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Research Article

Cucumber Production System as a Reservoir for Clinically Important Extended-Spectrum Beta-Lactamase- and AmpC Beta-Lactamase-Producing *Escherichia coli* and *Klebsiella pneumoniae* Strains

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This study evaluated clinically significant extended-spectrum β-lactamase (ESBL)- and AmpC β-lactamase (AmpC)- producing *Escherichia coli* and *Klebsiella pneumoniae* in a cucumber production system, focusing on their potential sources and public health implications. Samples from water ($n = 16$) and cucumber ($n = 12$) yielded 40 potentially pathogenic isolates (*E. coli*: $n = 17$, *K. pneumoniaen* = 23), identified through matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) analysis. All *E. coli* and *K. pneumoniae* isolates were phenotypically (Kirby Bauer disk diffusion) and genotypically [whole genome sequencing (WGS) (Illumina MiSeq)] characterized as multidrug resistant. Clinical relevance of these isolates is underscored by the pathogen probabilities (0.925–0.944 for *E. coli* and 0.534-0.897 K. *pneumoniae* isolates). The detection of *K. pneumoniae* ST985 in both the water source (Hartebeespoort Dam and preparation reservoir) and on the irrigated cucumbers highlights the importance of identifying the source of origin for more effective management and shows the potential that agricultural production environments can serve as conduits for the dissemination of clinically relevant antimicrobial-resistant bacteria. Understanding the presence of these clinically relevant organisms and antimicrobial resistance within the food system can lead to improved mitigation, on farm management and public health protection.

Keywords: antimicrobial resistance; cucumbers; Enterobacterales; food safety; multidrug resistance; one food; one health; whole genome sequencing

1. Introduction

The widespread emergence of antimicrobial resistance (AMR) is currently considered one of the most serious global public health concerns [1]. In addition, the diversity of antimicrobial-resistant bacteria (ARB) as well as the variety of sources from where they have been isolated further exacerbate the global threat to public health. Antimicrobial resistance genes (ARGs) are readily disseminated between different bacterial species by mobile genetic elements (MGEs) such as plasmids and insertion sequences (IS) [2, 3]. As a result, bacterial strains showing broader resistance emerge, making it more difficult for clinicians to select antibiotics to treat multidrug-resistant human pathogenic bacterial infections [2–4].

Escherichia coli (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) are pathogens that cause healthcare-associated infections such as pneumonia, nosocomial, and urinary tract infections, sepsis, meningitis, and respiratory infections [5, 6]. Clinically significant antibiotic-resistant *E. coli* and *K. pneumoniae* include extended-spectrum β-lactamase (ESBL)- and AmpC β-lactamase (AmpC)-producing

strains. These strains are often implicated in multidrug-resistant nosocomial infections and can hydrolyze penicillins, cephalosporins, and aztreonam [5]. In addition, genetically diverse and highly mobile ESBL gene families (bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$) have increasingly been reported as dominant [7, 8]. Furthermore, these strains are no longer restricted to healthcare systems but are now more frequently being isolated from fresh produce, agricultural environments and informal settlements [7, 9]. As an example, ESBL- and AmpC-producing *E. coli* ST10 and ST58, harboring ESBL and AmpC genes have been isolated from irrigation water and spinach [10, 11]. The World Health Organization (WHO) has listed ESBL-producing Enterobacterales (including *E. coli* and *K. pneumoniae*) as pathogens of critical importance in research and development programs [7, 12].

Globally, proactive solutions to slow down drug resistance include governments placing emphasis on the improvement of surveillance systems by evaluating and enhancing current diagnostic tools and methods (Centers for Disease Control and Prevention [CDC]; [13]). Until recently, surveillance studies were mostly based on phenotypic (e.g., double-disk synergy testing) and genotypic (e.g., PCR) characterization methods [14, 15]. However, the accuracy and efficiency of these methods in providing information about the genes conveying resistance or the MGEs associated with these genes were found to be insufficient [15, 16]. Whole genome sequencing (WGS) and high throughput bioinformatic analysis are therefore increasingly used to characterize bacteria (CDC; [16]), as complete bacterial genotypic characterization can provide more rapid and accurate information. Moreover, the potential for bacteria to express AMR and the routes of transmission of ARGs across an environment can be more readily determined with accurate tracking of ARGs and MGEs [14, 15]. However, access to affordable sequencing facilities and extensive training in bioinformatics and data interpretation required for WGS may be a constraint in some countries, particularly developing ones [12].

In South Africa (SA), information about the occurrence and dissemination of ESBL- and AmpC-producing bacteria and ARGs in fresh produce production and processing systems has been reported [17–19]. However, more data on the occurrence of AMR bacteria and ARGs in the agroecosystem is needed. This study aims to characterize ESBL-producing *E. coli* and ESBL- and/or AmpC-producing *K. pneumoniae* isolates from a cucumber production system using WGS to determine their AMR, serotypes, MGEs, pathogenicity probability and multilocus sequence types (MLSTs). This study therefore aimed to isolate, characterize, and determine the potential sources of AMR isolates within a cucumber production system.

2. Experimental Procedures

2.1. Study Area. This scoping study focused on a commercial cucumber production system in the North-West Province SA. The farm produced cucumbers in a greenhouse and had an on-site packhouse. The site was selected due to its dependence on irrigation water from the Hartebeespoort Dam, which is known to exceed irrigation water quality guidelines and is regularly exposed to untreated sewage effluents [20]. The commercial farm draws water from the dam before being channeled to a main holding reservoir via a cement canal. From the main holding reservoir, water is pumped into a holding preparation reservoir prior to irrigation. Postharvest processing of cucumbers on the farm included hand sorting (no washing), followed by packing, using plastic shrink wrap and then cold storage (10°C, ≤ 24 h), before transportation to the retail distribution center in Johannesburg, Gauteng Province, SA.

2.2. Sample Collection. A total of 16 water and 12 cucumber samples (three cucumbers per sample) were collected from the farm. The site was visited twice (in June and November 2018). Samples were collected according to what was available on the day of the visit. Four water samples (1 L each) were collected in sterile plastic bottles from each of the following points, which fed into the farm's irrigation system: the Hartbeespoort Dam, canal, holding reservoir, and the preparation reservoir. A cucumber sample was made up of a composite of three individual cucumbers. Three cucumber samples were collected from the greenhouses, six from the farm packhouse before packing, and three after wrapping. The cucumbers were collected aseptically using disposable sterile gloves and placed in brown paper bags. All the samples collected were transported cooled in cooler boxes and were stored at 4°C until further processing, which was within 24 h.

2.3. Sample Processing. Water samples (1 L × 16) were filtered through 0.45 μm nitrocellulose filters (Sartorius Stedim Biotech, Göttingen, Germany). The filters were then placed in 50 mL of buffered peptone water (BPW) (Biolab, Johannesburg, SA) for pre-enrichment purposes. Each cucumber sample (150 g) was placed in a sterile polyethylene strainer stomacher bag containing 150 mL BPW (1:1 weight-to-volume ratio) [21] and macerated for 5 min in a Stomacher 400 circulator paddle blender (Seward Ltd., West Sussex, United Kingdom).

2.4. Identification of ESBL- and/or AmpC-Producing E. coli and K. pneumoniae. The samples in BPW were incubated for 3–4 h at 37°C after which 1 mL was added to 9 mL Enterobacterales enrichment broth (Oxoid Ltd., Basingstoke, United Kingdom) according to ISO 21528-1:2004 and incubated overnight at 30°C [18, 22]. Presumptive ESBL- and/or AmpC-producing microorganisms were detected by streaking 10 μL of each of the enriched samples onto chromogenic ESBL ChromID media (Biomerieux, Johannesburg, SA), followed by overnight incubation at 30°C [18, 22]. All presumptive positive ESBL- and/or AmpC-producing *E. coli* and *K. pneumoniae* colonies based on colony color were isolated and purified on ChromID.

The identities of presumptive ESBL- and/or AmpC-producing isolates were confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) (Bruker, Bremen, Germany) analysis [18, 23]. Briefly, a single colony of an isolate grown and purified on ESBL ChromID and grown on nutrient agar (Biolab) was

transferred to the MALDI-TOF polished steel target plate and further analyzed according to manufacturer's instructions (AOAC-OMA#2017,09), following calibration with the bacterial test standard [18].

2.5. Antimicrobial Susceptibility Testing. After identification, 40 presumptive ESBL- and/or AmpC-producing Enterobacterales (including *E. coli* and *K. pneumoniae*) isolates were selected for further analysis. All isolates were screened for ESBL- and/or AmpC- production by the double-disk synergy test using 30 μg cefotaxime with and without 10 μg clavulanic acid, 30 μg ceftazidime with and without 10 μg clavulanic acid, and 30 μg cefpodoxime with and without 10 μg clavulanic acid (Davies Diagnostics (Pty), Randburg, SA), according to the manufacturer's instructions (European Committee on Antimicrobial Susceptibility Testing (EUCAST), [24]). Production of ESBLs was confirmed using the cefepime ESBL disc set (Cefepime 30 μg, cefepime-clavulanic acid 30 μg/10 μg,). Isolates showing resistance to cefoxitin and cefotaxime or ceftazidime were regarded as phenotypic indicators of AmpC production [24]. The AmpC production was confirmed using the AmpC detection set (Davies Diagnostics) [25, 26]. *K. pneumoniae* ATCC700603, *E. coli* NCTC13351, and *Enterobacter cloacae* NCTC1406 were used as positive controls and *E. coli* ATCC25922 was included as a negative control for ESBL- and AmpC- production [18].

Further evaluation of the isolates' antibiotic susceptibility was performed using the Kirby–Bauer disk diffusion method, following the guidelines of the Clinical and Laboratory Standards Institute [25]. Eight additional antibiotic classes were tested: ampicillin (10 μg), amoxicillin-clavulanic acid/augmentin (20/10 μg), amoxicillin (10 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), cefoxitin (30 μg), cefepime (30 μg), imipenem (10 μg), tetracycline (30 μg), gentamicin (10 μg), and chloramphenicol (30 μg) (Davies Diagnostics) [18, 25]. Breakpoints were interpreted according to CLSI [25] and the European Committee on Antimicrobial Susceptibility Testing [26] guidelines for Enterobacteriaceae. Based on these, results were categorized as susceptible, intermediate, or resistant. Isolates showing resistance to three or more antibiotic classes were classified as multidrug-resistant (MDR) [18].

2.6. WGS of Selected Isolates. The 40 isolates (17 *E. coli*—one from cucumbers and 16 from irrigation water samples and 23 K. *pneumoniae*—four from cucumbers and 19 from irrigation water samples) were further characterized with WGS. These were cultured overnight in TSB at 37°C (Oxoid) and gDNA extraction was performed using the DNeasy PowerSoil Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). The concentration of the gDNA was measured using the double stranded DNA Broad Range Assay and a Qubit 2.0 fluorometer (Life Technologies, Phoenix, SA) and purity using Nanodrop (ThermoFisher Scientific, Johannesburg) according to manufacturer's instructions.

2.7. Genome Sequencing and Analysis. Multiplexed paired-end libraries (2 × 300 bp) were prepared using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, United States) and sequenced on the Illumina MiSeq platform at 100× coverage at the National Institute of Communicable Diseases Sequencing Core Facility [11]. Raw reads were quality-checked, trimmed, and *de novo* assembled into contigs using CLC Genomics Workbench version 10 (QIAGEN).

The detection of ARGs, MLSTs, pathogen probability, and MGEs were determined by querying the databases ResFinder, MLST 2.0, PathogenFinder, and MGE, respectively, on the Centre for Genomic Epidemiology (CGE) (https://genomicepidemiology.org/services/). Additionally, *E. coli* and *in silico* serotypes were identified by querying the CGE SeroTypeFinder databases, respectively. Moreover, the capsule polysaccharide-based serotyping (K-type) of *K. pneumoniae* isolates was conducted using Kaptive Web [27].

3. Results

3.1. Microbiological Analysis. Of the *E. coli* ($n = 17$) and *K. pneumoniae* ($n = 23$) from the water and cucumber samples, 87.50% ($n = 35$) of the isolates were from water and 12.50% ($n = 5$) were from cucumbers. Isolates obtained from water were from Hartebeespoort Dam ($n = 20$), the canal ($n = 11$), the holding reservoir ($n = 2$), and preparation reservoir ($n = 2$) (Figures 1 and 2). Of the 35 water isolates, 16 were *E. coli* and 19 were *K. pneumoniae*. *E. coli* ($n = 1$) and *K. pneumoniae* ($n = 4$) isolates were from cucumbers before packing.

3.2. Phenotypic AMR Profiling of E. coli and K. pneumoniae Isolates. In total, 70% ($n = 28$) of the isolates tested positive for ESBL-production, while 27.50% ($n = 11$) of the isolates tested positive for both ESBL- and AmpC-production, and only 2.50% ($n = 1$) isolate was positive for AmpC-production (Figures 1 and 2).

All *E. coli* were classed as MDR (resistance to ≥ 3 antibiotic classes), with 100% resistant to amoxicillin, ampicillin, tetracycline, cefpodoxime, cefotaxime, and cefepime (Figure 1). High levels of resistance (94.12%) to ceftazidime-avibactam and trimethoprim sulfamethoxazole/cotrimoxazole were seen. Furthermore, resistance to gentamycin (70.59%), chloramphenicol (52.94%), amoxillin-clavulanic acid (41.18%), and imipenem (5.88%) were recorded.

All *K. pneumoniae* were classed as MDR, with 100% resistant to amoxycillin, ampicillin, cefpodoxime, cefotaxime, and cefepime (Figure 2). All *K. pneumoniae* were not resistant to imipenem. High levels of resistance to tetracycline and trimethoprim sulfamethoxazole/cotrimoxazole (95.65%) were recorded. Furthermore, resistance to ceftazidime-avibactam and gentamycin (82.61%), amoxillin-clavulanic acid (69.57%), and chloramphenicol (43.48%) was also found in the *K. pneumoniae* isolates.

3.3. Detection of Antibiotic Resistance Genes. Water samples from the Hartebeespoort dam, canal, and holding reservoir together with a cucumber sample from the packhouse before packing contained 17 β-lactam producing *E. coli* isolates (Figures 1 and 3). The β-lactam genetic determinants (e.g.,

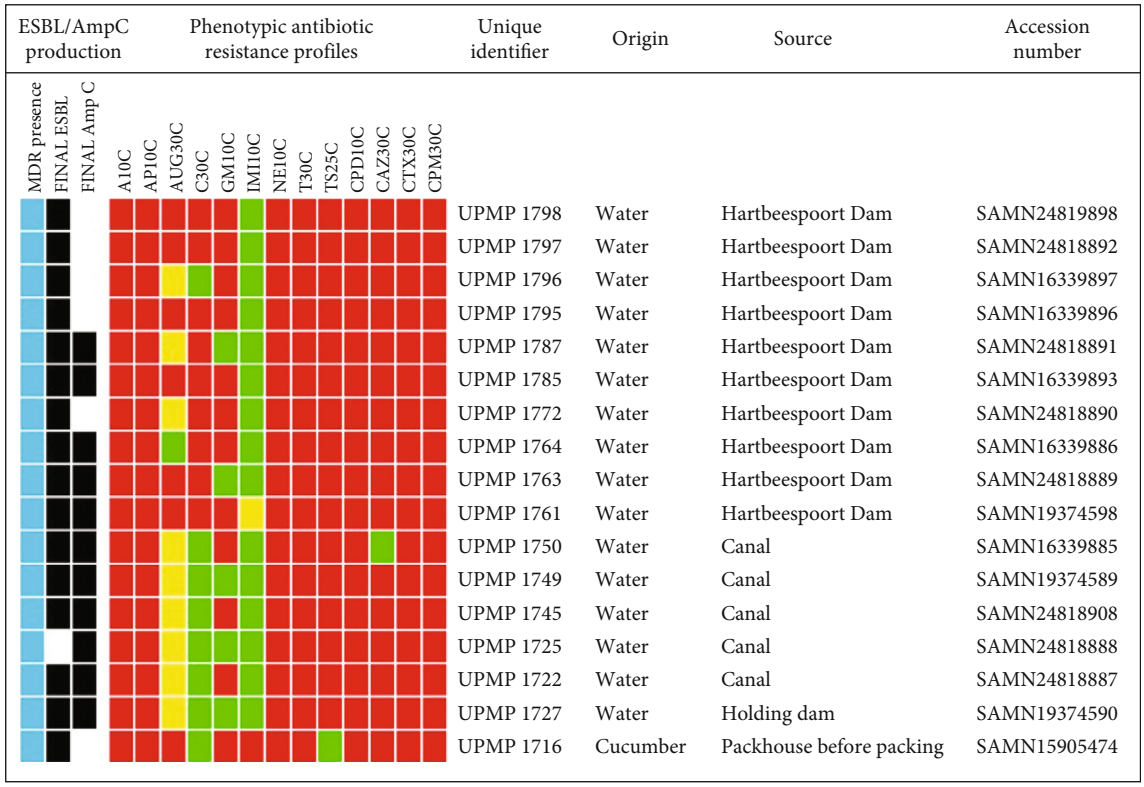
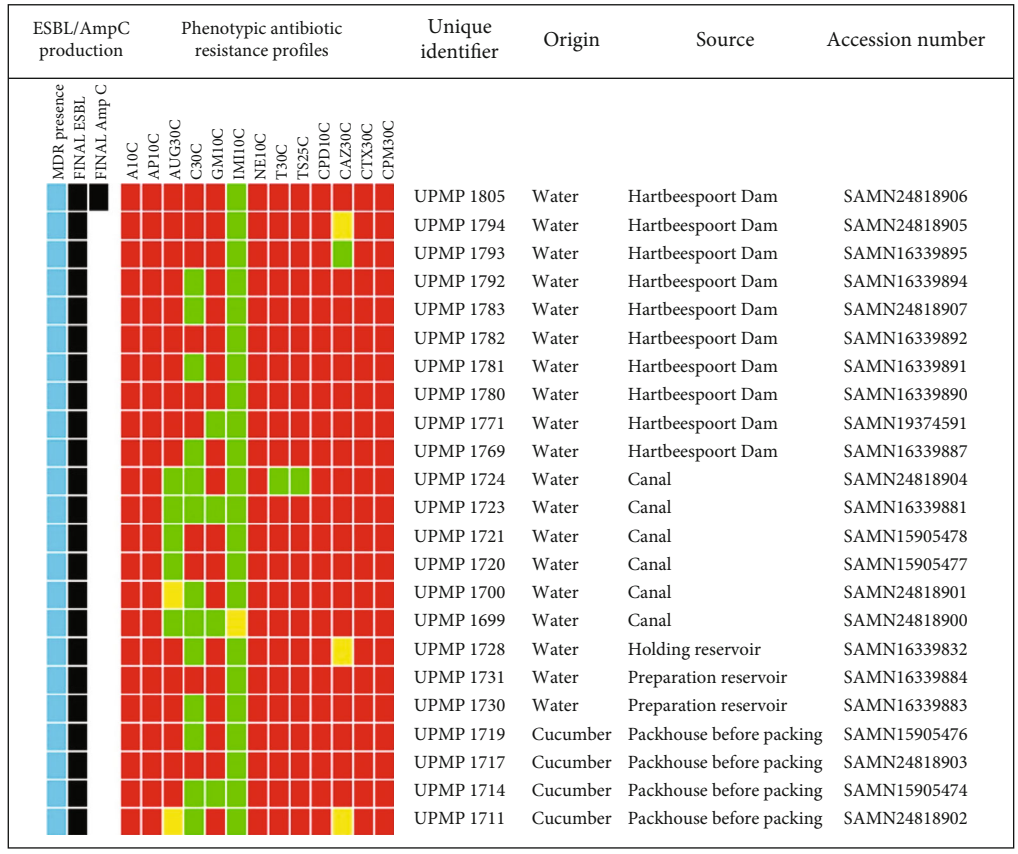


Figure 1: Summary of the *Escherichia coli* isolated from a cucumber agroecosystem. White indicates a negative phenotype for multidrug resistant and ESBL- or AmpC- production, blue a multidrug resistant phenotype, black an ESBL- or AmpC- producing phenotype. Red indicates a resistant phenotype, yellow an intermediate phenotype and green a susceptible phenotype.

blaCTX-M-14, blaCTX-M-15, and *blaTEM-1B)* were predominantly detected in the *E. coli* isolates from water samples (Figures 3 and 4). From the 10 *E. coli* isolates from Hartebeesport Dam, *blaCTX-M-15* was the most prevalent *β*-lactam gene detected (Figure 4). One isolate from Hartebeesport Dam (UPMP1796) contained 10 different *β*-lactam genes (Figure 4). Moreover, in the five *E. coli* isolates from the canal water, *blaCTX-M-14* predominated (Figure 4). The one *E. coli* isolated from cucumbers (UPMP1716) contained only *blaCTX-M-15* (Figure 4). Resistance to Aminoglycosides was determined in 12 isolates (Figure 3), although aminoglycoside resistance determinants were detected in 13 isolates. These ARGs included *aadA1*, *aadA2b*, *aph (3″)-Ib*, and *aph (6)-Id* (Figure 4). Fosfomycin resistance gene (*fosA3*) was detected in only five *E. coli* isolates from all water sources (Figures 3 and 4). Phenicol gene *cmlA1* was identified only in Hartebeespoort Dam water isolates (Figures 3 and 4). Seven isolates demonstrated co-occurrence of *cmlA1*, *sul3*, *tetA*, and *dfrA1*, all these isolates originated from Hartebeespoort Dam water (Figure 4). All 17 isolates were phenotypically resistant to tetracycline; however, only 10 isolates carried tetracycline resistant genes (*tetA*) (Figure 4).

The Hartebeespoort Dam, canal, holding reservoir, and preparation reservoir water together with cucumbers before packing contained 23 *β*-lactam producing *K. pneumoniae* isolates. Antibiotic resistance genes for *β*-lactam, Aminoglycoside, Quinolones, Fosfomycin, Phenicols, Sulfonamide, Tetracycline, and Trimethoprim were found in isolates from all sample type (Figure 5). The ARGs harbored by *K. pneumoniae* isolates have been detailed in Figure 6. Predominant *β*-lactam resistance genes in the *K. pneumoniae* isolates included *blaTEM-1B* followed by *blaCTX-M-15* and *blaSHV-187* as the most frequent genes detected. Five canal water *K. pneumoniae* isolates showed co-occurrence to multiple *blaTEM* genes. Only 10 *K. pneumoniae* isolates from Hartebeespoort Dam demonstrated a phenotypic resistance to the Aminoglycoside tested (gentamycin). Overall, 21 *K. pneumoniae* isolates demonstrated co-occurrence of Aminogylcoside resistance genes *aph (3″)-Ib* and *aph (6)-Id*. Moreover, co-occurrence of Quinolones resistance genes *oqxA*, *oqxB*, and *qnrB1* was seen in 16 isolates. Fosfomycin resistance gene (*fosA*) was present in 22 *K. pneumoniae* isolates. Phenicol ARGs were the least frequently harbored by isolates, solely originating from Hartebeespoort Dam (Figure 6). Whereas chloramphenicol ARGs were harbored by six *K. pneumoniae* isolates originating from Hartebeespoort Dam (four also demonstrated phenotypical resistance to chloramphenicol). The majority of *K. pneumoniae* isolates (22/23) harbored at least one tetracycline ARG and 22 were phenotypically resistant to tetracycline. Similarly, 22 *K. pneumoniae* isolates harbored at least one trimethoprim ARG and 22 were phenotypically resistant to trimethoprim sulfamethoxazole/cotrimoxazole. A total of 21 isolates harbored at least one sulfonamide ARG.



Unique identifier	Origin	Source	Accession number
UPMP 1805	Water	Hartbeespoort Dam	SAMN24818906
UPMP 1794	Water	Hartbeespoort Dam	SAMN24818905
UPMP 1793	Water	Hartbeespoort Dam	SAMN16339895
UPMP 1792	Water	Hartbeespoort Dam	SAMN16339894
UPMP 1783	Water	Hartbeespoort Dam	SAMN24818907
UPMP 1782	Water	Hartbeespoort Dam	SAMN16339892
UPMP 1781	Water	Hartbeespoort Dam	SAMN16339891
UPMP 1780	Water	Hartbeespoort Dam	SAMN16339890
UPMP 1771	Water	Hartbeespoort Dam	SAMN19374591
UPMP 1769	Water	Hartbeespoort Dam	SAMN16339887
UPMP 1724	Water	Canal	SAMN24818904
UPMP 1723	Water	Canal	SAMN16339881
UPMP 1721	Water	Canal	SAMN15905478
UPMP 1720	Water	Canal	SAMN15905477
UPMP 1700	Water	Canal	SAMN24818901
UPMP 1699	Water	Canal	SAMN24818900
UPMP 1728	Water	Holding reservoir	SAMN16339832
UPMP 1731	Water	Preparation reservoir	SAMN16339884
UPMP 1730	Water	Preparation reservoir	SAMN16339883
UPMP 1719	Cucumber	Packhouse before packing	SAMN15905476
UPMP 1717	Cucumber	Packhouse before packing	SAMN24818903
UPMP 1714	Cucumber	Packhouse before packing	SAMN15905474
UPMP 1711	Cucumber	Packhouse before packing	SAMN24818902

Figure 2: Summary of the *Klebsiella pneumoniae* isolated from a cucumber agroecosystem. White indicates a negative phenotype for multidrug resistant and ESBL- or AmpC- production, blue a multidrug resistant phenotype, black an ESBL- or AmpC- producing phenotype. Red indicates a resistant phenotype, yellow an intermediate phenotype and green a susceptible phenotype.

3.4. Detection of MGEs and Association to Antibiotic Resistance Genes. MGEs associated with *E. coli* isolates are outlined in Table 1. Plasmid IncFII (pRSB107) and insertion sequences IS1380 were both found associated with 35.29% of *E. coli* isolates and were the most abundant MGEs. Insertion sequences IS91 and IS5 were the predominant MGEs identified in 29.41% *E. coli* isolates. The plasmid IncFII (pRSB107) and insertion sequence IS91 were associated with *tet (A)*, *aadA2b*, and *cmIA1*. In addition, the insertion sequence IS1380 contained *blaTEM-1B*, *blaCTX-M-15*, *aph (6)-Id*, *aph (3″)-Ib*, *suI2*, and *dfrA1*. Furthermore, IS5 carried *blaCTX-M-27*. Only 5.88% of *E. coli* isolates were associated with ISSbo1, IS102, ISKpn19, IS6, and TN5403.

MGEs associated with *K. pneumoniae* isolates are outlined in Table 2. In 52.17% and 43.48% of *K. pneumoniae* isolates, insertion sequences IS1380 and IS6 were found, respectively. These were associated with various ARG from different classes. MGEs carrying *tet (A)* were found in *K. pneumoniae* isolates on IncFII (K) (34.78%) and on IS5 (30.43%). One additional plasmid associated with 8.7% of *K. pneumoniae* isolates included incFIA (HI1), which carried *tet (D)*. Additional insertion sequences within *K. pneumoniae* isolates included IS6100 (21.74%), ISEc9 (17.39%), IS110 and IS5075 (13.04% each), IS3 and IS91 (8.70% each), and ISVsa3 (3.35%). Two isolates were associated with a transposon (Tn5403) which harbored *qnrB1*.

3.5. In Silico Analysis of MLSTs, Serotypes, and Pathogenicity. Different sequence types of the 17 *E. coli* isolates were identified, including ST10, ST58, ST69, ST162, ST752, ST753, and ST9583 (Table 3). *E. coli* isolates from water included ST10, ST69, ST162, ST752, ST753, and ST9583. While the one *E. coli* was characterized from cucumbers was ST58. Seven sequence types of the 23 *K. pneumoniae* included ST13, ST14, ST29, ST628, ST873, ST985, and ST3609 (Table 4). The *K. pneumoniae* ST985 was found in water (Hartebeespoort Dam and preparation reservoir) and in cucumber (packhouse before packing) samples. The remaining *K. pneumoniae* sequence types were all present in water samples (Table 4).

Serotyping performed *in silico* using WGS data demonstrated different *E. coli* (Table 3) and *K. pneumoniae* serotypes based on their surface antigens or proteins (Table 4). The predominant *E. coli* serotypes included O101: H9 ($n = 7$) isolated from the dam water, which corresponded to *E. coli* ST10. These isolates had a predicted pathogenicity probability of 0.925–0.932. Two isolates of *E. coli* ST162 were also from the dam water, which were classified as H19 with a predicted pathogenicity probability of between 0.925 and 0.932. *E. coli* O75:H9 (ST58) was the only *E. coli* isolated from cucumbers, with a predicted pathogenicity probability of 0.944. Moreover, the following serotypes were identified O123:H40/O186:H40 ($n = 2$) corresponding to

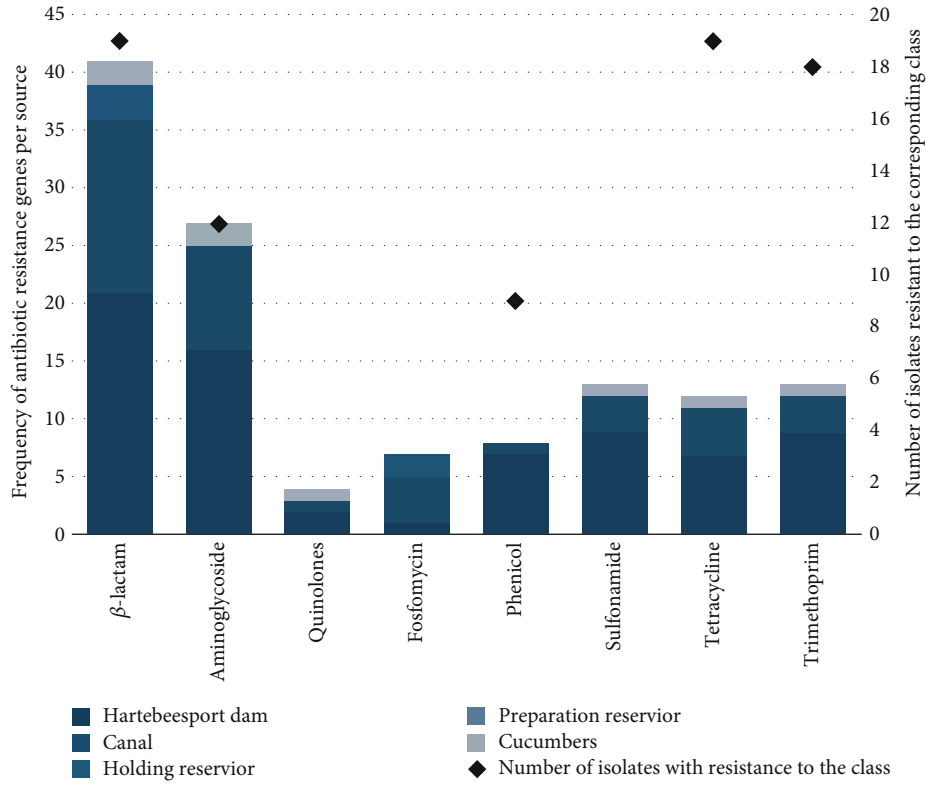


Figure 3: Frequency of antibiotic resistance genes in *Escherichia coli* isolated from a cucumber agroecosystem.

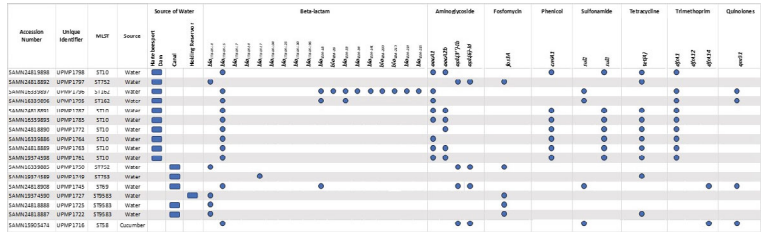


Figure 4: Antibiotic resistance genes detected in 17 *Escherichia coli* isolated from the cucumber agroecosystem.

ST752, H39 ($n = 3$) corresponding to ST9583, O15:H18 ($n = 1$) corresponding to ST69 and O27:H30 ($n = 1$) corresponding to ST753.

The predicted pathogenicity probability associated with *K. pneumoniae* isolates was lower than that for the *E. coli* isolates. *K. pneumoniae* ST985 ($n = 9$) associated with O1:K39 was isolated from cucumbers. In addition, *K. pneumoniae* ST985 associated with O1 and K39 were isolated from water (the dam and preparation reservoir) (predicted pathogen probability ranged from 0.534 to 0.888). Three *K. pneumoniae* ST873 (O101:K52) were isolated from the dam water; these isolates had a predicted pathogen probability range of 0.896–0.897. Moreover, from the dam water, three *K. pneumoniae* ST13 (O1:K30) were isolated with 0.883 to 0.888 predicted pathogen probability, while from the canal water, five *K. pneumoniae* ST29 (O1:K30) were isolated with a predicted pathogen probability range of 0.891–0.892. Moreover, O1:K2 ($n = 1$) associated with ST14, *K. pneumoniae* ST3609 (K117) ($n = 1$), and *K. pneumoniae* ST628 (O3b) ($n = 1$) were isolated from water (canal, holding reservoir, and the dam).

4. Discussion

This study explored the occurrence and characterization of clinically relevant Enterobacterales, *E. coli*, and *K. pneumoniae*, isolates producing ESBL and/or AmpC within a cucumber production system. Results demonstrate that cucumbers could serve as a conduit for the introduction of MDR bacteria of clinical relevance to human health. Results also showed the involvement of irrigation water as a potential carrier within the food system. These results further added to the current body of knowledge regarding the potential impact of irrigated fresh produce using contaminated water and the prevalence of ARB and ARGs within the agroecosystem, which could raise potential human health concerns. This study clearly provided scientific evidence that irrigation water can be a source of contamination,

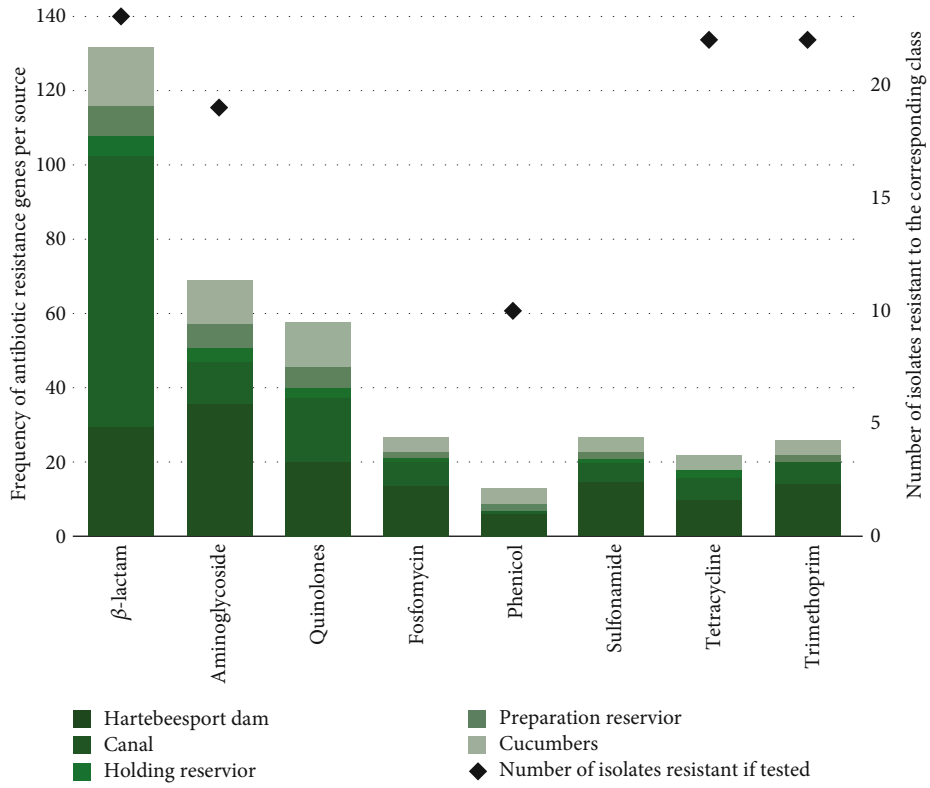


Figure 5: Frequency of antibiotic resistance genes in *Klebsiella pneumonia* isolated from a cucumber agroecosystem.

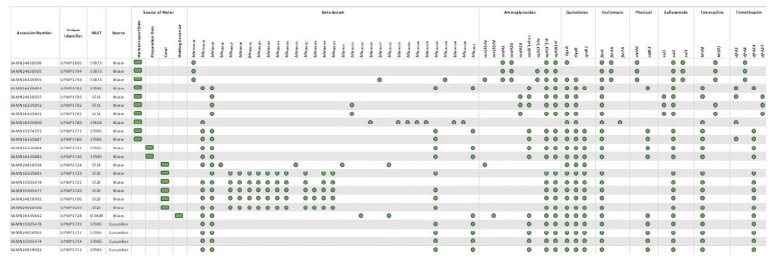


Figure 6: Antibiotic resistance genes detected in 23 *Klebsiella pneumonia* isolated from the cucumber agroecosystem.

TABLE 1: Summary of the mobile genetic elements in *Escherichia coli* isolates from irrigation water isolated from the cucumber agroecosystem.

Mobile genetic elements type	Mobile genetic elements	Number of isolates	Isolates with element present (%)	Resistant genes associated with MGEs
Plasmid	IncFII(pRSB107)	6	35.29	*tet(A), aadA2b and cmIA1*
Insertion sequence	IS1380	6	35.29	$bla_{TEM-1B,}$ $bla_{CTX-M-15}$, *aph(6)-Id, aph(3")-Ib, suI2, dfrA1*
Insertion sequence	IS91	5	29.41	*tet(A), aadA2b and cmIA1*
Insertion sequence	IS5	5	29.41	$bla_{CTX-M-27}$
Insertion sequence	ISEc9	3	17.65	$bla_{TEM-1B;}$ $bla_{CTX-M-15}$, *qnrS1; aph(6)-Id; aph(3")-Ib; sul2*
Insertion sequence	ISSbo1	1	5.88	*tet(A), aadA2b and cmIA1*
Insertion sequence	IS102	1	5.88	$bla_{CTX-M-27}$
Insertion sequence	ISKpn19	1	5.88	*qnrS1,* $bla_{CTX-M-15}$, bla_{TEM-1B}, *sul2, aph(6)-Id and aph(3")-Ib.*
Insertion sequence	IS6	1	5.88	*dfrA14*
Transposon	Tn5403	1	5.88	*qnrB1*

TABLE 2: Summary of the mobile genetic elements in *Klebsiella pneumonia* isolates from irrigation water isolated from the cucumber agroecosystem.

Mobile genetic elements type	Mobile genetic elements	Number of isolates	Isolates with element present (%)	Resistant genes associated with MGEs
Insertion sequence	IS1380	12	52.17	bla_{TEM-1B}, bla_{TEM-30}, bla_{TEM-70}, $bla_{TEM-104}$, $bla_{TEM-148}$, $bla_{TEM-176}$, $bla_{TEM-198}$, $bla_{TEM-207}$, $bla_{TEM-217}$, $bla_{TEM-220}$, $bla_{TEM-230}$, $bla_{TEM-234}$, $bla_{CTX-M-15}$, *sul2, aph(6)-Id, aph(3")-Ib, aac(3)-Id* and *qnrB1*
Insertion sequence	IS6	10	43.48	*dfrA14, dfrA27, aadA16, sul1 and aac(6')-Ib-cr*
Plasmid	IncFII(K)	8	34.78	*tet(A)*
Insertion sequence	IS5	7	30.43	*tet(A)*
Insertion sequence	IS6100	5	21.74	*sul1, aadA16, aac(6')-Ib-cr, dfrA14 and dfrA27*
Insertion sequence	ISEc9	4	17.39	bla_{TEM-1B}; bla_{TEM-30}; bla_{TEM-70}; $bla_{TEM-104}$; $bla_{TEM-148}$; $bla_{TEM-176}$; $bla_{TEM-198}$; $bla_{TEM-207}$; $bla_{TEM-217}$; $bla_{TEM-220}$; $bla_{TEM-230}$; $bla_{TEM-234}$; $bla_{CTX-M-15}$
Insertion sequence	IS5075	3	13.04	bla_{TEM-1B}, $bla_{CTX-M-15}$, *sul2; aph(6)-Id and aph(3")-Ib.*
Insertion sequence	IS110	3	13.04	bla_{TEM-1B}, $bla_{CTX-M-15}$, *sul2, aph(6)-Id, aph(3")-Ib;*
Insertion sequence	IS91	2	8.70	*suI2*
Insertion sequence	IS3	2	8.70	bla_{SHV-26}, bla_{SHV-78}, bla_{SHV-98}, $bla_{SHV-145}$, $bla_{SHV-179}$, $bla_{SHV-194}$, $bla_{SHV-199}$, *and dfrA1*
Plasmid	IncFIA(HI1)	2	8.70	*tet(D)*
Transposom	Tn5403	2	8.70	*qnrB1*
Insertion sequence	ISVsa3	1	4.35	*suI2*

TABLE 3: Multilocus sequence types, serotypes, and pathogenicity probability of *Escherichia coli* isolates from a cucumber agroecosystem.

Accession number	Unique ID	Source	MLST	O:H typing	Pathogenicity probability
SAMN24819898	UPMP1798	Water	ST10	O101:H9	0.932
SAMN24818891	UPMP1787	Water	ST10	O101:H9	0.925
SAMN16339893	UPMP1785	Water	ST10	O101:H9	0.925
SAMN24818890	UPMP1772	Water	ST10	O101:H9	0.927
SAMN16339886	UPMP1764	Water	ST10	O101:H9	0.925
SAMN24818889	UPMP1763	Water	ST10	O101:H9	0.925
SAMN19374598	UPMP1761	Water	ST10	O101:H9	0.928
SAMN16339897	UPMP1796	Water	ST162	H19	0.932
SAMN16339896	UPMP1795	Water	ST162	H19	0.925
SAMN15905474	UPMP1716	Cucumbers	ST58	O75:H9	0.944
SAMN24818908	UPMP1745	Water	ST69	O15:H18	0.934
SAMN24818892	UPMP1797	Water	ST752	O123:H40/O186:H40	0.932
SAMN16339885	UPMP1750	Water	ST752	O123:H40/O186:H40	0.933
SAMN19374589	UPMP1749	Water	ST753	O27:H30	0.936
SAMN19374590	UPMP1727	Water	ST9583	H39	0.931
SAMN24818888	UPMP1725	Water	ST9583	H39	0.931
SAMN24818887	UPMP1722	Water	ST9583	H39	0.931

introducing ESBL- and AmpC-producing organisms to crops.

It is important to note that isolates in this study were selectively cultured on media containing a third-generation cephalosporin to target clinically relevant Enterobacterales. This approach inherently selects isolates resistant towards β-lactams and potentially multidrug-resistant (MDR) strains, which explains the high prevalence of MDR among the recovered isolates. Consequently, the observed prevalence of ESBL- and AmpC-producing *E. coli* and *K. pneumoniae* may overrepresent resistant populations relative to the overall microbial community in the farm environment.

TABLE 4: Multilocus sequence types, serotypes, and pathogenicity probability of Klebsiella, pneumoniae isolates from a cucumber agroecosystem.

Accession number	Unique ID	Source	MLST	K:O typing	Pathogenicity probability
SAMN16339891	UPMP1781	Water	ST13	O1	0.883
SAMN16339892	UPMP1782	Water	ST13	K30	0.888
SAMN24818907	UPMP1783	Water	ST13	O1	0.888
SAMN24818904	UPMP1724	Water	ST14	O1:K2	0.886
SAMN24818900	UPMP1699	Water	ST29	O1:K30	0.892
SAMN24818901	UPMP1700	Water	ST29	O1:K30	0.891
SAMN15905477	UPMP1720	Water	ST29	K30	0.892
SAMN15905478	UPMP1721	Water	ST29	O1:K30	0.891
SAMN16339881	UPMP1723	Water	ST29	K30	0.891
SAMN16339832	UPMP1728	Water	ST3609	K117	0.879
SAMN16339890	UPMP1780	Water	ST628	O3b	0.885
SAMN24818906	UPMP1805	Water	ST873	O101	0.896
SAMN16339895	UPMP1793	Water	ST873	O101: K52	0.896
SAMN24818905	UPMP1794	Water	ST873	K52	0.897
SAMN24818902	UPMP1711	Cucumbers	ST985	O1:K39	0.885
SAMN15905474	UPMP1714	Cucumbers	ST985	O1:K39	0.884
SAMN24818903	UPMP1717	Cucumbers	ST985	O1:K39	0.888
SAMN15905476	UPMP1719	Cucumbers	ST985	O1:K39	0.885
SAMN16339883	UPMP1730	Water	ST985	K39	0.534
SAMN16339884	UPMP1731	Water	ST985	K39	0.885
SAMN16339887	UPMP1769	Water	ST985	O1	0.881
SAMN19374591	UPMP1771	Water	ST985	K39	0.885
SAMN16339894	UPMP1792	Water	ST985	O1	0.884

To our knowledge, previous studies in similar farm settings did not use this selective culturing method, highlighting that our results specifically reflect the subset of bacteria capable of growing under these selective conditions. While this selective approach was necessary to focus on clinically important Enterobacterales, the results should be interpreted with caution, as they reflect the subset of bacteria capable of growing under these selective conditions rather than the full microbial diversity present in irrigation water or produce samples.

In this study, 100% of the ESBL- and/or AmpC- producing *E. coli* and *K. pneumoniae* isolates associated with irrigation water and cucumbers were multidrug resistant. This was higher than the previously reported (98%) ESBL/AmpC-producing isolates that were MDR in a spinach production systems study in SA [19] and higher than a similar study on vegetables (including cucumbers), soil and water of a farm environment in Tunisia (93.3%) [28]. All ESBL- and/or AmpC-producing *E. coli* and *K. pneumoniae* isolates in this study were resistant to amoxicillin, ampicillin, cefpodoxime, cefotaxime, and cefepime. Moreover, the predicted pathogenicity probability for *E. coli* and *K. pneumoniae* ranged from 0.925 to 0.944 and 0.534 to 0.897, respectively, compounds the risk of infections from these organisms due to limited options of currently available antibiotics.

The co-occurrence of ARGs has been reported in previous studies, where β-lactamase encoding genes were reported to be associated with other genes conferring resistance to aminoglycoside, fosfomycin, phenicol, sulfonamide, tetracycline, trimethoprim, and quinolone antibiotics [7, 15, 29]. The trimethoprim (*dfrA14*) and quinolone (*qnrS1*) resistance genes in *E. coli* isolates from irrigation water of three major Swiss vegetable growing areas were reported to be associated with β-lactamase genes [30]. In the current study, β-lactamase encoding genes co-occurred in *E. coli* with ARGs from at least one other antibiotic class. In addition, the co-occurrence of *blaCTX-M-15*, *clmA1*, *sul3*, *tet (A)*, and *dfrA1* was seen in all ESBL- and/or AmpC-producing *E. coli* O101: H9 (ST10) from Hartebeespoort Dam, which were MDR to at least 12 antibiotics tested. This *E. coli* serotype has previously been reported to be associated with enterotoxigenic and Shiga toxin *E. coli* disease symptoms in diarrheal calves in a Chinese farm [31], human clinical infections, and wastewater samples [32]. The co-occurrence of ARGs and previous reports of *E. coli* O101:H9 (ST10) association with clinical isolates raise clinical or public health concerns because these isolates had a predicted pathogenicity of 0.925–0.932.

When exploring ESBL- and/or AmpC-producing *K. pneumoniae* isolates, β-lactamase encoding genes were found to be co-occurring with other genes conferring resistance to aminoglycoside (22/23), fosfomycin (22/23), phenicol (13/23), sulfonamide (21/23), tetracycline (22/23), trimethoprim (22/23), and quinolone (22/23) antibiotics.

Similarly, tetracycline resistance genes (*tetA* and *tetD*) were detected in isolates from fresh fruit and vegetables (including cucumbers) obtained from markets in Algeria [29]. Interestingly, *K. pneumoniae* isolates from hospitalized patients in KwaZulu-Natal Province (SA similarly harbored ARG conferring resistance to aminoglycosides (*aadA1*), fluoroquinolones (*oqxB*, *oqxA*), fosfomycin (*fosA3*), and sulphonamides (*sul2*) [7]. Finally, *K. pneumoniae* isolates from wastewater treatment plants in southern Romania harbored β-lactamase encoding genes as well as aminoglycosides (*aadA1*) and sulphonamides (*sul2*) ARGs [1]. The co-occurrence of ARGs and previous reports of *K. pneumoniae* association with clinical isolates raises clinical or public health concerns because these isolates had a predicted pathogenicity of 0.534–0.897.

The most prevalent ESBL-producing *K. pneumoniae* serotype [K39 (ST985)] was present in the Hartebeespoort Dam and on farm preparation reservoir (for irrigation) water and cucumber samples. These isolates were resistant to at least 11 antibiotics tested. The co-occurrence of 14 ARGs (*blaCTX-M-15*, *blaTEM-1B*, *blaSHV-187*, *acc(6')-Ib-cr*, *aph (3'')-Ib*, *aph (6)-Id*, *oqxA*, *oqxB*, *qnrB1*, *fosA*, *catB3*, *sul2*, *tet(A)*, and *dfrA14*) was seen in all *K. pneumoniae* O1:K39 (ST985). The *K. pneumoniae* ST985 was previously detected in water from Austrian rivers and clinical isolates from Austrian hospitals [33]. In addition, it was associated with nosocomial infections in Israel [34]. In this study, the predicted probability of pathogenicity was on average 0.85. Therefore, the association of *K. pneumoniae* ST985 with environmental (e.g., aquatic), clinical and fresh produce (cucumber or spinach) isolates can indicate the potential spread of clinically relevant strains in the water-fresh produce, potentially impacting the public health domain. Moreover, the most prevalent serogroup (O1) in *K. pneumoniae* isolates in this study was similarly reported to be isolated from pediatric patients hospitalized between 2010 and 2013 in Mexico City [35], confirming its ability to cause disease [36]. Similarly, the predicted pathogenicity probability for these isolates was high (average: 0.867). These reported studies aligned with work reported here and provide a classical one health dynamic with reported similar pathogen serotypes within human and environmental health.

The β-lactamase encoding gene *blaCTX-M-15* was previously reported to be the most widely distributed *bla*CTX-M variant worldwide [37, 38]. This *bla*CTX-M-15 was found to be predominant in *E. coli* (11/17) and *K. pneumoniae* (16/23) isolates from irrigation water and cucumber samples. Similarly, *blaCTX-M-15* was reported predominantly in nosocomial *K. pneumoniae* in KwaZulu-Natal Province (SA), Edo state (Nigeria), and Bucharest (Romania) [1, 7, 9, 29]. In addition, *bla*CTX-M-15 was found in *K. pneumoniae* isolates from fresh fruit and vegetables at markets in Bejaia (Algeria) and in wastewater treatment plants from Bucharest (Galati) and Targoviste (Romania) [1, 7, 9, 29]. Furthermore, *blaCTX-M-15* carrying *K. pneumoniae* isolates were also reported in a spinach production system in SA [11]. This study provides further evidence that the *blaCTX-M-15* gene is the most widely distributed *blaCTX-M* variant. The gene's wide dissemination could be attributed to the extensive and uncritical use of β-lactam antibiotics and its spread through MGEs.

In this study, *blaCTX-M-15* was carried on insertion sequences associated with both *E. coli* and *K. pneumoniae* (IS1380 and ISEc9), with *K. pneumoniae* only (IS5075 and IS110) and with *E. coli* only (ISKpn19). Further *E. coli* isolates carried one plasmid [IncFII (pRSB107)], eight insertion sequences (IS1380, IS91, ISEc9, ISKpn19, ISSbo1, IS5, IS102, IS6) and one transposon (Tn5403) all associated with ARGs. *K. pneumoniae* isolates carried two plasmids [IncFII (K) and IncFIA (HI1)], 10 insertion sequences (IS91, IS1380, IS6, IS5075, IS6100, IS3, IS5, IS110, ISEc9, ISVsa3) and one transposon (Tn5403) all associated with ARGs. Insertion sequences IS91, IS1380, ISEc9, IS5, IS6 and transposon Tn5403 were present in both *E. coli* and *K. pneumoniae* isolates, although insertion sequences were not associated with the same ARGs. The transposons in both species were associated with *qnrB1*. The treatment of ARB infections is complicated by the mode of dissemination of ARGs between bacteria in different habitats, which is often associated with the spread of MGEs carrying the resistance genes [29].

Multilocus Sequence Typing was used to get the genealogy of the *E. coli* isolates. This classification of *E. coli* isolates resulted in seven different sequence types discovered, with ST10, ST58, ST69, ST162, ST752, ST753, and ST9583 in both water and cucumber samples. The detected human-associated extra-intestinal pathogenic *E. coli* strains ST58 and ST10 were both previously reported in isolates from a farm environment in Tunisia [28], food animals in South Korea [39], and spinach production systems in SA [11]. In addition, the *E. coli* strain ST58 was detected previously in cattle in Pakistan [40] and store-bought produce in Germany [10]. The *E. coli* ST752 identified in the cucumber study has previously been reported in retail foods and environmental water sources in China [41, 42]. The presence of MDR *E. coli* isolates (ST58, ST10, and ST752) in farm and store settings, with similarities to human associated pathogens highlights the contribution of anthropogenic activities to the spread of ARGs [11].

The presence of *E. coli* and *K. pneumoniae* (typical fecal contaminants) in irrigation water may be driven by anthropogenic and animal activities upstream from the farm and therefore serving as a water contamination source. Livestock production is a likely contributor, as a cattle farm located upstream of the study site could introduce contamination through surface runoff. Human activity in the surrounding area may also play a role, as the presence of waste materials such as litter and vegetable debris were observed in the irrigation canal, suggesting the possibility of broader improper waste disposal. Although not directly observed during sampling, wildlife intrusion cannot be excluded, as animals moving through agricultural waterways may further contribute to fecal contamination. These findings are consistent with broader evidence indicating that both humans and hoofed livestock are major sources of fecal contamination in irrigation water systems [43]. Overall, it is likely that a combination of livestock, human, and wildlife sources collectively impacted the microbial quality of irrigation water at this farm.

In conclusion, when using typing methods based on WGS the link between irrigation water and crop contamination was highlighted in this study. This scoping study highlighted the likelihood that, due to the use of contaminated irrigation water, fresh produce could pose a potential threat to the safety of the food system. Therefore, cucumbers produced under such conditions may represent a public health concern, as this vegetable is customarily consumed raw and not cooked prior to consumption. In addition, this study showed the potential of using WGS in source tracking studies. This case study further contributes to the new body of knowledge of the prevalence of AMR in the One Health context and reflects on a typical hazard circulating in the food system that represents the context of One Food–One Health.

Data Availability Statement

The assembled sequences were submitted to GenBank and annotated via the National Center for Biotechnology Information (NCBI) prokaryotic genome annotation pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/). Data are available in the NCBI BioProject database (PRJNA642017) [11]. Accession numbers for the isolates are shown in Figures 1 and 2.

Conflicts of Interest

The authors declare no conflicts of interest.

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