disease outbreaks *(10, 12, 41)*.

An additional concern is the increasing levels of antibiotic resistance, specifically the extended-spectrum β-lactamase (ESBL)/AmpC resistance reported for *E. coli* isolates from fresh vegetables and the production environment from farm to fork *(6, 44)*. Although *E. coli* is a natural inhabitant of the intestinal human gut *(23),* other strains of *E. coli* are a source of transfer of virulence and antimicrobial resistance genes to other enteric commensal and pathogenic bacteria *(38)*. Moreover, ESBL- and/or AmpC-producing *Enterobacteriaceae,* including *E. coli,* have been categorized as the most serious human health threats by the World Health Organization (WHO) *(51)*. Consequently, 2.8 million antibiotic-resistant bacterial infections were reported each year in the United States, resulting in 35,000 deaths *(11)*.

In addition to determining the antibiotic resistance profiles, virulence gene profiling and identifying the source of contamination by using phylotyping and enterobacterial repetitive intergenic consensus (ERIC)-PCR is also essential to develop science-based mitigation strategies *(13, 54)*. The quadruplex PCR method has proved very useful in assigning *E. coli* strains into one of these main phylogenetic groups: A, B1, B2, or D as proposed by Clermont et al. *(14)* and Herzer et al. *(25)*. Strains in each of the four groups differed according to their phylogenetic characteristics, with A and B2 predominantly associated with animals and the B1 strain most predominant in plants *(13)*. Extraintestinal pathogenic strains belong to groups B2 and D *(15, 29, 43)*; intestinal pathogenic strains belong to groups A, B1, and D; and commensal strains belong to groups A and B1 *(32, 43)*. In addition to phylogenetic grouping of *E. coli,* ERIC-PCR was found to be a useful method in establishing genetic diversity in various studies *(1, 30, 31)*. Abakpa et al. *(1)* found a link when characterizing *E. coli* isolates from leafy green vegetables, irrigation water, manure, and soil samples from vegetable-producing regions in the Kano and Plateau states in Nigeria by using ERIC-PCR. A limited number of studies exist for South Africa that link fresh produce and environmental sources through phenotypic and genotypic characterization of *E. coli* isolates. Moreover, because there is no fresh produce foodborne disease outbreak–reporting structure in South Africa and subsequently no data, this scoping study was conducted to determine the contribution of the environment (irrigation water and soil) to the occurrence and dissemination of potential human pathogenic bacteria such as ESBL/AmpC-producing *Enterobacteriaceae,* included in the WHO critical priority list of pathogens. The current study aimed to address this issue through determining antibiotic resistance profiles, virulence genes, and phylogenetic groups of *E. coli* and ESBL/AmpC-producing *E. coli* isolates from two commercial lettuce and spinach supply chains. An additional aim was to determine whether a link between *E. coli* isolates from irrigation water and leafy green vegetables could be established using ERIC-PCR fingerprinting.

MATERIALS AND METHODS

Site selection. Samples were collected from two commercial farms with onsite packhouses. All the sites were GlobalG.A.P. certified and produced both lettuce and spinach. Farm A was situated in a peri-urban area in Gauteng Province and drew its irrigation water from the Klip River, which is frequently contaminated with sewage spillage from an upstream municipal treatment plant. Farm B was situated in the North West Province and drew its irrigation water from the Hartbeespoort dam known to be contaminated with feces as a result of the presence of informal settlements with inadequate sanitation along the Jukskei River, which feeds into the dam. The samples were collected on two sampling occasions on each farm from May 2017 to June 2018, whereas the water, soil, and leafy greens were collected on the same day. Fresh produce (lettuce and spinach) sample collection points included the production field (at harvest) and in the packhouse. In the field, spinach was aseptically harvested using a sterile (with 70% ethanol) knife and made up as spinach bunches. In the packhouses, spinach bunches were washed in borehole water–containing wash bath (75 to 80 ppm of chlorine); thereafter, spinach was cut, dried, packed, labeled, and stored in a cold room (4°C) for ≤24 h. For lettuce, only the outer leaves were removed, cut, washed, dried, packaged, labeled, and stored in a cold room (4°C) for ≤24 h. After cold storage, spinach and lettuce were transported to the specific retailers or retailer distribution centers in Gauteng and North West provinces.

Sample collection. In total, 239 samples, including 68 lettuce and 68 spinach samples, 63 water samples, and 40 soil samples, were collected from farms A and B. Five samples of lettuce and spinach at harvest were aseptically collected in a zigzag pattern across the field, whereas three samples of each were collected at the entrance to the packhouse (at receive) and placed in brown paper bags. In addition, three samples of modified atmosphere–packaged lettuce and spinach were collected; these packages contained 98% nitrogen. Three modified atmosphere packages of lettuce and spinach also were purchased from retail outlets. The water samples were aseptically collected in three 1-L 70% ethanol-sterilized, air-dried plastic bottles *(18)* from farm A at four different points, which included river, reservoir, overhead irrigation pivot, and onsite packhouse washing water. Similar to farm A, water samples from farm B were collected at three different points that included reservoir, overhead irrigation pivot, and onsite washing water. Soil samples were aseptically collected in a zigzag pattern, corresponding to fresh produce collected in the field. All samples were transported cooled in a cooler box and stored at 4°C until analysis, within 24 h.

Microbiological analysis. The water samples were filtered through cellulose nitrate filters (0.45-μm pore size; Sartorius, Johannesburg, South Africa) that were subsequently placed into 50 mL of buffered peptone water (BPW; 3M, Johannesburg, South Africa), incubated at 37°C for 24 h, and used for further selective enrichment. A composite sample of 25 g consisting of 5 g from each of the five soil samples collected in the field was transferred into 225 mL of BPW. The soil sample in 225 mL of BPW was incubated at 37°C for 24 h and used for further selective enrichment. Individual samples were composed of three lettuce heads or three spinach bunches. The outer and inner leaves of each lettuce head and the spinach stalks were aseptically removed. Subsequently, lettuce and spinach were aseptically chopped and weighed, and 50 g of the leaf material was placed into a sterile polyethylene stomacher bag containing 200 mL of BPW and

macerated for 5 min at 230 rpm in a Stomacher 400 circulator (Seward Ltd., London, UK) *(52)*. The lettuce and spinach in the 200 mL of BPW was incubated at 37°C for 24 h and used for further selective enrichment.

Selective enrichment, isolation, and identification of *E. coli* and ESBL/AmpC-producing *E. coli* isolates. Each of the BPW samples was incubated at 37°C for 3 to 4 h, whereafter 1 mL of each of the samples was transferred to 9 mL of *Enterobacteriaceae* enrichment broth and incubated at 30°C for 24 h for selective enrichment of ESBL/AmpC-producing *E. coli* (Oxoid Ltd., Johannesburg, South Africa). A loopful of the enriched broth was subsequently streaked onto ChromID ESBL agar plates (bioMérieux, Midrand, South Africa) and incubated at 30°C for 24 h, as described by Blaak et al. *(7)*. All presumptive-positive ESBL/AmpC-producing *Enterobacteriaceae* colonies were isolated from the selective chromogenic media and purified. The macerated lettuce and spinach, irrigation water, and soil samples in BPW were incubated at 37°C for 24 h. Subsequently, a loopful (10 μL) of enriched sample was streaked out in duplicate on eosin methylene blue differential agar (Merck, Darmstadt, Germany) for the detection and isolation of *E. coli*. Isolates were purified, streaked onto nutrient agar, and incubated at 37°C for 24 h for identification purposes.

MALDI-TOF identification of purified isolates. Purified isolate identities, to species level, were determined using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF; Bruker Daltonics, Bremen, Germany) *(46)*. In brief, purified bacterial cultures grown on nutrient agar were transferred in duplicate directly to the MALDI-TOF–polished steel target plate and overlaid with α-cyano-4-hydroxycinnamic acid (Bruker Daltonics). The target plate was analyzed using microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics) in conjunction with Biotyper automation software and library (Bruker Daltonics). A score value between 1.700 and 1.999 indicated a reliable genus identification, a value of 2.0 indicated a reliable genus and probable species identification, and a value of 2.3 indicated a reliable genus and species identification.

Antibiotic resistance. The confirmed *E. coli* isolates ($n = 51$) were subjected to antibiotic susceptibility testing using the Kirby Bauer disk diffusion technique as described in the guidelines of Clinical and Laboratory Standard Institute (CLSI) *(16)*. Isolates were cultured in 9 mL of brain heart infusion broth (Merck) and incubated at 37°C for 24 h. Subsequently, 100 μL of each bacterial suspension was spread plated onto Mueller-Hinton agar plates; thereafter, antimicrobial disks were added and the plates were incubated at 37°C for 24 h (Merck). The *E. coli* isolates were screened for resistance against nine antibiotics belonging to six classes. Antibiotics used included ampicillin (10 μg), amoxicillin–clavulanic acid (20 μg/10 μg), amoxicillin (10 μg), trimethoprim-sulfamethoxazole/cotrimoxazole (1.25 μg/23.75 μg), imipenem (10 μg), tetracycline (30 μg), gentamycin (10 μg), and chloramphenicol (30 μg) (Mast Diagnostics, Randburg, South Africa) and neomycin (10 μg) *(26)*, and the cephalosporin class (cefpodoxime [10 μg], cefotaxime [30 μg], ceftazidime [30 μg], cefoxitin [30 μg], alone or in combination with clavulanic acid [10 μg]). Inhibition zone diameters were measured and compared with predetermined breakpoints according to the CLSI protocol *(16)* and European Committee on Antimicrobial Susceptibility Testing guidelines *(20)*. Results were then interpreted as sensitive, intermediate, and resistant. Isolates that were resistant to three or more different antibiotic classes were defined as demonstrating multidrug resistance (MDR).

DNA extraction for genotypic characterization. Single colonies of *E. coli* isolate were cultured in tryptone soy broth (Oxoid Ltd.) at 37°C for 24 h. Biomass was subsequently collected by centrifugation at 15,000 × *g* for 5 min. The DNA was extracted using the Zymo Quick-gDNA MiniPrep kit (Inqaba Biotech, Johannesburg, South Africa) according to manufacturer's instructions. The DNA concentration of each isolate was determined using Qubit Broad Range dsDNA kit in combination with a fluorometer (Thermo Fisher Scientific, Johannesburg, South Africa), according to the manufacturer's instructions.

Phylogenetic grouping. A quadruplex PCR was used to determine the phylogenetic groups of the *E. coli* strains, as described by Clermont et al. *(14)* using primers for the targeted gene fragments *chuA, yjaA, TspE4.C2,* and *arpA* (Table 1). The primers used were synthesized by Inqaba Biotech. For *E. coli* strains classified into group A or C or into group D or E by using the quadruplex method, a second PCR was performed to confirm whether the isolate belonged to group C (*chuA, yjaA, TspE4.C2,* and allele-specific *trpA*) or to group D (*chuA, yjaA, TspE4.C2,* and allele-specific *arpAgpE*) as described by Lescat et al. *(33)* (Table 1). In addition, the *Escherichia* clades I and II were determined using the *chuA, yjaA, Tspe4.C2,* and additional gene target *arpA*. The 25-μL PCR master mix reactions contained 1× Dream *Taq* green (Thermo Fisher Scientific), primers as outlined in Table 1, and 80 to 100 ng of DNA template. The following cycling conditions were used: initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 5 s, and annealing-extension at 59°C (quadruplex and group C) or 57°C (group E) for 20 s, and a final extension at 72°C for 5 min *(14)*. Thereafter, the amplicons were visualized using gel electrophoresis on a 2% agarose gel stained with Condasafe nucleic acid staining solution (Separations, Johannesburg, South Africa) at 120 V for 30 min followed by molecular imaging under UV in conjunction with the Image Lab software (Bio-Rad, Johannesburg, South Africa). The *E. coli* strains were assigned to phylogenetic groups as described by Clermont et al. *(13)* and Carlos et al. *(9)*.

Presence of virulence genes. The same *E. coli* isolates used for determining antibiotic resistance were tested for the presence of diarrheagenic virulence genes and the house-keeping *mdh* gene (internal PCR reaction control) as described by Omar and Barnard *(40)* (Table 1). The *E. coli* reference strains used as positive controls and primers (synthesized by Inqaba Biotech) used in the PCR reactions are outlined in Table 1. The PCR reactions in a 25-μL final reaction volume composed of 1× Dream *Taq* green PCR master mix (Thermo Fisher Scientific), primers, and 80 to 100 ng of extracted DNA template. The reactions were subjected to the following thermocycling conditions: initial denaturation step at 95°C for 15 min, followed by 35 cycles consisting of denaturing at 94°C for 45 s, annealing at 55°C for 45 s, extension at 68°C for 2 min, and final elongation at 72°C for 5 min. The PCR amplicons were visualized in a 2% agarose gel (TopVision agarose, Thermo Fisher Scientific), stained, and visualized as previously described.

Enterobacterial repetitive intergenic consensus: PCR fingerprinting. The ERIC-PCR fingerprints were generated for the same *E. coli* isolates used for antibiotic susceptibility testing and virulence gene detection, including the two reference strains *E. coli* O157:H7 (ATCC 35150) and *E. coli* 25922. The forward

TABLE 1. *Primer sequences for each gene target in PCR methods described by Omar and Barnard (40)*

Gene target	Primer[a]	Primer sequence (5′–3′)	Primer concn (μM)	Size (bp)	Reference culture	Reference
Phylogenetic group (quadruplex PCR)						
chuA	chuA.1b	ATG GTA CCG GAC GAA CCA AC	0.36	288	*E. coli* O157, *E. coli* 25922	*13*
	chuA.2	TGC CGC CAG TAC CAA AGA CA				
yjaA	yjaA.1b	CAA ACG TGA AGT GTC AGG AG	0.36	211		*14*
	yjaA.2b	AAT GCG TTC CTC AAC CTG TG				
TspE4.C2	TspE4.C2.1b	CAC TAT TCG TAA GGT CAT CC	0.36	152		
	TspE4.C2.2b	AGT TTA TCG CTG CGG GTC GC				
arpA	AceK.f	AAC GCT ATT CGC CAG CTT GC	0.48	400		
	ArpA1.r	TCT CCC CAT ACC GTA CGC TA				
trpA	trpAgpC.1	AGT TTT ATG CCC AGT GCG AG	0.36	219	*E. coli* O157	*33*
	trpAgpC.2	TCT GCG CCG GTC ACG CCC				
arpAgpE	ArpAgpE 1	GAT TCC ATC TTG TCA AAA TAT GCC	0.36	301		
	ArpAgpE 2	GAA AAG AAA AAG AAT TCC CAA GAG				
Virulence gene (multiplex PCR)						
LT toxin	*LT* (F)	GGC GAC AGA TTA TAC CGT GC	0.48	410	DSM 10973, DSM 27503	*40*
	LT (R)	CGG TCT CTA TAT TCC CTG TT				
ST toxin	*ST* (F)	TTT CCC CTC TTT TAG TCA GTC AAC TG	0.24	160		
	ST (R)	GGC AGG ATT ACA AAG TTC ACA				
bfpA	*bfpA* (F)	AAT GGT GCT TGC GCT TGC	0.25	324	DSM 8703, DSM 8710	*49*
	bfpA (R)	GCC GCT TTA TCC AAC CTG GTA				
pCVD4321AA probe	*AA PR* (F)	CTG GCG AAA GAC TGT ATC AT	0.36	630	DSM 27502	*5*
	AA PR (R)	AAT GTA TAG AAA TCC GCT GTT				
mdh	*Mdh* (F)	GGT ATG GAT CGT TCC GAC CT	0.36	300	*E. coli* O157, *E. coli* 25922	*40*
	Mdh (R)	GGC AGA ATG GTA ACA CCA GAG T				
eae	*eaeA* (F)	CTG AAC GGC GAT TAC GCG AA	0.72	917		
	eaeA (R)	GAC GAT ACG ATC CAG				
stx_1	stx_1 (F)	ACA CTG GAT CTC AGT GG	0.36	614		
	stx_1 (R)	CTG AAT CCC CCT CCA TTA TG				
stx_2	stx_2 (F)	CCA TGA CAA CGG ACA GCA GTT	0.36	779		
	stx_2 (R)	CCT GTC AAC TGA GCA CTT TG				
ipaH	*ipaH* (F)	GTT CCT TGA CCG CCT TTC CGA TAC CGT C	0.36	600	DSM 9034	*5*
	ipaH (R)	GCC GGT CAG CCA CCC TCT GAG AGT AC				
ERIC PCR	ERIC-1 (R)	ATG TAA GCT CCT GGG GAT TCAC	0.4		*E. coli* O157, *E. coli* 25922	*48*
	ERIC-2 (F)	AAG TAA GTG ACT GGG GTG AGCG				

[a] F, forward; R, reverse.

and reverse primer sequences were used as outlined in Table 1 *(37)*. The 25-μL PCR reactions were composed of 1× Dream *Taq* green PCR master mix, each primer with a final concentration of 0.4 μM and 80 to 100 ng of extracted DNA template. Thereafter, the PCR reactions were performed using the following cycling conditions: initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 1 min and extension at 72°C for 8 min, and a final extension at 72°C for 15 min *(48)*. The PCR amplicons were visualized in a 2% agarose gel as previously described, but run at 45 V for 5 h.

Statistical analysis. The ERIC-PCR DNA fingerprints were compared and analyzed with BioNumerics 7.6 (Applied Maths, Saint-Marten-Latem, Belgium). Percent similarities of digitized bands were calculated using the Pearson correlation coefficient and the unweighted pair group arithmetic mean, and complete linkage algorithms were used to derive a dendrogram. Relationships between the DNA fingerprints of each of the isolates are depicted as dendrograms.

RESULTS

Prevalence of *Escherichia coli*. In total, 51 *E. coli* isolates (Fig. 1; Supplemental Table S1), including 28 *E. coli,* were obtained from 17.4% (11 of 63) of the water samples, 11.8% (16 of 136) leafy green samples, and 2.5% (1 of 40) of the soil samples. After selective enrichment and streaking onto chromogenic media, 23 ESBL/AmpC-producing *E. coli* isolates were recovered from 25.4% (16 of 63) of water samples and 5.15% (7 or 136) leafy green samples, whereas none were recovered from the soil samples.

Antimicrobial resistant profiles. Of the 51 *E. coli* isolates screened, all isolates showed resistance to neomycin (100%), ampicillin (70.6%), amoxicillin (68.6%), tetracycline (45%), trimethoprim-sulfamethoxazole (43%), chloramphenicol (25.5%), Augmentin (11.8%), and gentamicin (7.8%) (Fig. 1; Table S1). The number of antibiotics to which isolates were resistant, the most frequent antibiotic resistance patterns, and the number of antibiotic classes for *E. coli* and ESBL/AmpC-producing *Enerobacteriaceae* are summarized in Tables 2 and 3. Thirty-three (64.7%) of 51 *E. coli* isolates were multidrug resistant, with 100% (23 of 23) ESBL/AmpC-producing *E. coli* exhibiting MDR and 35.7% (10 of 28) *E. coli* exhibiting MDR.

Phylogenetic grouping. Phylotyping showed that 25.5% ($n = 13$) of the 51 *E. coli* isolates belonged to group B2, 19.6% ($n = 10$) to group B1, and 17.6% ($n = 9$) to group A, followed by 5.9% ($n = 3$) to group D and 3.9% to groups E and F ($n = 2$ for each group). In addition, 19.6% ($n = 10$) belonged to clade I or II, with 3.9% ($n = 2$) of unknown grouping (Fig. 1; Table S1).

Presence of virulence genes. Of the 51 *E. coli* isolates screened for the presence of virulence genes, only 2 (3.8%) harbored the *eae* virulence gene, with one *E. coli* isolate (1.9%) from a river sample and one isolate (1.9%) from an irrigation pivot water sample. In addition, no other *E. coli* isolates harbored any of the other diarrheagenic virulence genes tested.

ERIC-PCR fingerprinting of *E. coli* isolates. Seven *E. coli* clusters resulted, based on 70% similarity: five distinct clusters and two single clusters (Fig. 1). Cluster A composed of several *E. coli* isolates from river water (B46, B47, B1, L34, B2, and L32), reservoir water (B17), irrigation pivot water (B26, B28, B62, L80, L28, B27, and B51), lettuce (B34, L4, B25, B35, B30, B33, and B31), and spinach (B44, B52, B37, B43, B42, B41, B39, B38, and B49), which clustered at >70% similarity, with L80 harboring the *eae* gene. In addition, *E. coli* isolates from lettuce (B34 and L4) from farm A showed genetic relatedness at 97% similarity, and both belonged to phylogenetic group B1; however, one isolate was an *E. coli* and the other was an ESBL/AmpC-producing *E. coli* isolate. Similarity at 98% was observed between *E. coli* isolates from lettuce (B25) and irrigation pivot water (B26) on farm B, belonging to the same phylogenetic group B1, showing a link between the water and lettuce samples. *E. coli* isolates from spinach (B37 and B43) on farm A revealed genetic relatedness at 100% and also clustered with B2 at 95% similarity, whereas all three belonged to phylogenetic group clade I or II and were generic. Moreover, the *E. coli* isolates from spinach (B41), river water (L34), and irrigation pivot water (L28) on farm A revealed genetic relatedness at 98%. This showed a link between the river, irrigation pivot water, and the *E. coli* from spinach; however, the phylogenetic groups and antimicrobial profiles were different. Isolates from river water (B2) and spinach (B38) from farm A revealed genetic relatedness at 92% and were both generic and belonged to phylogenetic group A, whereas the river isolate was multidrug resistant. Farm A *E. coli* isolates from spinach (B49) and irrigation pivot water (B51) showed genetic relatedness at 98% and were both generic, although they belonged to different phylogenetic grouping.

Cluster B consisted of *E. coli* isolates from river water (L50), reservoir water (L9 and L11), irrigation pivot water (L52 and L56), lettuce samples (L6), and spinach samples (B40, L59, L16, L14, and L15), which clustered at 86 to 98% similarity. *E. coli* isolates from spinach (B40) and irrigation pivot water (L52) from farm A clustered at 98% and belonged to phylogenetic group B2 and D, respectively. Similarly, *E. coli* isolates from spinach samples (L14 and L15) on farm B, which clustered at 98% similarity, also belonged to the same phylogenetic grouping B2. Cluster C composed of two *E. coli* isolates from reservoir water (B6 and L65) on farm A, which clustered at 78% similarity. Although from different farms, *E. coli* isolates from the reservoir water (L10) and lettuce (L3) samples clustered in cluster D at 96% similarity. In addition, cluster E composed of *E. coli* isolates from irrigation pivot water (L26 and L54), river water (L8), and reservoir water (40) on farm A, which were >80% similar, belonging to the same phylogenetic grouping B2. One *E. coli* isolate from irrigation pivot water (L26) on farm A clustered at 94%, with an isolate from river

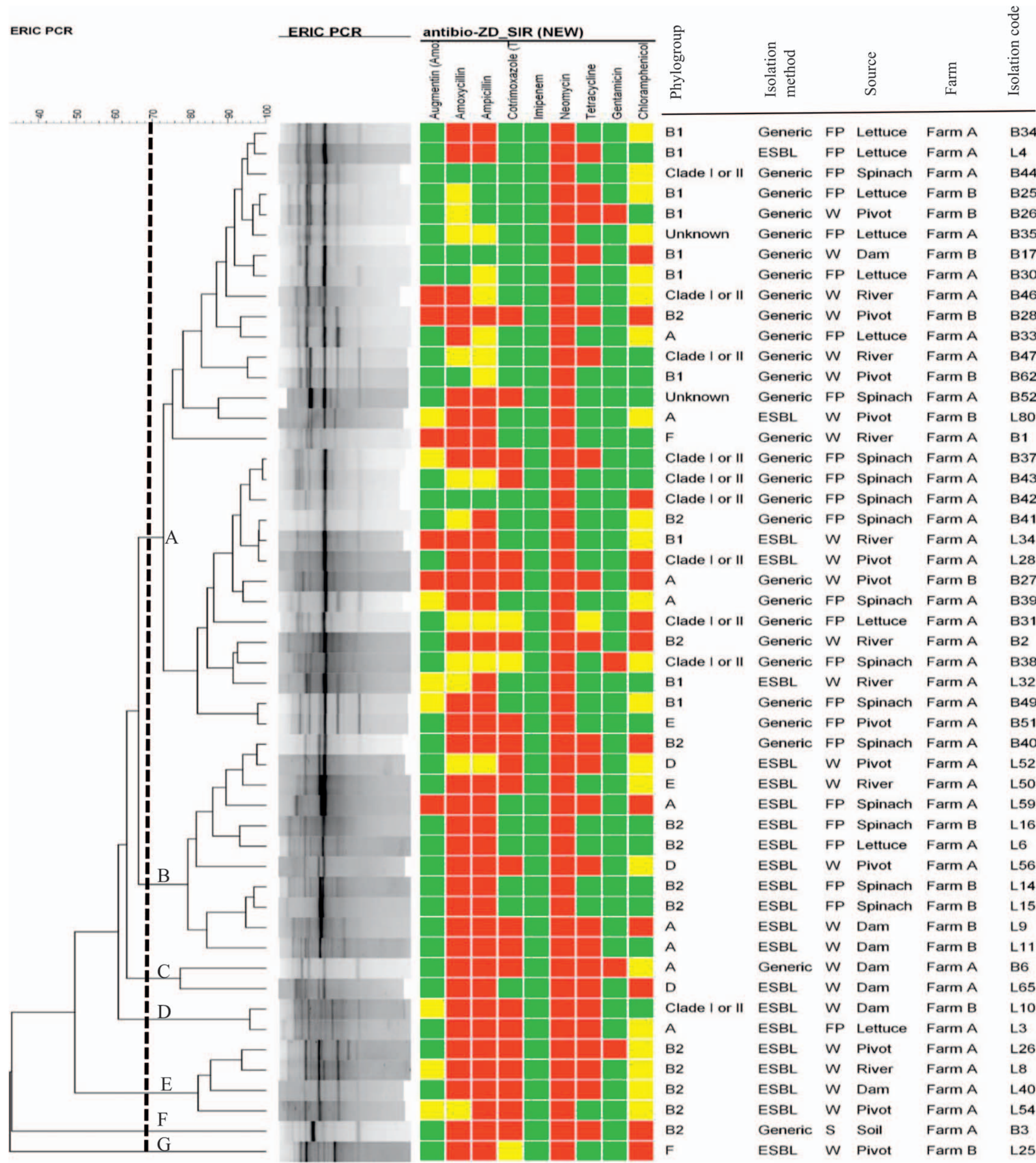

FIGURE 1. *Dendrogram showing the genetic relatedness of generic and extended spectrum- and AmpC β-lactamase–producing* Escherichia coli *isolated from the production environment (irrigation water and soil), lettuce, and spinach based on ERIC-PCR; the antibiotic resistance profiles; and the phylogenetic grouping of the isolates. Red block represents resistance; green, intermediate resistance; and yellow, susceptibility.*

water (L8) that harbored the *eae* gene. Clusters F and G were composed of single *E. coli* isolates from soil (B3) and irrigation pivot (L25), respectively.

DISCUSSION

In South Africa, several studies have focused mainly on the microbiological quality of leafy green vegetables at the point of sale *(19, 31, 45)*; however, it is important to understand the link between the *E. coli* isolated from leafy green vegetables and irrigation water used during production and processing. In this study, a high prevalence of *E. coli* was observed in irrigation water (52.9%), followed by leafy green vegetables (45.1%); prevalence was less frequent in soil samples (1.9%). The ERIC-PCR showed that *E. coli* isolates from irrigation water (river, reservoir, and irrigation pivot) and leafy green vegetables (lettuce and spinach) were closely related at similarity value >90%. Similar results were reported by Njage and Buys *(38),* who found a high degree of genetic relatedness between *E. coli* isolates from leafy green vegetables (lettuce) and irrigation water sources used in the vegetable production field. Njage and Buys *(38)* also suggested that genetic similarity between *E. coli* isolates from fresh produce clustering with isolates from irrigation water sources indicates possible contamination or pathway of transmission of pathogens. The findings in this study suggest a link and/or genetic relationship between *E. coli* isolates from irrigation water

TABLE 2. *Summary of the number of antibiotics to which* Escherichia coli *isolates from water, soil, and fresh produce were resistant, the most frequent antibiotic resistance patterns, and the number and details of antibiotic classes*

No. of antibiotics to which isolates were resistant	No. of isolates ($n = 28$)	No. of isolates/farm		No. of isolates with specific patterns	Most frequent antibiotic resistance patterns[a]	No. of antibiotic classes	Antibiotic class(es)
		Farm A	Farm B				
1	4	3	1	4	NE10C	1	Aminoglycosides
2	8	1	1	2	NE10C, T30C	2	Aminoglycosides, tetracycline
		2		2	NE10C, C30C	2	Aminoglycosides, phenicol
		1		1	A10C, NE10C	2	Penicillin, aminoglycosides
		1		1	NE10C, GE10C	1	Aminoglycosides
		1		1	AP10C, NE10C	2	Penicillin, aminoglycosides
		1		1	TS25C, NE10C	2	Sulfonamides, aminoglysides
3	6		1	1	NE10C, T30C, C30C	3	Aminoglycosides, tetracycline, phenicol
			1	1	NE10C, T30C, GE10C	2	Aminoglycosides, tetracycline
		3		3	A10C, AP10C, NE10C	2	Penicillin, aminoglycosides
		1		1	AUG30C, A10C, NE10C	2	Penicillin, aminoglycosides
4	3	2		2	A10C, AP10C, TS25C, NE10C	3	Penicillin, sulfonamides, aminoglycosides
		1		1	AUG30C, A10C, AP10C, NE10C	2	Penicillin, aminoglycosides
5	1	1		1	A10C, AP10C, TS25C, NE10C, T30C	4	Penicillin, sulfonamides, aminoglycosides, tetracycline
6	4	3		3	A10C, AP10C, TS25C, NE10C, T30C, C30C	5	Penicillin, sulfonamides, aminoglycosides, tetracycline, phenicol
		1		1	A10C, AP10C, TS25C, NE10C, T30C, GE10C	4	Penicillin, sulfonamides, aminoglycosides, tetracycline
7	2		2	2	AUG30C, A10C, AP10C, TS25C, NE10C, T30C, C30C	5	Penicillin, sulfonamides, aminoglycosides, tetracycline, phenicol

[a] NE10C, neomycin; T30C, tetracycline; C30C, chloramphenicol; AP10C, ampicillin; TS25C, trimethoprim-sulfamethoxazole; GE10C, gentamycin; A10C, amoxycillin; AUG30C, amoxycillin–clavulanic acid.

sources and leafy green vegetables, similar to results observed in previous studies *(1, 18, 30, 38)*.

The presence of the *eae* virulence gene from the river (farm A) and irrigation pivot water (farm B) was observed at a low percentage (3.8%), corresponding findings of du Plessis et al. *(18),* who detected the *eae* gene in irrigation water sources (river). Jongman and Korsten *(30)* detected 13.3% of stx_2 *E. coli* gene in irrigation water samples, although stx_1 and *eae* were not detected in leafy green vegetables and irrigation water samples. By contrast, none of the Shiga toxin–producing *E. coli* virulence genes (stx_1, stx_2, and *eae*) were detected from *E. coli* isolates tested in some studies that analyzed fresh produce and water samples *(8, 21)*.

Phylogenetic analysis showed that 25.5% of *E. coli* isolates were predominantly from phylogroup B2, although its distribution varied with sources (irrigation water at 9.8%, soil at 1.9%, and leafy green vegetables at 5.8% on farm A; on farm B, distribution was observed in 1.9% of irrigation water and 5.8% of leafy green vegetables) and less assigned in groups B1, A, and D. Similarly, Alwash and Al-Rafyai *(4)* found that group B2 *E. coli* isolates were most prevalent (50.8%) in irrigation water (Al-Hillah River), which supplies water in close-by agricultural sites. The phylogenetic groups B2 and A displayed phenotypes linked to animal-associated strains *(35),* suggesting that *E. coli* isolates distributed in human extraintestinal pathogenic strain B2 may have been as a result of fecal contamination *(9, 37)*. By contrast, du Plessis et al. *(19)* observed prevalence of 86% in *E. coli* isolates classified in phylogenetic group A obtained from spinach and cabbage purchased at vendors and retail stores in Johannesburg. Gritli et al. *(23)* also found that most of the *E. coli* isolates (74%) from leafy green vegetables (lettuce) belonged to phylogenetic groups A and B1. Therefore, the study suggests that the *E. coli* isolates grouped in phylogenetic group B2 obtained from leafy green vegetables (lettuce and spinach) could have originated from environmental sources (irrigation water sources and soil) in both commercial farms.

In this study, *E. coli* resistance to ampicillin and amoxicillin was similar to that documented by Faour-Klingbeil et al. *(21),* who observed 78% resistance of *E. coli* isolates against ampicillin (raw vegetables). This contrasts to a previous study conducted in Belgium where low resistance (7%) of *E. coli* isolates to ampicillin from lettuce samples, irrigation water, and soil were recorded *(27)*. Similar to our findings, du Plessis et al. *(19)* found 100% *E. coli* resistance to neomycin in spinach and cabbage from informal and retail market, whereas the current study found

TABLE 3. *Summary of the number of antibiotics to which ESBL/AmpC-producing* Escherichia coli *isolates from water, soil, and fresh produce were resistant, the most frequent antibiotic resistance patterns, and the number and details of antibiotic classes*

No. of antibiotics to which isolates were resistant	No. of isolates ($n = 23$)	No. of isolates/farm		No. of isolates with specific patterns	Most frequent antibiotic resistance patterns[a]	No. of antibiotic classes	Antibiotic classes
		Farm A	Farm B				
5	5		4	4	A10C, AP10C, NE10C, CPD10C, CTX30C	3	Penicillin, aminoglycosides, cephalosporins
		1		1	AP10C, NE10C, CPD10C, CAZ30C, CTX30C	3	Penicillin, aminoglycosides, cephalosporins
6	4	1		1	A10C, AP10C, NE10C, T30C, CPD10C, CTX30C	4	penicillin, aminoglycosides, tetracycline, cephalosporins
		1		1	A10C, AP10C, NE10C, CPD10C, CAZ30C, CTX30C	3	Penicillin, aminoglycosides, cephalosporins
		1		1	TS25C, NE10C, T30C, CPD10C, CAZ30C, CTX30C	4	Sulfonamides, aminoglysides, tetracycline, cephalosporins
		1		1	AP10C, TS25C, NE10C, CPD10C, CAZ30C, CTX30C	4	Penicillin, sulfonamides, aminoglycosides, cephalosporins
7	7	1	2	3	A10C, AP10C, TS25C, NE10C, T30C, CPD10C, CTX30C	5	Penicillin, sulfonamides, aminoglycosides, tetracycline, cephalosporins
		1		1	A10C, AP10C, TS25C, NE10C, CPD10C, CAZ30C, CTX30C	4	Penicillin, sulfonamides, aminoglycosides, cephalosporins
		1		1	A10C, AP10C, NE10C, T30C, C30C, CPD10C, CTX30C	5	Penicillin, aminoglycosides, tetracycline, phenicol, cephalosporins
			1	1	A10C, AP10C, NE10C, C30C, CPD10C, CTX30C, FOX30C	4	Penicillin, aminoglycosides, phenicol, cephalosporins
		1		1	AUG30C, A10C, AP10C, NE10C, CPD10C, CAZ30C, CTX30C	3	Penicillin, aminoglycosides, cephalosporins
8	3	1		1	A10C, AP10C, TS25C, NE10C, T30C, CPD10C, CAZ30C, CTX30C	5	Penicillin, sulfonamides, aminoglycosides, tetracycline, cephalosporins
			1	1	A10C, AP10C, TS25C, NE10C, T30C, C30C, CPD10C, CTX30C	6	Penicillin, sulfonamides, aminoglycosides, tetracycline, phenicol, cephalosporins
		1		1	A10C, AP10C, TS25C, NE10C, T30C, GE10C, CPD10C, CTX30C	5	Penicillin, sulfonamides, aminoglycosides, tetracycline, cephalosporins
9	4	1		1	AUG30C, A10C, AP10C, NE10C, T30C, C30C, CPD10C, CAZ30C, CTX30C	5	Penicillin, aminoglycosides, tetracycline, phenicol, cephalosporins
		2		2	A10C, AP10C, TS25C, NE10C, T30C, CPD10C, CAZ30C, CTX30C, FOX30C	5	Penicillin, sulfonamides, aminoglycosides, tetracycline, cephalosporins
		1		1	A10C, AP10C, TS25C, NE10C, C30C, CPD10C, CAZ30C, CTX30C, FOX30C	5	Penicillin, sulfonamides, aminoglycosides, phenicol, cephalosporins

[a] A10C, amoxycillin; AP10C, ampicillin; NE10C, neomycin; T30C, tetracycline; C30C, chloramphenicol; CPD10C, cefpodoxime; CTX30C, cefotaxime; FOX30C, cefoxitin; GE10C, gentamycin; AUG30C, amoxycillin–clavulanic acid; CAZ30C, ceftazidime; TS25C, trimethoprim-sulfamethoxazole.

resistance to neomycin in *E. coli* isolates throughout the leafy green supply chains. Neomycin is a drug that is often used to reduce risk during surgery and to treat other bacterial infections *(24)*. This is a concern because antibiotic resistance of *E. coli* is increasingly becoming a public health problem both in developed and developing countries *(21)*. The current study also found low prevalence of resistance to gentamicin at 7.8%, lower than the 22% resistance of *E. coli* isolated from raw vegetables (lettuce, parsley, cucumber, and tomato; mint) reported by Faour-

Klingbeil et al. *(21)*. Additional resistance of *E. coli* isolates to tetracycline (45%), trimethoprim-sulfamethoxazole (43%), and chloramphenicol (25.5%) was observed in the current study, although resistance was low against Augmentin (11.8%).

All strains in this study were resistant to one or more antibiotic classes, with 64.7% of the *E. coli* isolates exhibiting MDR throughout the supply chain (river, reservoir, irrigation pivot point, soil, and lettuce and spinach). An increase in *E. coli* strains becoming resistant to multiple antibiotics (35.7%) is emerging, and this has been observed by the MDR in this study. However, MDR prevalence of 100% observed in ESBL/AmpC-producing *E. coli* in this study calls for concern, because antibiotic resistance owing to ESBL/AmpCs is a major public health concern *(28, 53)*. This is because ESBL/AmpCs are capable of breaking down β-lactam antibiotics, thereby rendering them ineffective *(50)*. Faour-Klingbeil et al. *(21)* also reported 60% prevalence of *E. coli* strains from different points of the supply chain (irrigation water and vegetables) resistant to more than three antibiotics in a study carried out in Lebanon. A corresponding study found that 70.7% of *E. coli* isolated from irrigation water and leafy green vegetables were resistant to various antibiotics including ampicillin, cefoxitin, and nalidixic acid *(30)*. This clearly indicates that leafy green vegetables may become contaminated with antibiotic-resistant *E. coli* strains during production and harvesting *(27)*. The increase antibiotic resistance of gram-negative *E. coli* strains isolated throughout the food chain may result in bacterial infections that prove difficult to treat, leading to high treatment cost and creating a burden on the health care system *(28, 50)*. However, no noticeable association of results for the ERIC-PCR profiles, phylogenetic groups and antimicrobial resistance profiles of the isolates tested were evident, similar to the results reported by Adamus-Bialek et al. *(2)*.

The findings herein show a clear link between multidrug resistant, including ESBL/AmpC-producing *E. coli,* isolates detected in irrigation water and leafy green vegetable samples through the supply chains; thus, further investigations are needed with a greater number of samples and isolates. These results emphasize the role of irrigation water in the potential transmission of human pathogens to leafy green vegetables eaten raw, which may constitute a health risk to the consumer.

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SUPPLEMENTAL MATERIAL

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