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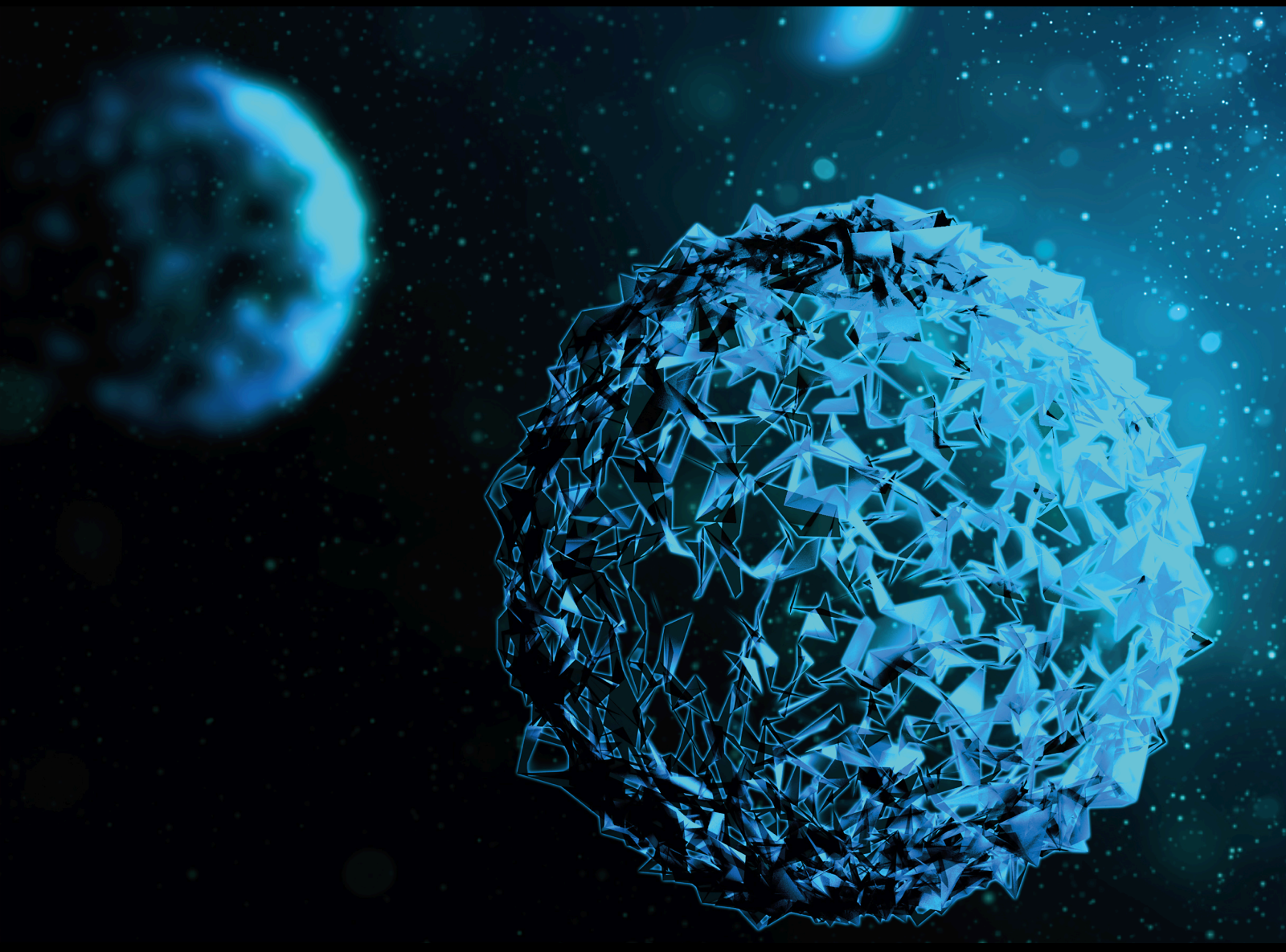

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Research Article

Antimicrobial Resistance Factors of Extended-Spectrum Beta-Lactamases Producing *Escherichia coli* and *Klebsiella pneumoniae* Isolated from Cattle Farms and Raw Beef in North-West Province, South Africa

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Background. Extended spectrum beta-lactamases (ESBLs) producing Enterobacteriaceae cause severe infections in humans which leads to complicated diseases. There is increasing evidence that cattle contribute to the development and spread of multidrug resistant pathogens and this raises public health concern. Despite this, data on the concurrence of ESBL producing pathogens in cattle, especially in the North-West province are rare. Therefore, the aim of the present study was to isolate, identify and characterise ESBL producing *E. coli* and *K. pneumoniae* species from cattle faeces and raw beef samples. *Results.* A total of 151 samples comprising 55 faeces samples and 96 raw beef samples were collected and 259 nonreplicative potential isolates of Enterobacteriaceae were obtained. One hundred and ninety-six isolates were confirmed as *E. coli* (114; 44%) and *K. pneumoniae* (82; 32%) species through amplification of *uspA* and *uidA* and *ntrA* gene fragments, respectively. Antimicrobial susceptibility test revealed that large proportions (66.7–100%) of the isolates were resistant to Amoxicillin, Aztreonam, Ceftazidime, Cefotaxime, and Piperacillin and were multidrug resistant isolates. Cluster analysis of antibiotic inhibition zone diameter data revealed close similarities between isolates from different sources or species thus suggested a link in antibiotic exposures. The isolates showing phenotypic resistance against ESBL antimicrobial susceptibility tests were screened for the presence of ESBL gene determinants. It was observed that 53.1% of the isolates harboured ESBL gene determinants. The *blaTEM*, *blaSHV* and *blaCTX-M* genes were detected in *E. coli* isolates (85.5%, 69.6%, and 58%, respectively) while *blaCTX*-M and *blaOXA* were detected in *K. pneumoniae* (40% and 42.9%, respectively). All the genetically confirmed ESBL producing *E. coli* and *K. pneumoniae* isolates were subjected to Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR analysis. Fingerprinting data revealed great similarities between isolates from different areas and sources which indicates cross-contamination between cattle and beef. *Conclusion.* This study revealed that cattle and its associated food products, beef in particular, harbour ESBL producing pathogens. And this warrants a need to enforce hygiene measures and to develop other mitigation strategies to minimise the spread of antibiotic resistant pathogens from animals to human.

1. Introduction

Extended spectrum beta-lactamases (ESBL) are enzymes that can hydrolyse various β-lactam antibiotics and thus mediate resistance to penicillins, 3rd and 4th generation cephalosporins [1]. The genes encoding for those enzymes are commonly found both in the chromosomes and plasmids among the species belonging to Enterobacteriaceae family. As a result, ESBLs have emerged as a cause of resistance in Enterobacteriaceae, particularly *E. coli* and *Klebsiella* species. This phenomenon was first reported in 1980s in Europe and subsequently in USA [2]. Different antibiotic groups are applied at both therapeutic and subtherapeutic levels in the management of farm animals. Beta-lactam antibiotics are widely used in veterinary medicine

precisely due to their high specificity, perfect selective toxicity, and potent killing effects [3, 4]. Thus excessive use of these antibiotics in veterinary medicine exacerbated the emergence and dissemination of genetic determinants, particularly in *E. coli* and *K. pneumoniae* species [5].

ESBL producing *E. coli* and *Klebsiella* species cause severe infections in humans even in countries with advanced public health and health care facilities [5, 6]. And most of infections are associated with cross contamination in hospital and clinic settings. Despite this, community acquired infections have also been reported in several countries worldwide [2, 7, 8]. Given that the natural hosts of *E. coli* and *Klebsiella* species are ruminants especially cattle, community acquired infections have been linked to the consumption of contaminated food particularly meat [3, 9]. ESBL producing *E. coli* and *Klebsiella* species may be transmitted to meat if standard operating procedures as well as proper hygiene practices are not implemented in the farms and abattoirs, respectively [9–12]. Despite the public health significance of ESBL producing strains globally, and the need to assess their occurrence in food producing animals [9–11, 13], only one report has been in the Eastern Cape Province of South Africa [14].

Agriculture contributes about 2.6% to the total GDPR and 19% to formal employment in the North-West province and it is of extreme importance to the inhabitants [15]. Despite the fact that the North-West province is known to be an important food basket in South Africa, with Maize and sunflower as the most important crops, the province is well known for cattle farming and it is sometimes referred to as the Texas of South Africa. The largest percentage of grazing land and cattle herds are seen in Stella and near Vryburg. In addition, a wide range of livestock farming, which includes cattle, sheep, goats, and chicken farming, is practiced in the Marico region. This kind of farming contributes a substantial percentage towards the economic growth of the area. Meat and dairy products are the main products produced, with commercial game ranching also contributing through the supply of meat.

The frequent use of antibiotics to enhance animal productivity provides opportunities for bacteria that harbour resistance determinants to be released into the environment through the discharge of faecal matter from animals, and this poses severe epidemiological implications on humans [16], given that the province is registered as the forth in terms of the prevalence HIV/AIDS in the country. In this paper, we report on the antimicrobial resistance profiles of ESBL producing *E. coli* and *Klebsiella* species isolated from faecal samples collected from cattle farms as well as meat from randomly selected supermarkets in the North-West province of South Africa as part of a larger study designed to use bacteriophages as biocontrol agents for multiple antibiotic resistant bacteria in the environment and the South African food chain.

2. Materials and Methods

2.1. Sample Collection. Sampling was done between August 2014 and May 2015. A total of 55 faeces samples were collected from 2 commercial cattle farms in Mafikeng District of the North-West province for the study. Faecal samples were collected directly from the rectum of individual animals using sterile arm-length gloves and in order to avoid duplication of sampling, the cattle were looked in their respective handling pens. In addition, 96 raw beef samples were collected from butcheries, supermarkets, and retails in 11 major cities of the four Districts of the North-West province. After collection, samples were transported on ice to the North-West University Molecular Microbiology laboratory for immediate processing. Data on antibiotic type and treatment history were collected for the purpose of understanding antibiotic exposure histories of isolates from the study population.

2.2. Isolation and Identification of E. coli and Klebsiella Species. Approximately, two grams (2 g) of each meat sample was washed in 5 mL of 2% (w/v) buffered peptone water (BPW) obtained from Biolab, South Africa while 2 g of faecal samples was dissolved in 5 mL of tryptic soya broth (TSB) (Merck (Pty) Ltd, South Africa). Ten-fold serial dilutions of the samples were prepared using 2% (w/v) buffered peptone water as the diluent [7]. Aliquots of 100 μL from each dilution were spread-plated on MacConkey agar (with crystal violet) (Merck (Pty) Ltd, South Africa) plates and plates were incubated aerobically at 37°C for 24 hours. After incubation, single pale, colourless, and pink colonies were sub-cultured onto MacConkey agar (with crystal violet) plates. One colony per plate was picked into a sterile tryptic soya broth and further incubated aerobically at 37°C for 24 hours for glycerol stock preparation and isolates were preserved at −80°C for future use.

2.3. Preliminary Identification Tests. The pure colonies were subjected to Gram-staining [18] and the oxidase test, TSI test [19] as well as the Simmons' citrate agar test to screen for characters of *E. coli* and *Klebsiella* species.

2.4. Phenotypic Screening of Isolates for ESBL Production. All isolates were screened for ESBL production on Brilliance ESBL Agar plates [20] obtained from Oxoid, Basingstoke, United Kingdom. In order to perform this, isolates were subcultured in Brilliance ESBL Agar and plates were incubated aerobically at 37°C for 24 hours. ESBL-producing *E. coli* and *Klebsiella* species were identified by the presence of blue or pink and green colonies, respectively. For quality control, *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 13883) were used as a negative control. Potential *E. coli* and *Klebsiella* isolates were further subjected to the API 20E analytical assay following the manufacturer's instructions (BioMériux, France).

2.5. Extraction of Genomic DNA. Bacteria chromosomal DNA was extracted from all presumptive isolates using Zymo Research Genomic DNA™–Tissue MiniPrep Kit following the manufacturer's instructions (Inqaba Biotechnical Industry Ltd, Pretoria, South Africa). The quality and quantity of the extracted DNA was determined using a UV–Vis Thermo Scientific™ NanoDrop Lite Spectrophotometer (model S-22, Boeco, Germany). The DNA samples were stored at −80°C for future use.

2.6. Molecular Identification of E. coli and Klebsiella Species by PCR Analysis. As an internal control all DNA samples were screened for bacterial 16S rRNA gene fragments using the 27F

Table 1: Oligonucleotide primer sequences used for amplification of 16S rRNA universal, *uspA*, *uidA*, and *ntrA* genes and PCR cycling conditions used.

Primers	Sequence (5′–3′)	Targeted gene	Amplicon size (bp)	PCR conditions and cycles	Reference
27F	AGAGTTTGATCATGGCTCAG	*16S rRNA*	1420	1 cycle of 3 minutes at 94°C, 25 cycles of 1 minute at 94°C, 1 minute at 55°C, 2 minutes at 72°C; 1 cycle of 10 minutes at 72°C	[21]
1492R	GGTACCTTGTTACGACTT				
uspAF	CCGATACGCTGCCAATCAGT	*uspA*	884	1 cycle of 5 minutes at 95°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 30 seconds at 72°C; 1 cycle of 5 minutes at 72°C	[22]
uspAR	ACGCAGACCGTAGGCCAGAT				
uidAF	CTGGTATCAGCGCGAAGTCT	*uidA*	556	1 cycle of 10 minutes at 95°C, 35 cycles of 45 seconds at 95°C, 30 seconds at 59°C, 1 minute 30 seconds at 72°C; 1 cycle of 10 minutes at 72°C	[23]
uidAR	AGCGGGTAGATATCACACTC				
ntrA	CATCTCGATCTGCTGGCCAA	*ntrA*	90	1 cycle of 10 minutes at 95°C, 35 cycles of 45 seconds at 95°C, 30 seconds at 55°C, 1 minute 30 seconds at 72°C; 1 cycle of 10 minutes at 72°C	
ntrA	GCGCGGATCCAGCGATTGGA				

and 1492R universal oligonucleotide primer sequences [21] and were synthesised by Inqaba Biotechnical Industries, [PTY] Ltd, South Africa), and are shown in Table 1.

2.7. E. coli and Klebsiella pneumoniae Species Specific PCR Identification Tests. Amplification of the *uidA* and *uspA* housekeeping genes specific to *E. coli* species and *ntrA* gene fragments related to *K. pneumoniae* specific sequences was performed following previous protocols [22, 23], with minor modifications. PCR reaction mixtures were prepared as standard 25 μL volumes that constituted 12.5 μL of 2X DreamTaq Green Master Mix, 11 μL RNase free PCR water, 0.5 μL mixture of the forward and reverse primers (0.25 μL of each primer) and 1 μL of template DNA. All the PCR reagents were Fermentas USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. Amplifications were performed using DNA thermal cycler (model-Bio-Rad C1000 Touch TM Thermal Cycler) obtained from Bio-Rad Laboratories, Inc. USA, oligonucleotide primer sequences synthesised by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa and conditions that appear in Table 1. PCR amplicons were stored at 4°C until electrophoresis.

2.8. Sequencing of PCR Amplicons. Bacterial 16S rRNA gene fragments were sequenced by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa and sequences were subjected to a Blast Search Tool (http://blast.ncbi.nlm.nmih.gov/Blast.cg) in order to confirm the identities of the isolates.

2.9. Antimicrobial Susceptibility Test. An *in-vitro* antimicrobial susceptibility test was performed on all isolates according to the Kirby-Bauer disk-diffusion method [24] in order to determine antibiotic resistant profiles and making use of antibiotic discs (Mast Diagnostics, Merseyside, UK) which were placed on inoculated Muller Hinton agar (MHA) plates. The following antibiotic impregnated discs were used: Amoxicillin (10 μg), Piperacillin (100 μg), Cephalothin (30 μg), Cefotaxime (30 μg), Ceftazidime (30 μg), Cefepime (30 μg), Cefoxitin (30 μg), Aztreonam (30 μg), and Ertapenem (10 μg), and these antibiotic discs contained the CLSI approved concentrations [25]. Antibiotic growth inhibition zone diameter data were compared with standard reference values in order to classify the isolates as sensitive, intermediate resistance or resistant to a particular antibiotic [25]. In the evaluation of the results, strains displaying intermediate resistance were regarded as resistant. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883 strains were used as positive controls.

2.10. Detection of ESBL Genes in E. coli and Klebsiella Species by Multiplex PCR Analysis. All confirmed *E. coli* and *Klebsiella* isolates were screened for the presence of the *blaCTX-M*, *blaOXA*, *blaSHV*, *blaTEM*, *blaCMY*, *blaCMY-1*, and *blaCMY-2* ESBL producing genes determinants using previously described PCR protocol [26, 27]. The primer sequences, targeted genes, amplicon sizes, and PCR conditions are listed in Table 2. The reactions were prepared in standard 25 μL reaction volumes that comprised 12.5 μL of a 2X DreamTaq Green Master Mix, 11 μL nuclease free water, 0.25 μL set of each primer, and 1 μL of template DNA. All the PCR reagents were Fermentas USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. The amplifications were performed using DNA thermal cycler (model-Bio-Rad C1000 Touch TM Thermal Cycler) obtained from Bio-Rad Laboratories, Inc. USA and cycling conditions indicated in Table 2. PCR amplicons were held at 4°C until resolved by electrophoresis.

2.11. ERIC-PCR Genetic Typing of ESBL Producing E. coli and K. pneumoniae Isolates. All ESBL producing *E. coli* and *K. pneumoniae* isolates were subjected to ERIC-PCR in order to determine the genetic similarities between the isolates. ERIC-PCR was performed using a single oligonucleotide primer ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) as previously described [28]. The ERIC fingerprints were compared and analysed for the presence, absence, and intensity of band data obtained.

2.12. Electrophoresis of PCR Products. PCR products were separated by electrophoresis on a 2% (w/v) agarose gel containing ethidium bromide (0.1 μg/mL) (Sambrook et al., 1989). Depending on the size of the amplicons, a 1 Kb

Table 2: Oligonucleotide primer sequences used for detection of ESBL genes and PCR cycling conditions used.

Primer	Primer sequence (5′–3′)	Target gene	Amplicons size (bp)	PCR conditions and cycles	Reference
blaTEMF	AAACGCTGGTGAAAGTA	*blaTEM*	822	1 cycle of 5 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 1 minute at 45°C, 1 minute at 72°C; 1 cycle of 10 minutes at 72°C	[27]
blaTEMR	AGCGATCTGTCTAT				
blaSHVF	ATGCGTTATATTCGCCTGTG	*blaSHV*	753		
blaSHVR	TGCTTTGTTATTCGGGCCAA				
blaCTX-MF	CGCTTTGCGATGTGCAG	*blaCTX-M*	550		
blaCTX-MR	ACCGCGATATCGTTGGT				
blaOXAF	ATATCTCTACTGTTGCATCTCC	*blaOXA*	619		
blaOXAR	AAACCCTTCAAACCATCC				
blaCMY-1F	GTGGTGGATGCCAGCATCC	*blaCMY-1*	915	1 cycle of 3 minutes at 94°C, 25 cycles of 1 minute at 94°C, 1 minute at 58°C, 1 minute at 72°C; 1 cycle of 10 minutes at 72°C	[26]
blaCMY-1R	GGTCGAGCCGGTCTTGTTGAA				
blaCMY-2F	GCACTTAGCCACCTATACGGCAG	*blaCMY-2*	758		
blaCMY-2R	GCTTTTCAAGAATGCGCCAGG				

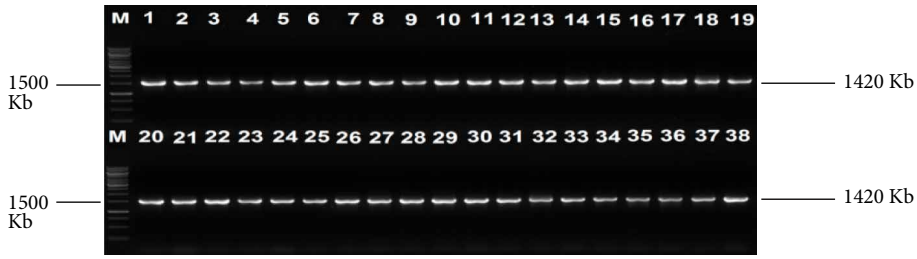

Figure 1: A 2% (w/v) agarose gel of 16S rRNA gene fragments amplified from *E. coli* and *Klebsiella* isolates as well as *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 13883) control strains. Lane M = DNA marker (1 kb O'GeneRuler DNA marker), Lane 1 = *E. coli* (ATCC 25922), Lane 2 = *K. pneumoniae* (ATCC 13883), Lanes 3–19 = 16S rRNA gene fragments of *E. coli* isolates from cattle faeces and beef samples, Lanes 20–38 = 16S rRNA gene fragments of *K. pneumoniae* isolates from cattle faeces and beef samples.

or 100 bp DNA molecular weight marker (Fermentas, USA) was included in each gel. Electrophoresis was conducted in a horizontal Pharmacia biotech equipment system (model Hoefer HE 99X, Amersham Pharmacia biotech, Sweden) for 1 hour at 80 V using 1X (v/v) TAE buffer. A ChemiDoc Imaging System (Bio-RAD ChemiDocTM MP Imaging System, UK) was used to capture the image using Gene Snap (version 6.00.22) software (GSL Biotech Chicago, USA).

2.13. Statistical Analysis. Statistical analysis of antibiotic resistance data was performed using the Minitab Release software (version 13.31) produced by Minitab, LLC, Pennsylvania, USA. Correlations between antibiotic resistant isolates from the various sources were determined using the percentage antibiotic resistance for each antibiotic the Pearson's product of moment and scored as significant if $p \leq 0.05$. Furthermore, cluster analysis of isolates from the different stations was determined using bacterial growth inhibition zone diameter data obtained from antibiotic susceptibility tests on Statistica version 12 (Statsoft, US). Analysis was performed using Wards algorithm and Euclidean distances [34].

3. Results

3.1. Detection of E. coli and Klebsiella pneumoniae Isolated from Faecal and Beef Samples. One hundred and fifty-one (55 cattle faeces and 96 raw beef) samples were collected and analysed. A total of 259 nonreplicative presumptive isolates were selected based on differences in colonial morphologies. All the isolates were Gram-negative rod shaped bacteria that were oxidase positive and hydrolysed the substrates glucose, sucrose and lactose at sample concentrations of 0.1, 1.0, and 1.0%, respectively in the TSI medium. Out of 259 isolates that were subjected to API 20E assay, 145 (56%) *E. coli* and 114 (44%) *Klebsiella* species were positively identified. Large proportions 196 (76.4%) of these isolates were confirmed as ESBL producing strains based on activity on Brilliance ESBL agar and this comprised 114 (58.2%) *E. coli* (blue colonies) and 82 (41.8%) *Klebsiella* species (green colonies). As an internal control the 16S rRNA gene fragment was successfully amplified in all the 259 isolates and Figure 1 indicates a 2% (w/v) agarose gel image of the bacterial 16S rRNA gene fragments. Large proportions 44% of these isolates were confirmed as *E. coli*, while 32% were positive for *Klebsiellapneumoniae* through amplification of *uidA* and *uspA* and *ntrA* gene fragments, respectively. Figures 2–4 indicate 2% agarose gel images of the *uidA*, *uspA* and *ntrA* gene fragments amplified in the study. A total of 63 (24.3%) isolates that were negative for *E. coli* and *Klebsiella pneumoniae* specific sequences were classified as others (Figure 5).

3.2. 16S rRNA Gene Sequencing. The 16S rRNA gene sequence data indicated that *E. coli* isolates possessed great (97% to

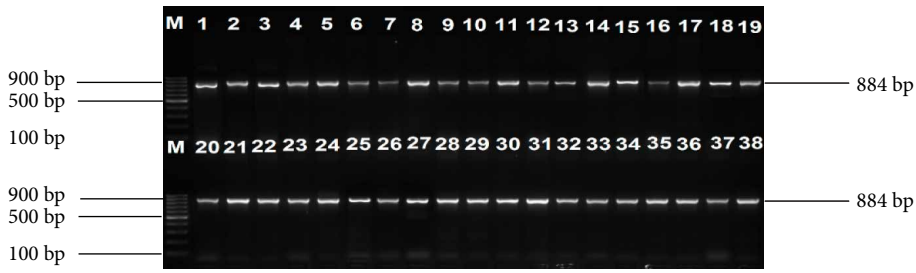

Figure 2: A 2% (w/v) agarose gel image showing the *uspA* gene fragments amplified from all *E. coli* isolates and *E. coli* (ATCC 25922) control strain. Lane M = 100 bp DNA Ladder, Lane 1 = *uspA* gene fragments amplified from *E. coli* (ATCC 25922) control strain, Lanes 2–38 = *uspA* gene fragments amplified from *E. coli* isolates from cattle faeces and beef samples.

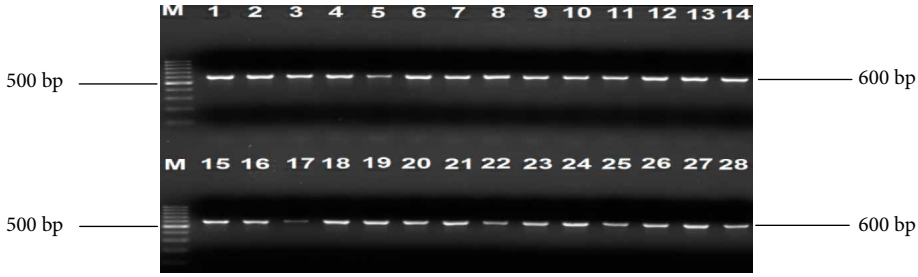

Figure 3: A 2% (w/v) agarose gel image showing *uidA* gene fragments amplified from all *E. coli* isolates and *E. coli* (ATCC 25922) control strain. Lane M = 100 bp DNA Ladder, Lane 1 = *uidA* gene fragments amplified from *E. coli* (ATCC 25922) control strain, Lanes 2–28 = *uidA* gene fragments amplified from *E. coli* isolates from cattle faeces and beef samples.

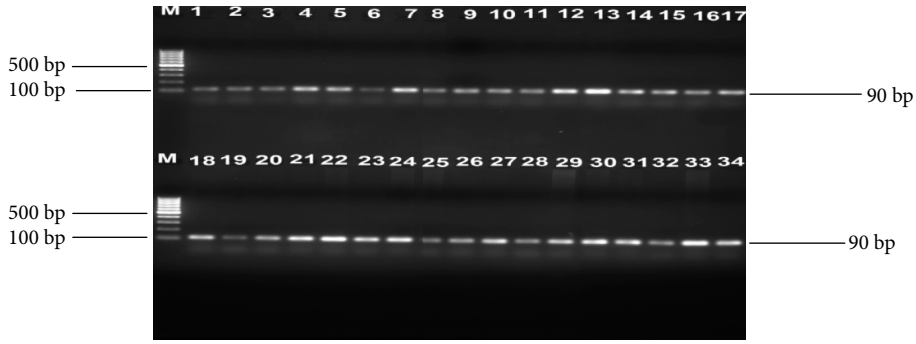

Figure 4: A 2% (w/v) agarose gel image showing *ntrA* gene fragments amplified from all *K. pneumoniae* isolates and *K. pneumoniae* (ATCC 13883) control strain. Lane M = 100 bp DNA Ladder, Lane 1 = *ntrA* gene fragments amplified from *K. pneumoniae* (ATCC 13883) control strain and Lanes 2–34 = *ntrA* gene fragments amplified from *K. pneumoniae* isolates from cattle faeces and beef samples.

99%) similarities to *E. coli* strain O157:H6 (Accession No: CP007592.1), *E. coli* strain C15 (Accession No: CP011018.1) and *E. coli* strain SUS3EC (Accession No: KF991476.1) 16S ribosomal RNA gene, partial sequence. In addition, *K. pneumoniae* isolates possessed 95% sequence similarities to *K. pneumoniae* strain QLR-1 (Accession No: KM096433.1) and a *K. pneumoniae* strain (Accession No: HG416956.1) 16S ribosomal RNA gene, partial sequence.

3.3. Antibiotic Susceptibility Profiles of Isolates. A total of 196 PCR confirmed *E. coli* and *Klebsiellapneumoniae* isolates that revealed ESBL traits on Brilliance ESBL agar were subjected to antimicrobial susceptibility test in order to evaluate their resistance patterns. The number of isolates that was resistant to the different antimicrobial agents was translated into percentages in Table 3. Large proportions (85–100%) of the isolates from all the sampling sites except for those from samples from Mafikeng (54.5%) and Boshoek (66.7%) were resistant to Ampicillin. In addition, significant proportions (66.7–100%) of the isolates from Stella and Boshoek were resistant to Cefotaxime, Piperacillin, Ceftazidime, and Aztreonam. Similarly, large proportions (90%) of the isolates from Potchefstroom were resistant to Amoxicillin, Cephalothin, and Piperacillin. Despite the fact that some isolates obtained in the study displayed low levels of resistance to some of the antibiotics tested, the detection of multi-drug resistant isolates was a cause for concern since they may pose severe health complications on humans.

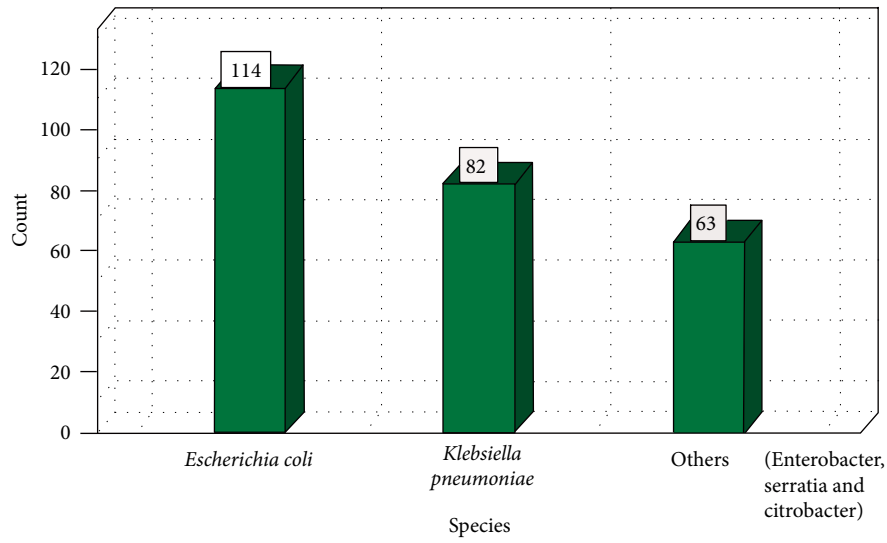

Figure 5: Distribution of *E. coli* and *K. pneumoniae* isolated from beef and faeces samples based on genus specific PCR analysis.

Table 3: Percentage antibiotic resistance pattern of the isolates from cattle faeces and beef samples.

Area	Antibiotics resistance (%)								
	FOX	CTX	KF	ETP	CPM	PRL	CAZ	ATM	A
Boshoek	33.3	100	66.7	33.3	33.3	66.7	66.7	66.7	66.7
Brits	16.7	50	16.7	33.3	33.3	33.3	16.7	16.7	100
Carletonville	16.7	66.7	33.3	33.3	33.3	50	16.7	50	100
Lichtenburg	28.6	14.3	57.1	14.3	14.3	42.9	28.6	14.3	85.7
Mafikeng	12.1	43.4	30.3	17.2	15.2	37.4	17.2	23.2	54.5
Marikana	11.1	55.6	66.7	33.3	55.6	88.9	33.3	44.4	100
Potchefstroom	10	50	90	30	30	90	40	50	90
Rustenburg	28	52	60	28	32	60	28	36	96
Stella	33.3	100	33.3	33.3	33.3	100	66.7	66.7	100
Ventersdorp	14.3	42.9	42.9	14.3	28.6	57.1	14.3	42.9	100
Vryburg	10	70	65	15	35	70	30	45	85

FOX = Cefoxitin, CTX = Cefotaxime, KF = Cephalothin, ETP = Ertapenem, CPM = Cefepime, PRL = Piperacillin, CAZ = Ceftazidime, ATM = Aztreonam, A = Amoxicillin.

3.4. Cluster Analysis of E. coli and Klebsiella pneumoniae Species for Antibiotic Resistance Relationship. A total of 77 multi-drug resistant isolates that comprised 40 *E. coli* and 37 *Klebsiella pneumoniae* were randomly selected from all sampling locations and subjected to cluster analysis using their antibiotic growth inhibition zone diameter data. Random selection was due to the fact that the software can only accommodate a maximum of 77 isolates. A total of 52 isolates formed reliable cluster patterns and two main clusters (Cluster 1 and Cluster 2) were identified (Figure 6). The largest cluster (Cluster 1) contained 43 isolates and it was divided into two subclusters (Cluster 1A = 39) and (Cluster 1B = 9). The clusters were analysed for patterns of association of isolates from different sources and/or locations and data are presented in Table 4.

Subcluster 1A was considered a mixed cluster since it contained isolates from all the 11 sampling sites and large proportions (28.2% and 20.5%) of these isolates were obtained from beef and cattle faeces samples, respectively. On the contrary, subcluster 1B as well as cluster 2 did not contain any of the isolates obtained from cattle faeces in the study. In addition, a significant proportion (33.3%) of the isolates from samples collected in Mafikeng were dominant in cluster 2 as well as small proportions (11.1%) of isolates from the other six (6) sampling sites. Subcluster 1B comprised 25% and 50% of the isolates from Brits and Mafikeng, respectively. The great similarities in the antimicrobial resistant profiles of the isolates from different sampling sites clearly indicate similarities in antibiotic exposure histories.

3.5. Molecular Detection of ESBL Determinant Genes in E. coli and K. pneumoniae Isolates. All 196 isolates that comprised 114 *E. coli* and 82 *K. pneumoniae* were screened by multiplex PCR analysis for ESBL gene determinants (*blaTEM*, *blaSHV*, *blaCTX-M*, *blaOXA*, and *blaCMY-2*). The proportion of isolates that were positive for the respective genes are shown in Table 5, while Figures 7 and 8 indicate a 2% (w/v) gel

Table 4: Percentage representation of *E. coli* and *K. pneumoniae* isolated from various areas and/or sources within different clusters.

Sampling area	Source	Cluster 1A $N = 39$	Cluster 1B $N = 4$	Cluster 2A $N = 9$
Brits	Beef	2 (5.1%)	1 (25%)	1 (11.1%)
Boshoek	Beef	1 (2.6%)	0 (0%)	1 (11.1%)
Carletonville	Beef	5 (12.8%)	0 (0%)	0 (0%)
Lichtenburg	Beef	2 (5.1%)	0 (0%)	0 (0%)
Mafikeng	Beef	11 (28.2%)	2 (50%)	3 (33.3%)
	Cattle faeces	8 (20.5%)	0 (0%)	0 (0%)
Marikana	Beef	0 (0%)	0 (0%)	0 (0%)
Potchefstroom	Beef	2 (2.6%)	0 (0%)	1 (11.1%)
Rustenburg	Beef	4 (10.3%)	1 (25%)	1 (11.1%)
Stella	Beef	1 (2.6%)	0 (0%)	1 (11.1%)
Ventersdorp	Beef	2 (2.3%)	0 (0%)	1 (11.1%)
Vryburg	Beef	1 (2.3%)	0 (0%)	0 (0%)

Table 5: Proportion of ESBLs genes detected from isolates obtained from cattle faeces and raw beef samples.

Bacteria species	No of isolates positive for ESBL activity	No. of isolates positive for ESBL associated genes					
		bla_{TEM}	bla_{SHV}	$bla_{CTX\text{-}M}$	bla_{OXA}	$bla_{CMY\text{-}1}$	$bla_{CMY\text{-}2}$
E. coli	69 (66.3%)	59 (85.5%)	48 (69.6%)	40 (58%)	20 (29%)	0 (0%)	7 (10.1%)
K. pneumoniae	35 (33.7%)	8 (22.9%)	12 (34.3%)	14 (40%)	15 (42.9%)	0 (0%)	0 (0%)
Total	104 (100%)	67 (64.4%)	60 (57.7%)	54 (51.9%)	35 (33.7%)	0 (0%)	7 (6.7%)

image of the *blaTEM* (1100 bp), *blaSHV* (740 bp), *blaCTX-M* (550 bp) and *blaOXA* (470 bp). Large proportions 53.1% of the isolates harboured the ESBL genes targeted in the study. In addition, ESBL gene determinants were frequently detected in *E. coli* (35%) isolates than in *K. pneumoniae* (18%) (Table 5). Moreover, all ESBL genes investigated were detected among the *E. coli* isolates and this *blaTEM* gene fragment was dominant (85.5%). Despite the fact the *blaTEM* gene was detected in 64.4% isolates, only a small proportion (22.8%) of *K. pneumoniae* isolates harboured this gene.

The *blaSHV* and *blaCTX-M* were detected at proportions of 57.7% and 51.9%, respectively, among the *E. coli* and *K. pneumoniae* isolates. The *blaSHV* gene was dominant (69.6%) among *E. coli* species when compared to *K. pneumonia* (34.3%) isolates. Similarly, a larger proportion (58%) of *E. coli* isolates harboured the *blaCTX-M* gene than *K. pneumonia* (40%) isolates. As depicted in Figure 9, the *blaCMY-2* gene determinant was the least detected among all the ESBL genes targeted and was harboured by only 7 (10%) *E. coli* isolates while none of the *K. pneumoniae* isolates was positive for this gene.

3.6. Genotypic Typing of ESBL Producing E. coli and Klebsiella pneumoniae Isolated from Faecal and Beef Samples. The genetic relatedness of 196 (114 *E. coli* and 82 *K. pneumoniae*) isolates was determined by subjecting them to ERIC PCR analysis. Results indicated great genetic similarities among isolates and fingerprinting patterns of *E. coli* isolates possessed 4 to 9 bands per isolate ranging between 0.25 kb and 10 kb (Figure 10). However, a large proportion of the *E. coli* isolates had fingerprints characterised by 6 bands per isolate. *K. pneumoniae*, produced genetic fingerprinting patterns that were characterised by 2–8 bands per isolate ranging from 0.25 kb to over 10 kb (Figure 11). Similarly, large proportions of the *K. pneumoniae* isolates produced six bands per isolate. The great genetic relatedness among *K. pneumoniae* and *E. coli* isolates detected in samples obtained from different sample sites coupled with the fact that they were recovered from both cattle and raw beef samples indicates some form of cross contamination in the food chain particularly in abattoirs, but does not exclude contamination during handling and packaging of meat. This also indicates the need to improve farm management practices in the area.

4. Discussions

Food-producing animals are known to be reservoirs for ESBL-producing strains, especially *E. coli* [9, 14, 29, 30]. Animals colonised with ESBL producing strains have been reported to serve as potential sources of *E. coli* and *Klebsiella pneumoniae* infections in humans particularly in rural communities [31] and this was a course for concern. The primary objective of the present study was to isolate and identify *E. coli* and *Klebsiella pneumoniae* isolates from cattle faeces and beef samples. In the present *E. coli* and *Klebsiella pneumoniae* species were successfully isolated and confirmed using biochemical tests, genus specific PCR analysis and 16S rRNA gene sequencing. The use of *uidA* and *uspA* and *ntrA* housekeeping genes to confirm the identities of *E. coli* and *Klebsiella pneumoniae* species, respectively have been applied in other studies [22, 23]. In our study, *E. coli* was dominant among isolates recovered from cattle faeces and raw beef samples when compared to *Klebsiella pneumoniae*. Similar observations have

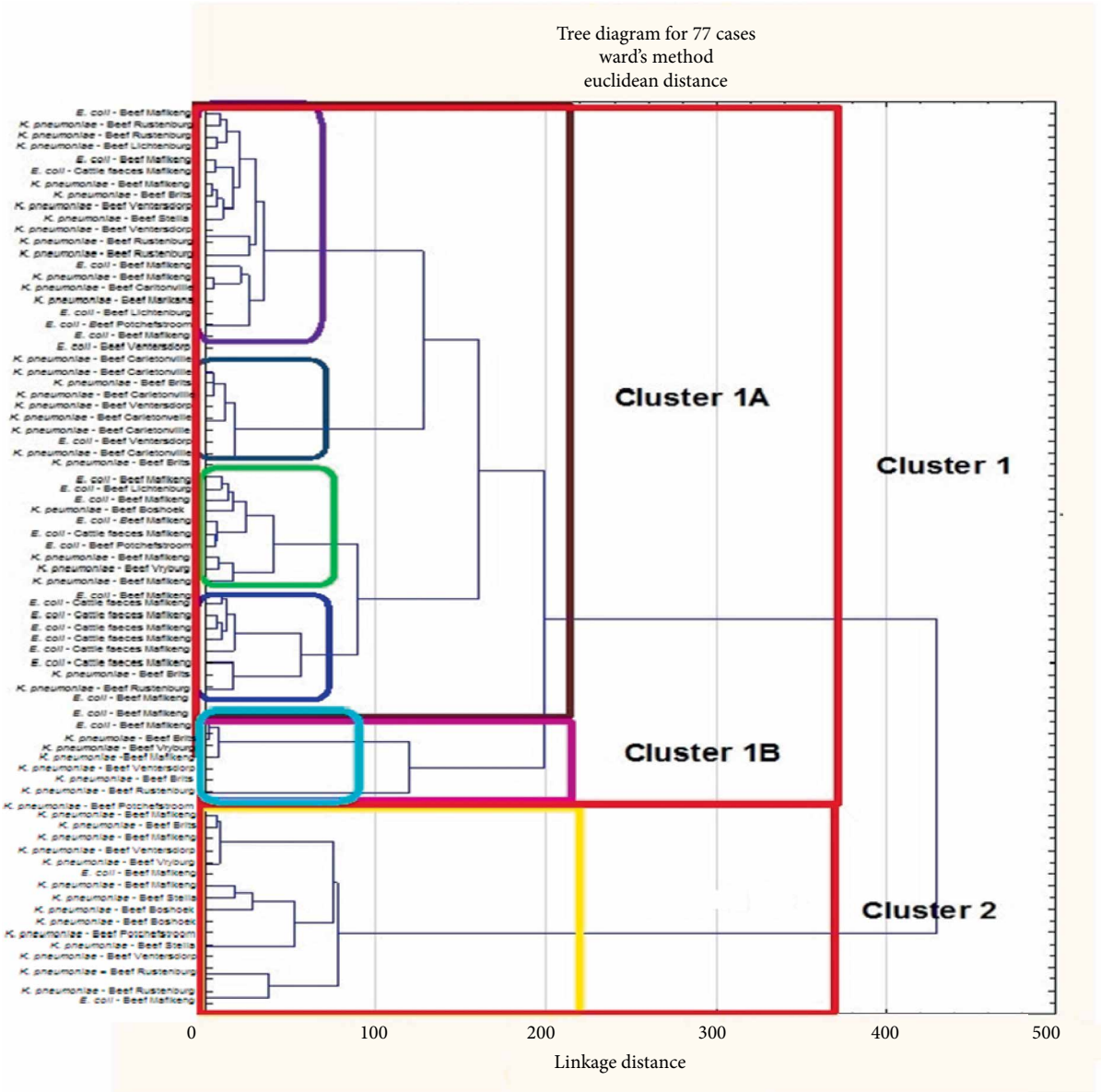

Figure 6: Dendrogram showing the relationship between isolates from cattle faeces and raw beef samples based on antimicrobial inhibition zone diameter data.

been reported [11, 12, 23] and these findings are in agreement with the generalisation that *E. coli* are highly prevalent in the gastrointestinal track of ruminants when compared to other members of the family Enterobacteriaceae [23]. This also explains why *E. coli* species are approved for use in contamination source tracking investigations.

Another objective of the study was to determine the proportion of *E. coli* and *Klebsiella pneumoniae* isolates that possessed ESBL resistant phenotypes as well as their resistant determinants. This was motivated by the fact that ESBL-producing *E. coli* and *Klebsiella pneumoniae* have been frequently found to produce extended-spectrum β-lactamases (ESBLs) and thus making them resistant to cephalosporin antibiotics, as well as a number of other classes of antibiotics [6]. In addition, infections caused by ESBL-producing pathogens are problematic due to the potential of harbouring coresistant determinants to other antimicrobial agents hence present severe challenge to public health practitioners resulting from limited antibiotic treatment options. In general, a large proportion 196 (76.4%) of these isolates tested in the study produced phenotypic ESBL activities on Brilliance ESBL agar. These results are in accordance with previous findings [10, 11, 14, 32, 33]. Although the results from these studies varied significantly, it has also been observed that the occurrence of pathogenic *E. coli* strains was higher in cattle faeces than in beef samples [17]. This was not surprising since *E. coli* strains are known occur as a normal flora of ruminants, especially cattle [12]. However, the most susceptible host in a given area cannot be assumed without thorough analysis since data from our previous findings revealed that pigs rather than cattle are the principal host

Figure 7: A 2% (w/v) agarose gel image showing ESBL [bla_{TEM}(1100 bp), bla_{SHV}(740 bp), $bla_{CTX\text{-}M}$ (550 bp) and bla_{OXA} (470 bp)] gene fragments amplified from *E. coli* isolates. Lane M = 1 kb DNA marker, Lane 1 = negative control, Lanes 5, 6, and 10 = bla_{TEM} gene fragments, Lanes 2, 9, and 12 = bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{OXA} gene fragments, Lane 3 = $bla_{CTX\text{-}M}$ gene fragments and Lanes 4, 7, 8, 11, and 13 = bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ gene fragments amplified from *E. coli* isolates.

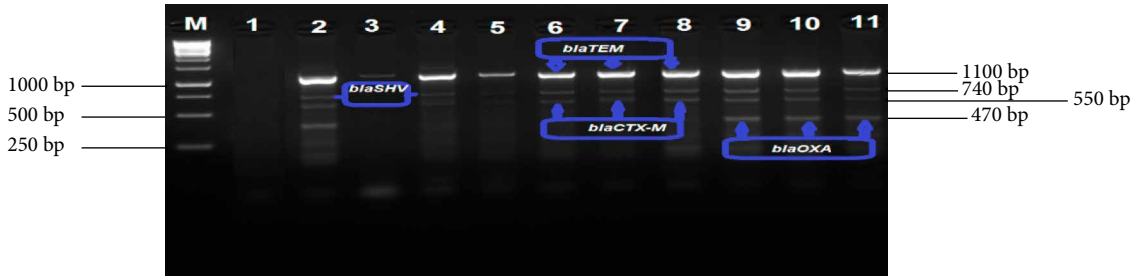

Figure 8: A 2% (w/v) agarose gel image showing ESBL [bla_{TEM} (1100 bp), bla_{SHV} (740 bp), $bla_{CTX\text{-}M}$ (550 bp) and bla_{OXA} (470 bp)] gene fragments amplified from *K. pneumoniae* isolates. Lane M = 1 kb DNA Ladder (O'GeneRuler), Lane 1 = negative control, Lanes 2, 9, 10, and 11 = bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{OXA} gene fragments, Lane 3 = bla_{TEM} gene fragments, Lanes 4, 5, 6, 7, and 8 = bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ gene fragments.

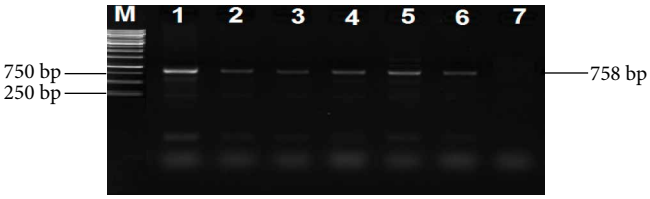

Figure 9: A 2% (w/v) agarose gel image showing $bla_{CMY\text{-}2}$ ESBL gene fragments amplified from *E. coli* isolates. Lane M = 1 kb DNA Ladder. Lanes 1–6 = $bla_{CMY\text{-}2}$ gene fragments amplified from *E. coli* isolates and Lane 7 = negative control.

for *E. coli* O157 strains in the North-West province of South Africa [17, 34].

Antibiotic resistance is currently a very serious problem that has received great attention from the larger scientific community due to its impact on both hospital as well as community settings [35]. This, therefore, implies that rapid detection of resistant determinants in bacteria within diagnostic laboratories and knowledge of the antibiotic resistant profiles of circulating strains is very essential for the judicious recognition of the impact of these organisms to humans in a given geographical location. Within the family Enterobacteriaceae, the production of extended spectrum beta-lactamases is currently on the increase particularly among *E. coli* and *K. pneumoniae* species [35, 36], and these enzymes mediate in cellular processes that impede on treatment of infections caused by these pathogens [35, 37].

In the present study, large proportions (54.5–100%) of the isolates were resistant to Amoxicillin. In addition, 33.3–100% of the isolates were also resistant to Piperacillin while 14.3–100% were resistant to Cefotaxime. Similar observations had been reported [14, 38]. Furthermore, significantly larger proportions (16.7–90% and 16.7–66.7%) were frequently resistant to Cephalothin and Ceftazidime, respectively. Isolates from Brits and Stella were most often resistant to these drugs when compared to those from the other sampling sites. Previous findings as well as antimicrobial usage surveillance data in the study area revealed that the frequent utilization of beta-lactam antibiotics in the treatment of bacterial infections continue to be the prominent cause of high levels of beta-lactam resistance among Gram-negative bacteria [6]. In addition, the potential of ESBL-producing organisms to resistant destruction when exposed to beta-lactam antibiotics is also enhanced by frequent mutations that occur in the gene sequences encoding for beta-lactamases [6, 7]. This could account for the high levels of resistance observed against Amoxicillin since this antimicrobial agent is extensively used as the preferred drug in the treatment of bacterial infections in both veterinary and human medicine [14]. Cluster analysis of antibiotic growth inhibition zone diameter data revealed that subcluster 1A was a mixed cluster that contained isolates from all the different sample sites. These data indicate that the antibiotypes of isolates within subcluster 1A were similar and this might be due to similar antimicrobial exposure histories. Changes in the antimicrobial phenotypes may be associated with environmental factors or the acquisition of plasmids. However, the utilization of an antibiotic phenotypic typing method in which isolates are clustered based on raw antibiotic growth inhibition diameter data was efficient in the typing and nosocomial

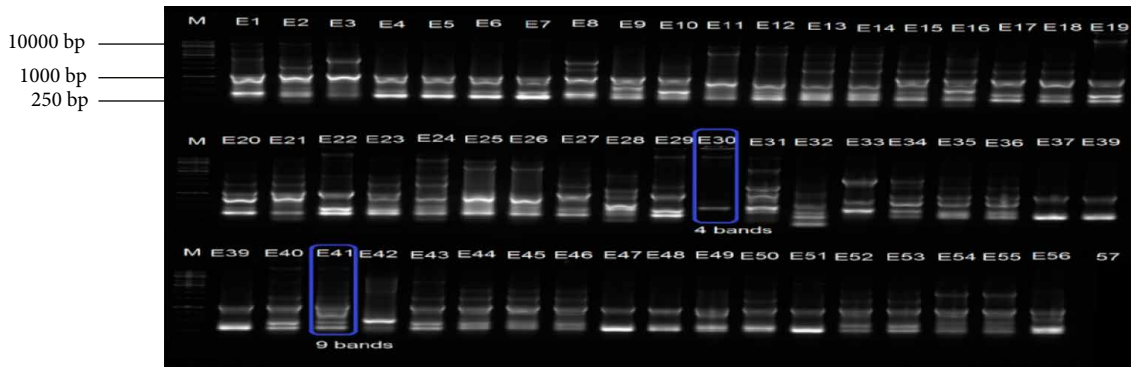

Figure 10: A 2% (w/v) agarose gel image depicting Enterobacterial Repetitive Intergenic Consensus (ERIC) fingerprints of representative *E. coli* isolates. Lane M = 1 kb DNA ladder, Lanes 1–32 = ERIC fingerprints of *E. coli* isolates from beef samples, Lanes 33–56 = ERIC fingerprints of *E. coli* isolates from cattle faeces and Lane 57 = Negative control.

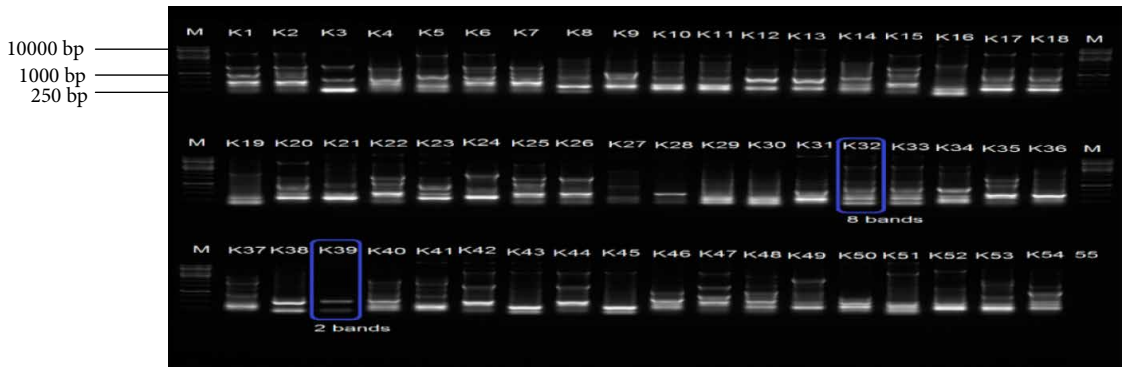

Figure 11: A 2% (w/v) agarose gel image depicting Enterobacterial Repetitive Intergenic Consensus (ERIC) fingerprints of representative *K. pneumoniae* isolates. Lane M = 1 kb DNA ladder, Lanes 1–54= ERIC fingerprints of *K. pneumoniae* isolates from beef samples and Lanes 55 = Negative control.

infection surveillance of Methicillin Resistant *Staphylococcus aureus* [39]. The natural hosts of *E. coli* and *K. pneumoniae* species are ruminant animals especially cattle indicating these antimicrobial resistance pathogens can easily contaminate food products and be transmitted to humans. Therefore, an investigation of the ESBL profiles among *E. coli* and *K. pneumoniae* strains in animals is of paramount importance, and data generated may assist in limiting cross-contamination [17].

A further objective of the study was to screen *E. coli* and *K. pneumoniae* isolates for ESBL antibiotic resistance gene determinants. Despite the fact that ESBL resistance genes have most frequently been detected among *E. coli* and *K. pneumoniae* isolated from humans, clinical care and hospital facilities, a number of studies have also reported that ESBL resistance genes are harboured by *E. coli* and *K. pneumoniae* strains from food-producing animals, meat products as well as vegetables [9, 11, 14, 38, 40–44]. In most studies, ESBL genes (*blaCXT-M*, *blaTEM*, *blaSHV*, *blaOXA* and *blaCMY*) were the most frequently detected resistant determinants in *E. coli*, *K. pneumoniae* and *Salmonella* species [6, 14, 37]. ESBL isolates that possess these resistant determinants particularly *blaCXT-M* may pose severe public health complications to humans if consumption in contaminated food products [11, 14, 40–44].

In the present study, large proportions (53.1%) of the isolates possessed ESBL genes and the *blaCTX-M*, *blaSHV* and *blaTEM* genes most frequently detected in *E. coli* than in *K. pneumoniae* isolates. These results are in accordance with the findings of a previous study conducted in the Eastern Cape Province, South Africa as well as in India [14, 41–44]. Of great concern ESBL gene determinants detected in the present study was higher than those of a similar study previously conducted in Nigeria [38]. It is hereby suggested that the proportion of ESBL resistant gene determinants reported among bacteria isolates may be dependent on differences in geographical locations, sensitivity of the methods used to either isolate bacteria or detect resistance genes as well as the source and nature of specimens analysed.

The last objective of this study was to determine the genetic relatedness of ESBL-producing *E. coli* and *K. pneumoniae* isolates using ERIC PCR analysis. ERIC PCR analysis was usually employed to amplify various regions of DNA flanked by conserved sequences in order to generate isolate specific genetic fingerprints [28]. ERIC typing results revealed that isolates from different sources and/or locations shared similar genetic fingerprinting patterns and in particular those from cattle faeces and beef samples. The great similarities in genetic fingerprints indicate that the isolates may have originated from a common ancestral strain. ERIC-PCR band patterns of *K.*

pneumoniae isolates from beef samples were very similar despite the differences in their sampling stations and this was in accordance with a previous report [45]. The findings of several studies have revealed that ERIC fingerprinting is a reliable tool for discriminating among isolates from different sources and hence considered to be a powerful tool for surveillance and control of antibiotic resistant bacteria [28, 46, 47]; these data indicate the need to improve on farm management techniques as well as standard operational procedures in abattoirs. In addition, contamination of raw beef with these multi-drug resistant pathogens may have also occurred at sale points and this, therefore, amplifies the need to enforce proper hygiene practices in supermarkets.

5. Conclusion

To the best of our knowledge, this is the first study to report the occurrence of ESBL producing *E. coli* and *K. pneumoniae* in cattle faeces and their associate food products (beef) in the study area. Large proportions (66.7–100%) of multidrug resistant isolates were present in the samples and these isolated clustered together based on their antibiotic inhibition zone diameter data suggesting a very close link in antibiotic exposure histories. Large proportions of these *E. coli* and *K. pneumoniae* isolates displayed ESBL phenotypic resistance traits, while 53.1% harboured ESBL gene determinants. The *blaTEM*, *blaSHV* and *blaCTX-M* genes were detected in *E. coli* isolates (85.5%, 69.6%, and 58%, respectively) while *blaCTX*-M and *blaOXA* were detected in *K. pneumoniae* (40% and 42.9%, respectively). In conclusion these findings indicate that, tracking and monitoring the spread of ESBL producing strains in food producing animals and beef at farms and sales points are urgently needed to improve public health in South Africa.

6. Limitations and Suggestion

There are several members of Enterobacteriaceae living in the intestines of animals and isolated from their faeces. However, in the study focus was directed at isolating *E. coli* and *Klebsiella pneumoniae* and this was a limitation for the study. Another limitation of the study was that cattle faeces were collected from one district (Mafikeng), whereas beef samples were collected from all the four districts of the North-West province. Thus, the data obtained from cattle faeces might not completely reflect the actual prevalence of ESBL producing organisms in cattle. Despite this, it is suggested that further studies should be carried out to determine the linkage of ESBL producers in animals and human and to address factors that contributes to the successful dissemination of ESBL producing strains to human.

Data Availability

No access to data on the antimicrobials used in the investigated farms; unavailability or scarcity of data from meat wholesale suppliers and the Department of Agriculture, Forestry and Fisheries of South Africa.

Conflicts of Interest

The authors declare that there have no conflicts of interest regarding the publication of this paper.

Author's Contributions

Kotsoana Peter Montso, Sicelo Beauty Dlaminia, Ajay Kumar, and Collins Njie Ateba are contributed equally to this work.

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Supplementary Materials

Figure S1: a flow chart illustrating sample collection and bacterial characterization. (*Supplementary Materials*)

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Research Article

Extended-Spectrum β-Lactamases among Enterobacteriaceae Isolated from Urinary Tract Infections in Gaza Strip, Palestine

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Background. Extended-spectrum β-lactamase-producing organisms causing urinary tract infections are increasing in incidence and pose a major impendence to health-care facility, having limited therapeutic options. This study aimed to assess the prevalence of ESBLs in Enterobacteriaceae isolates causing urinary tract infections in Gaza strip, Palestine, and to characterize β-lactamase types and associated resistance genes. *Methods*. Eighty-five Enterobacteriaceae isolates were recovered from urinary tract infections within three months in Gaza Strip hospitals. The characterization of β-lactamase genes and the genetic environments of CTX-M, the identification of associated resistance genes, and the presence and characterization of integrons were tested by PCR and sequencing. *Results*. The occurrence rate of ESBL among tested isolates was 30 (35.3%), and among ESBL-positive isolates, $bla_{\text{CTX-M}}$ was the highest followed by bla_{TEM}. ESBL-CTX-M-1 group was confirmed in 93.3%, and the remaining carried CTX-M-9 group. CTX-M-15, CTX-M-3, CTX-M-1, CTX-M-14, CTX-M-27, and CTX-M-37 enzymes were demonstrated among the isolates with the majority (73%) being CTX-M-15. IS*Ecp-1* was demonstrated in 27 (90%, high incidence) of ESBL isolates. Class 1 integrons have been detected in higher rates (53.3%) in ESBL-positive isolates in comparison with non-ESBL isolates (6, 33.3%). Cassettes of integron-1 contain (*aadA1*, *aadA2*, *aadA5*, *dfrA5*, *dfrA7*, *dfrA12*, and *dfrA17*) genes. The *aac(6′)-Ib-cr* gene was demonstrated in 36.7% of ESBL-positive isolates. *Conclusions*. This study indicates that $bla_{\text{CTX-M-15}}$ was the most prevalent β-lactamase in this region. Our study demonstrates for the first time in Palestine the identification of $bla_{\text{CTX-M-15}}$ in *P. rettgeri* and *S. liquefaciens*, also $bla_{\text{CTX-M-37}}$ in *E. cloacae*. The coexpression of multiple β-lactamase genes with *aac(6′)-Ib-cr* and *qnr* in the presence of IS*Ecp-1* and integrons in individual strains will increase the dissemination of highly resistant strains. ESBL producers were more resistant than non-ESBLs producers for almost all tested antibiotics.

1. Introduction

Urinary tract infection (UTI) is one of the most common widespread infections, mainly caused by Enterobacteriaceae, especially *Escherichia coli*, that are encountered by hospitalized and outpatients [1]. Normally, UTIs are treated with different classes of antibiotics such as β-lactams, β-lactam/β-lactamase inhibitors, carbapenems, and fluoroquinolones [2]. However, recent data worldwide reveal that these uropathogens have become resistant to most conventional drugs [3].

Extended-spectrum β-lactamases (ESBLs) are bacterial enzymes that hydrolyze oxyimino-cephalosporins and confer resistance to broad-spectrum cephalosporins and aztreonam [4]. Enterobacteriaceae harboring ESBLs is a global problem with limited available treatment options [5]. ESBL-producing bacteria are related with infections that are consequences of bad clinical facilities, inappropriate

antibacterial therapy, prolonged hospital stays, and greater hospital costs [6]. In the past years, there has been an increase in widespread dissemination of β-lactamase-mediated resistance with high significance in the prevalence of ESBL-producing Enterobacteriaceae [7].

ESBL-producing Enterobacteriaceae have become a major concern in clinical setting worldwide, causing outbreaks related to enzymes of CTX-M class being most common [8]. Recently, ESBLs of CTX-M type have been replaced by SHV- and TEM-ESBLs types worldwide among various members of Enterobacteriaceae [9]. Currently, CTX-M β-lactamases are categorized into five classes according to their amino acid sequences and include the CTX-M-1, -2, -8, -9, and -25 clusters [10]. Presently, the dominants of CTX-M-1 and CTX-M-9 are most prevalent in geographical distributions [11–13], with many exhibiting CTX-M-15, which is the most widely distributed CTX-M enzyme worldwide [14–16].

Early investigation of patients infected with ESBL-producing urinary pathogens is necessary to prescribe the most efficient therapy and minimize the dissemination of infection by applying preventive measures. Therefore, the aim of this study was to detect the occurrence of ESBL-producing Enterobacteriaceae and investigate the molecular characteristics of β-lactamase-producing isolates obtained from three hospitals in Gaza Strip, Palestine.

2. Materials and Methods

2.1. Isolation and Identification. Eighty-five Enterobacteriaceae isolates were recovered from urinary tract infection samples obtained in various units of three hospitals in Gaza Strip, Palestine, during the period of three months in 2013. Only one bacterial isolate per patient was included in this study. All collected specimens were sent to the microbiology laboratory to be processed for bacterial isolation and identification. Standard methods for isolation and identification were used [17]. The isolates were confirmed to genus and species level by amplification and sequencing of 16S rRNA gene.

2.2. Antibiotic Susceptibility Testing. Susceptibility testing for all isolates to 15 antibiotic agents was performed by the disk diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [18] using commercial antibiotic disc panels comprising (μg/disk) ampicillin (10), cefoxitin (30), ceftazidime (30), cefotaxime (30), gentamicin (10), amikacin (30), tobramycin (10), amoxicillin-clavulanic acid (20/10), nalidixic acid (30), ciprofloxacin (5), imipenem (10), kanamycin (30), trimethoprim-sulfamethoxazole (1.25/23.75), tetracycline (30), and chloramphenicol (30).

2.3. Identification of β-Lactamase Genes and Genetic Environment of $bla_{CTX\text{-}M}$ Genes. PCR amplification and sequencing were used to determine the presence of the genes encoding TEM, SHV, OXA, and CTX-M type β-lactamases. The genetic environment surrounding the $bla_{\text{CTX-M}}$ genes was studied by the amplification of IS*Ecp-1*, *orf477*, and IS*903* [19].

2.4. Detection of Non-β-Lactam Resistance Genes. Isolates were screened for the genes associated with resistance to tetracycline (*tet(A)* and *tet(B)*), sulfamethoxazole (*sul1*, *sul2*, and *sul3*), and gentamicin (*aac(3)-I*, *aac(3)-II*, and *aac(3)-IV*). The genes *qnrA*, *qnrB*, *qnrS*, *qepA*, *and aac*(6′)-1b were studied by PCR and sequencing to identify the variants according to Jouini et al. [20].

2.5. Detection and Characterization of Integrons. The presence of *intI1* and *intI2* genes encoding for classes 1 and 2 integrase, respectively, and the presence of *qacED1-sul1* genes within 3′-conserved region of class 1 integrons was identified by PCR. The variable regions of class 1 and 2 integrons were studied in *intI1*- or *intI2*-positive isolates by PCR and sequencing to identify the gene cassettes [19].

Positive and negative controls were kindly provided from the Universite´ Tunis-El Manar, Tunis, and were used in all PCR and sequencing experiments.

2.6. Data Analysis. The data of antimicrobial resistance of non-ESBL and ESBLs of Enterobacteriaceae isolates were analyzed by SPSS version 20 software (IBM Corporation, Somers, NY) by applying a Pearson's chi-square test. Level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Bacterial Strains. In this study, we screened 85 Enterobacteriaceae isolates from urine from three Palestinian hospitals in Gaza Strip for the determination of β -lactamase-encoding genes and to characterize their type, genetic environments of CTX-M, and associated resistance genes.

A total of 85 urinary tract infection samples were obtained from Al-Shifa Hospital (37; 43.5%), Balsam Hospital (27; 31.8%), and Al-Remal Center (21; 24.7%). The samples collected from Al-Shifa and Balsam hospitals were from inpatients, and the Al-Remal Polyclinic samples were from outpatients. Out of 85 bacterial isolates, *Escherichia coli* was the predominant isolate (60; 70.6%) followed by *Klebsiella pneumoniae* (15; 17.6%), *Proteus mirabilis* (3; 3.5%), *Enterobacter cloacae* (3; 3.5%), *Serratia liquefaciens* (2; 2.4%), *Providencia rettgeri* (1; 1.2%), and *Morganella morganii* (1; 1.2%).

3.2. Antimicrobial Susceptibility and ESBL-Positive Isolates. Among the 85 isolates collected, extended-spectrum β-lactamases were confirmed in 30 isolates (35.3%); among these were *E. coli* (20 isolates), *K. pneumoniae* (5 isolates), *E. cloacae* (3 isolates), *S. liquefaciens* (1 isolates) and *P. rettgeri* (1 isolates).

The susceptibility to imipenem was 80.0% against ESBL-producing isolates. However, the resistance rate to cefotaxime and ampicillin was 100%, which was high among ESBL-producing isolates. Resistance to sulfamethoxazole/

trimethoprim, nalidixic acid, ciprofloxacin, kanamycin, and tetracycline among strains was 76.7, 66.7, 66.7, 60.0, and 60.0%, respectively. Low resistance in ESBL-producing isolates against gentamicin, chloramphenicol, cefoxitin, amikacin, and amoxicillin clavulanic acid was recorded, and the resistance rate was less than 40.0% (Table 1).

On comparing the antibiotic resistance of ESBL and non-ESBL Enterobacteriaceae, both showed low resistance to imipenem in comparison to other tested antibiotics. However, resistance to other antibiotics was higher among ESBL than non-ESBL isolates with statistical significance (Table 1). To the end, the resistant pattern of ESBL producers was significantly higher than that of non-ESBL producers for all 15 tested antibiotics except for imipenem (Table 1).

3.3. Identification of β-Lactamases. Molecular characterization of 30 ESBL-producing isolates revealed that all of them harbored $bla_{\text{CTX-M}}$ genes (100%). bla_{TEM}, bla_{OXA}, and bla_{SHV} genes were detected in 10 (33.3%), 1 (3.3%), and 1 (3.3%) of the isolates, respectively.

In this study, 28 of the 30 strains were found to be positive for the ESBL-CTX-M-1 group, 23 were confirmed to carry $bla_{\text{CTX-M-15}}$, 2 strains harbored $bla_{\text{CTX-M-3}}$, 2 isolates carried $bla_{\text{CTX-M-1}}$, and the remaining 1 isolate carried $bla_{\text{CTX-M-37}}$. Of the 2 remaining strains that are positive for the ESBL-CTX-M-9 group, 1 carried $bla_{\text{CTX-M-14}}$ and 1 harbored $bla_{\text{CTX-M-27}}$ (Table 2).

β-lactamase genes identified by PCR and sequencing among the 20 ESBL-positive *E. coli* strains are as follows: $bla_{\text{CTX-M-15}}$ ($n = 11$), $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$ ($n = 4$), $bla_{\text{CTX-M-1}}$ ($n = 2$), $bla_{\text{CTX-M-14}}$ ($n = 1$), $bla_{\text{CTX-M-27}}$ ($n = 1$), and $bla_{\text{CTX-M-3}}$ ($n = 1$). There were five ESBL-positive *K. pneumoniae* isolates; three isolates harbored ($bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{SHV-1}}$), one carried ($bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$), and one carried ($bla_{\text{CTX-M-3}}$). The three isolates of *E. cloacae* producing ESBL had the following β-lactamase genes: $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$ ($n = 1$), $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$ ($n = 1$), and $bla_{\text{CTX-M-37}}$ ($n = 1$). The $bla_{\text{CTX-M-15}}$ was identified in ESBL-producing *Serratia liquefaciens* and *Providencia rettgeri* (Table 2).

Non-ESBL β-lactamases were identified in 18 isolates: *E. coli* ($n = 10$), *K. pneumoniae* ($n = 6$), *P. mirabilis* ($n = 1$), and *Morganella* spp. ($n = 1$). The β-lactamases that are not classified as ESBL include genes encoding TEM-1, SHV-1, and OXA. β -lactamase genes identified among the 18 non-ESBL β-lactamase strains are as follows: $bla_{\text{TEM-1}}$ was the most predominate gene, which was detected in 12 isolates (nine *E. coli*, one *K. pneumoniae*, one *Morganella* spp., and one *P. mirabilis*). The SHV-encoding genes were found among five isolates of *K. pneumoniae* in two variants; $bla_{\text{SHV-1}}$ and $bla_{\text{SHV-11}}$ with the frequency of 2 and 3, respectively. Finally, $bla_{\text{OXA-1}}$ was detected in only one isolate of *E. coli* (Table 2).

3.4. Genetic Environments of $bla_{\text{CTX-M}}$ Genes. The genetic mechanisms that may be involved in the expression and mobilization of $bla_{\text{CTX-M}}$ genes and the genetic environments upstream and downstream of $bla_{\text{CTX-M}}$ genes were studied in ESBL-positive Enterobacteriaceae isolates by PCR and sequencing. The sequence of *orf477* was found downstream of the ESBL-CTX-M-1 group members ($bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-1}}$, and $bla_{\text{CTX-M-27}}$ genes) in twenty-seven isolates. The insertion sequence (IS*903*) was identified downstream of the $bla_{\text{CTX-M-14}}$ gene in one isolate (NT134), whereas the downstream region of $bla_{\text{CTX-M-27}}$ and $bla_{\text{CTX-M-37}}$ in *E. cloacae* and *E. coli*, respectively, was unknown. The insertion sequence (IS*Ecp-1*) was found upstream $bla_{\text{CTX-M}}$ genes in twenty-seven isolates; however, the upstream region of $bla_{\text{CTX-M-37}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{CTX-M-14}}$ in NT60, NT117, and NT134 isolates, respectively, was unknown.

3.5. Integrons and Arrangement of Gene Cassettes. Class 1 integron has been demonstrated in ten ESBL-positive *E. coli* isolates with the following gene cassette arrangements: *dhfr17 + aadA5* (6 isolates), *dhfrA7* (2 isolates), and *dfrA12 + aadA2* (one isolate); three of those integrons lacked the *qacEΔ1* and *sul1* genes. In *K. pneumoniae*, class 1 integrons were present in 3 of the 5 ESBL-producing isolates with two different genetic arrangements (*dfrA12 + aadA2)* and *dfrA5. Int1* was identified in two *ESBL-positive E. cloaca*, the gene cassette implicated in the resistance to streptomycin (*aadA1)* was detected in one isolate, and one of those integrons lacked the *qacEΔ1* and *sul1* genes. The cassette that conferred resistance to trimethoprim (*dfrA12*) and streptomycin (*aadA2*) was found in integron-1 among ESBL-producing *S. liquefaciens*.

Five of non-ESBL *E. coli* contained class 1 integron with gene cassette arrangement (*dhfr17 + aadA5*) found in one isolate. Class 1 integron has been demonstrated in non-ESBL-*Morganella morganii* Isolate (Table 2).

3.6. Non-β-Lactam Antimicrobial Agent-Coding Genes. A variety of genes which confer resistance to non-β-lactam antibiotics were observed among ESBL-producing Enterobacteriaceae: *tetA* and *tetB* (in 11 and 5, respectively, of tetracycline-resistant strains); eleven of the 30 ESBL isolates harbored *sul* genes (*sul1*: ($n = 7$); *sul1 + sul2*: ($n = 3$); and *sul1 + sul3*: ($n = 1$)). The *aac(3)-II* gene was found in 13 gentamicin-resistant isolates, and the *aac(6′)-Ib-cr* gene was detected in eleven isolates. The *qnrB1* gene was identified in two isolates, and *qnrA* and *qnrS1* genes were each identified in one isolate. Yet, five non-ESBL *E. coli* isolates harbored some non-β-lactam antibiotics coding genes such as *sul-1* ($n = 2$), *aac(3)II* ($n = 2$), *aac(6′)-Ib-cr* ($n = 1$), *tetA* ($n = 2$), *tetB* ($n = 1$), and *sul2* ($n = 1$). Non-ESBL-*Morganella* spp. carried *sul1* gene (Table 2).

4. Discussion

A total of 85 Enterobacteriaceae isolates obtained from urinary tract infections from in- and outpatient populations during a three-month time period were evaluated for the production of ESBL, β-lactamase enzymes, and associated resistance genes.

The finding showed that among 85 urine isolates, *E. coli* was the most prevalent representing 60 (70.6%) of isolates followed by *K. pneumoniae* 15 (17.6%). This is in agreement

Table 1: Comparison of antibiotics resistance in 30 ESBL and 55 non-ESBL Enterobacteriaceae.

Antibiotics	Resistance in ESBL-producing isolates (%)	Resistance in non-ESBLs-producing isolates (%)	P value
Cefotaxime	30 (100%)	6 (11%)	0.000
Ceftazidime	15 (50%)	4 (7.3%)	0.000
Gentamicin	10 (33.3%)	3 (5.5%)	0.001
Ampicillin	30 (100%)	32 (58.2%)	0.000
Imipenem	6 (20.0%)	7 (12.7%)	0.278
Nalidixic acid	20 (66.7%)	7 (12.7%)	0.000
Sulfamethoxazole/ trimethoprim	23 (76.7%)	18 (32.7%)	0.000
Tobramycin	15 (50.0%)	5 (9.1%)	0.000
Ciprofloxacin	20 (66.7%)	5 (9.1%)	0.000
Kanamycin	18 (60.0%)	9 (16.4%)	0.000
Tetracycline	18 (60.0%)	2 (3.6%)	0.000
Cefoxitin	10 (33.3%)	1 (1.8%)	0.000
Amikacin	10 (33.3%)	3 (5.5%)	0.001
Amoxicillin-clavulanic acid	10 (33.3%)	3 (5.5%)	0.001
Chloramphenicol	12 (40.0%)	7 (12.7%)	0.012

with other studies investigating Enterobacteriaceae causing urinary tract infections in Sri Lanka and Qatar [5, 21].

The prevalence of ESBL among our isolates was confirmed in 30 isolates (35.3%). This was similar to the findings of Liu et al., Giwa et al., and Caccamo et al. [22–24]where ESBL production among Enterobacteriaceae causing urinary tract infections was found to be 33.4%, 34.3%, and 36.0%, respectively.

Our findings demonstrated that imipenem was the most effective drug against ESBL isolates. Studies performed in Palestine from different clinical awards demonstrated the same as our findings about the effectiveness of imipenem [1, 25–27].

The results of our study showed that the frequency of antibiotic resistance among ESBL producers is higher than that of resistance in nonproducers; our findings correlate with other studies in Tanzania and India [28, 29]. The spread of ESBL producers in hospitals and their increased antibiotic resistance is alarming.

Resistance to imipenem was found to be low among ESBL producers (20.0%) and non-ESBL producers (12.7%) without statistical significance. The lower resistance to imipenem among ESBL and non-ESBL's was reported in India [30]. The mechanism of resistance to carbapenem occurs by bacterial production of β-lactamases and decreases the permeability of the antibiotics by changes in porin channels in the cell wall or reduced susceptibility of bacterial cell toward meropenem through upregulation of efflux pumps [31]. The imipenem resistance in our study could be due to production of carbapenemase. This may be because patients in Palestinian hospitals are treated with carbapenems which may have a role in development of multidrug-resistant strains. Detection of carbapenemase genes are beyond the aims of this study and will be our planned future work.

Molecular genotyping of ESBL-containing isolates revealed the highest presence of CTX-M genes (100%) followed by TEM genes, which is in agreement with previous reports from the region and around the globe [1, 32]. CTX-M enzymes have become dominant extended-spectrum β-lactamases in Europe [33] and in many Middle Eastern countries [34].

In this report, 93.3% of Enterobacteriaceae isolates from urine harbored ESBLs-CTX-M-1 group, including $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-1}}$, and $bla_{\text{CTX-M-37}}$ genes, whereas 6.7% of the isolates carried the ESBL-CTX-M-9 group, including $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-27}}$. This finding is in agreement with study in Qatar that showed among the ESBL-producing Enterobacteriaceae infections in urine 90% of the CTX-M belonged to CTX-M-1 group and 8% belonged to the CTX-M-9 group [5]. In a recent study in USA, among the ESBL-producing Enterobacteriaceae, 80% and 20% were positive for CTX-M-1 group and CTX-M-9 group, respectively [32].

Genotypic characterization of all ESBL-positive isolates revealed that the majority (73%) of the CTX-M type was CTX-M-15. A lot of reports worldwide described that CTX-M-15 is the most prominent distributed ESBL-CTX-M enzyme [14–16]. Rapid dissemination of CTX-M-15 enzyme is reported in many countries. The easy transfer of this gene is associated with the epidemic plasmid [35].

One of the significant findings of this study is that this is the first report of $bla_{\text{CTX-M-15}}$ in *P. rettgeri* and *S. liquefaciens* in the Middle East and also the first report of $bla_{\text{CTX-M-15}}$ in *E. cloacae* in Palestine. In the previous reports, $bla_{\text{CTX-M-15}}$ was identified in *P. rettgeri* causing urinary tract infections in Croatia [36], and $bla_{\text{CTX-M-15}}$ was reported *in E. cloacae* in Southern China [22], Egypt [11], and Yemen [37].

In our study, CTX-M-15, CTX-M-3, CTX-M-1, CTX-M-14, CTX-M-27, and CTX-M-37 enzymes were demonstrated among the isolates and indicated a diversity of the CTX-M groups in clinical Enterobacteriaceae isolates recovered from Palestinian hospitals. Enterobacteriaceae harboring CTX-M-15 with CTX-M-14 have been reported as the predominant CTX-M-ESBL types in clinical isolates worldwide [11–13]. In a Canadian study that was conducted in 2007, among CTX-M-producing isolates from 11 Canadian medical centers, CTX-M-15 was the most common (86.5%), followed by CTX-M-14, CTX-M-3, and CTX-M-2 [38]. CTX-M-3 was predominant in clinical isolates in Taiwan

Table 2: Characteristics of the 30 ESBL and 18 non-ESBL Enterobacteriaceae isolates recovered from urinary tract infections.

Class 1 integron			Non-β-lactamase genes	Genetic environment of bla_{CTX-M} gene		β-lactamase genes	Antimicrobial resistance pattern	Hospital	Species	Bacterial code
Variable region	*qacE*Δ1 + *sul1*	*Int1*		Upstream region	Downstream region					
				ESBLs						
–	–	–	*aac(3)II, aac(6′)-Ib-cr, sul2, QnrS1*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$, bla_{TEM-1}	CTX, GM, AMP, IMP, NAL, SXT, TOB, CIP	Al-Shifa	*K. pneumoniae*	NT13
dhfr17 + aadA5	+	+	*sul1*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$	CTX, AMP, SXT, CIP	Al-Shifa	*E. coli*	NT14
–	–	–	*aac(3)II, aac(6′)-Ib-cr, sul1, TetB*	IS*Ecp*	*orf477*	$bla_{CTX-M-15}$	CAZ, CTX, GM, AMP, KAN, NAL, SXT, TOB, CIP, TET	Balsam	*E. coli*	NT40
–	–	–	*qnrA, sul1*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$	CTX, AMP, NAL, SXT, CIP	Al-Shifa	*E. coli*	NT55
dfrA12 + aadA2	+	+	*aac(6′)-Ib-cr, sul1*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{SHV-1}	CAZ, CTX, AMP, KAN, NAL, AMK, SXT, TOB, CIP	Al-Shifa	*K. pneumoniae*	NT57
–	–	–	*sul1, TetA*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$	CAZ, CTX, AMP, IMP, NAL, SXT, CIP, TET	Al-Shifa	*E. coli*	NT58
–	–	+	*aac(3)II, qnrB1, TetA*	Unknown	Unknown	$bla_{CTX-M-37}$	AMC, CAZ, CTX, GM, AMP, IMP, KAN, NAL, AMK, SXT, FOX, TOB, CIP, TET	Al-Shifa	*E. cloacae*	NT60
–	–	–		IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1}	AMC, CAZ, CTX, GM, AMP, KAN, NAL, AMK, SXT, TOB, CIP, TET	Al-Shifa	*K. pneumoniae*	NT66
dfrA12 + aadA2	+	+	*aac(3)II, aac(6′)-Ib-cr, qnrB1, sul1, sul2, TetA*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$	CAZ, CTX, AMP, NAL, CHL, CIP, TET	Al-Shifa	*E. coli*	NT68
–	–	–		IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$, bla_{TEM-1}	AMC, CTX, AMP, FOX	Al-Shifa	*Enterobacter cloacae*	NT69
dhfr17 + aadA5	–	+	*aac(3)II, aac(6′)-Ib-cr, sul1, sul3, TetA, TetB*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$	AMC, CAZ, CTX, GM, AMP, KAN, NAL, SXT, FOX, TOB, CHL, CIP, TET	Al-Shifa	*E. coli*	NT71
dfrA12 + aadA2	+	+	*aac(3)II, aac(6′)-Ib-cr, sul1, TetA*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$	AMC, CAZ, CTX, GM, AMP, KAN, NAL, AMK, SXT, FOX, TOB, CHL, CIP, TET	Al-Shifa	*Serratia liquefaciens*	NT73
–	–	–		IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$	CTX, AMP, KAN, AMK, FOX, TOB	Al-Shifa	*E. coli*	NT75
–	–	–	*aac(3)II, sul1, sul2, TetA*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$, bla_{TEM-1}	CTX, GM, AMP, SXT, TOB, TET	Al-Shifa	*E. coli*	NT84
dhfr17 + aadA5	+	+	*aac(3)II, aac(6′)-Ib-cr, sul1, sul2*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-3}$	AMC, CAZ, CTX, AMP, KAN, NAL, SXT, FOX, TOB, CHL, CIP	Al-Shifa	*E. coli*	NT92
dhfr17 + aadA5	–	+	*aac(3)II, aac(6′)-Ib-cr, sul1, TetB*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$	AMC, CAZ, CTX, GM, AMP, KAN, NAL, SXT, FOX, TOB, CHL, CIP, TET	Al-Shifa	*E. coli*	NT98
–	–	+	*aac(6′)-Ib-cr, qnrS1, TetB*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1}	CAZ, CTX, AMP, IMP, KAN, SXT, CHL, CIP, TET	Balsam	*K. pneumoniae*	NT106

Table 2: Continued.

Class 1 integron			Non-β-lactamase genes	Genetic environment of bla_{CTX-M} gene		β-lactamase genes	Antimicrobial resistance pattern	Hospital	Species	Bacterial code
Variable region	*qacE*Δ1 + *sul1*	*Int1*		Upstream region	Downstream region					
–	–	–	*TetA*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$	AMC, CAZ, CTX, AMP, NAL, SXT, FOX, TET	Al-Remal	*E. coli*	NT108
dfrA5	–	+	*aac(3)II*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-3}$	AMC, CAZ, CTX, AMP, IMP, KAN, AMK, SXT, TOB, CHL	Al-Remal	*K. pneumoniae*	NT112
–	–	–	*TetB*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$, bla_{TEM-1}	CAZ, CTX, AMP, SXT, TET	Al-Remal	*E. coli*	NT116
dfrA7	–	+	*TetA*	Unknown	*orf477*	$bla_{CTX-M-15}$, bla_{TEM-1}	AMC, CTX, AMP, NAL, SXT, FOX, TET	Balsam	*E. coli*	NT117
dhfr17 + aadA5	–	+	*aac(3)II, aac(6′)-Ib-cr, sul1, TetA*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$	CAZ, CTX, AMP, KAN, NAL, AMK, SXT, TOB, CIP, TET	Balsam	*E. coli*	NT121
–	–	–	*TetA*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-1}$	CTX, AMP, KAN, NAL, CHL, CIP, TET	Balsam	*E. coli*	NT126
dhfr17 + aadA5	–	+		IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$	CTX, AMP, AMK, SXT	Balsam	*E. coli*	NT129
–	+	+	*aac(3)II, aac(6′)-Ib-cr, sul1*	Unknown	*IS903*	$bla_{CTX-M-14}$	CTX, GM, AMP, KAN, NAL, AMK, SXT, TOB, CHL, CIP, TET	Balsam	*E. coli*	NT134
dhfrA7	+	+	*sul1*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$, bla_{TEM-1}	CTX, AMP, KAN, NAL, SXT, CIP	Balsam	*E. coli*	NT138
–	–	–		IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$	CTX, AMP, CHL	Balsam	*Providencia rettgeri*	NT146
–	–	–	*TetA*	IS*Ecp-1*	Unknown	$bla_{CTX-M-27}$	CTX, AMP, KAN, NAL, AMK, CIP, TET	Balsam	*E. coli*	NT147
–	–	–	*TetA*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-1}$	CTX, AMP, KAN, NAL, CHL, CIP, TET	Balsam	*E. coli*	NT151
aadA1	+	+	*aac(3)II, sul1, sul2*	IS*Ecp-1*	*orf477*	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{OXA-1}	CTX, GM, AMP, IMP, KAN, SXT, FOX, TOB, CHL	Al-Shifa	*Enterobacter cloacae*	NT157
					Non-ESBLs					
–	–	–				bla_{TEM-1}	CAZ, CTX, AMP, CHL	Al-Shifa	*E. coli*	NT4
–	+	+	*aac(3)II, sul1*			bla_{TEM-1}	GM, AMP, KAN, NAL, SXT, TOB, CIP	Al-Shifa	*E. coli*	NT16
						bla_{SHV-11}	AMP	Al-Remal	*K. pneumoniae*	NT25
–	–	+	*sul1, sul2*			bla_{TEM-1}	AMP, NAL, SXT, CIP	Al-Remal	*E. coli*	NT28
dhfr17 + aadA5	+	+	*sul1*			bla_{TEM-1}	AMP, SXT	Al-Remal	*E. coli*	NT29
–	–	–				bla_{TEM-1}	AMP	Al-Remal	*E. coli*	NT30
–	–	–				bla_{SHV-11}	AMP	Balsam	*K. pneumoniae*	NT36

Table 2: Continued.

Class 1 integron			Non-β-lactamase genes	Genetic environment of bla_{CTX-M} gene		β-lactamase genes	Antimicrobial resistance pattern	Hospital	Species	Bacterial code
Variable region	*qacE*Δ1 + *sul1*	*Int1*		Upstream region	Downstream region					
–	–	–	*aac(3)II, aac(6′)-Ib-cr, TetA, TetB*			bla_{OXA-1}	GM, AMP, KAN, NAL, TOB, CIP, TET	Al-Shifa	*E. coli*	NT64
–	+	+	*sul1*			bla_{TEM-1}	AMC, AMP, IMP, SXT	Al-Shifa	*Morganella morganii*	NT89
–	–	–				bla_{SHV-11}	AMP	Al-Remal	*K. pneumoniae*	NT102
–	–	+				bla_{TEM-1}	AMC, AMP, KAN, SXT, CHL	Al-Remal	*E. coli*	NT107
–	–	–				bla_{SHV-1}	AMP	Al-Remal	*K. pneumoniae*	NT114
–	–	+				bla_{TEM-1}	AMP, NAL, SXT	Al-Remal	*E. coli*	NT115
–	–	–				bla_{TEM-1}	CTX, AMP, SXT	Balsam	*E. coli*	NT123
–	–	–				bla_{TEM-1}	AMP, IMP, KAN, CHL	Balsam	*Proteus mirabilis*	NT125
–	–	–				bla_{TEM-1}	AMP	Balsam	*E. coli*	NT130
–	–	–				bla_{TEM-1}	CAZ, CTX, AMP, IMP	Balsam	*K. pneumoniae*	NT131
–	–	–				bla_{SHV-1}	CAZ, CTX, AMP, IMP	Balsam	*K. pneumoniae*	NT149

[39]; this enzyme was described in clinical isolates of Enterobacteriaceae in Korea and France [40, 41]. Sequencing the amplification of PCR products from the two *E. coli* isolates detected the CTX-M-encoding gene as $bla_{\text{CTX-M-1}}$; this gene has been demonstrated in clinical Enterobacteriaceae in France and Italy [41, 42]. CTX-M-27 enzyme has been reported in nosocomial outbreaks caused by *Salmonella enterica* in a neonatal unit in Tunisia [43].

Our study reported for the first time the presence of CTX-M-37 in clinical Enterobacteriaceae isolates in the Middle East. Its presence was recently reported in clinical strains of *E. cloacae* in Mongolia [44] and was also identified in *S. enterica* serotype Isangi from South Africa [45].

Our results showed that ten $bla_{\text{CTX-M-15}}$-containing isolates carried more than one β-lactamase gene, and three *K. pneumoniae* isolates harbored $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-1}}$, and $bla_{\text{TEM-1}}$. One isolate coexpressed $bla_{\text{CTX-M-15}}$ with other β-lactamase gene, and *E. cloacae* carried $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, and $bla_{\text{OXA-1}}$. In previous studies, Enterobacteriaceae isolates carrying multiple β-lactamase genes have been reported [34, 46, 47]. The occurrence of multiple β-lactamase genes in individual strains is a concern because it will enhance coresistance and greater resistance to various classes of antibiotics.

Insertion sequences and integrons have played an important role in the dissemination of $bla_{\text{CTX-M}}$. IS*Ecp-1* is the most common insertion element associated with $bla_{\text{CTX-M}}$. Moreover, the role of IS*Ecp-1* is mobilization of the $bla_{\text{CTX-M}}$ gene, and it acts as a strong promoter which enhances the expression of $bla_{\text{CTX-M}}$ genes [48]. IS*Ecp-1* was demonstrated in high incidence (90%) among our isolates which is a growing problem of high resistance in our hospitals that can carry high risk through dissemination of $bla_{\text{CTX-M}}$ between patients. IS*Ecp-1* insertion sequence was found upstream of CTX-M enzyme in Enterobacteriaceae isolates from Tunisia and Croatia [19, 36].

In the present study, class 1 integrons were detected and included resistance genes in the variable region encoding resistance to streptomycin (*aadA1*, *aadA2*, and aadA5) and trimethoprim (*dfrA5*, *dfrA7*, *dfrA12*, and *dfrA17*) which increases the risk of the dissemination of antimicrobial resistance by horizontal transmission by integrons. The integrons lacking *qacEΔ1* and *sul1* genes have been previously reported in other countries [19, 49].

ESBL-positive isolates have been demonstrated to carry higher rates (16; 53.3%) of class 1 integrons compared to non-ESBL isolates (6; 33.3%). The integron contribution to transfer extended-spectrum cephalosporin resistance has been established [50]. The frequency of occurrence of integrons among our ESBL-positive isolates was 53% which is less than the rate of isolates from Europe [51]; in contrast to this, the integron frequency was 73% and 92% in isolates from Australia and India [50, 52]. In our study, the percentage of integron in non-ESBL-producing isolates was almost similar to the results obtained from studies performed in Spain and India [52, 53]. May be there are possibilities of transferring the integrons carrying ESBL genes and other drug-resistant determinants from ESBL to non-ESBL-producing isolates that make non-ESBL isolates more resistant and complicate antibiotic resistance problems in our hospitals.

The non-β-lactam antibiotic resistance pattern of the isolates showed that the *sul* and *aac(3)-II* genes were the most prominent identified in our isolates. This is in agreement with the findings of a study conducted in Spain [54]. The variant *aac(6′)-Ib-cr* gene has the ability to reduce susceptibility to aminoglycosides and ciprofloxacin [16]. This gene prevalence rate in our study was 36% among ESBL-producing Enterobacteriaceae isolates in comparison with 25% in a previous study in Palestine [55]. A significant association between resistance to broad-spectrum cephalosporins and resistance to quinolones was reported [56]. The association of *qnrs1* gene with $bla_{\text{CTX-M}}$ identified in this study was similar to the findings of a study in Algeria [57]. We also observed the associations between *qnrB1* and $bla_{\text{CTX-M}}$ which is in agreement with previously reported results in Tunisia [58]. Moreover, the correlation between *qnrA* and $bla_{\text{CTX-M}}$ demonstrated here was also reported in Yemen [37]. The presence of β-lactamases in association with other resistance genes, *qnr* gene and *aac(6′)-Ib-cr*, in the same strain could complicate the treatment of these pathogens. These results support previous reports that suggest larger dissemination of *aac(6′)-Ib-cr* with *qnr* determinants, in ESBL-producing isolates harboring $bla_{\text{CTX-M-15}}$ gene [58–60].

Regarding the comparison of the occurrence of non-β-lactam genes within ESBL and non-ESBL isolates, the non-β-lactam genes were confirmed in 83% and 33.3% in ESBL and non-ESBL isolates, respectively. These findings could complicate the resistance problem and limit the antimicrobial drug therapy of infections caused by ESBL producers.

5. Conclusions

Our study highlights the high incidence of ESBL in Enterobacteriaceae recovered from urinary tract infections in Gaza hospitals, Palestine, with the increase in antibiotics resistance for most commonly used antibiotics in our hospitals except for imipenem that showed the highest activity against ESBL isolates. ESBL producers were found to be more resistant than non-ESBLs producers for almost all tested antibiotics. This study indicates that $bla_{\text{CTX-M-15}}$ was the most prevalent β-lactamase in Gaza Strip hospitals. To our knowledge, this is the first report on identification of $bla_{\text{CTX-M-15}}$ in *P. rettgeri* and *S. liquefaciens* and also $bla_{\text{CTX-M-37}}$ in *E. cloacae* in the Middle East.

The associations between multiple β-lactamase genes in individual strains with other resistance genes such as *aac(6′)-Ib-cr* and *qnr* in the presence of IS*Ecp-1* and integrons increase the dissemination of highly resistant strains and complicate the problem of clinical conditions and treatment failure in our hospitals.

Data Availability

All data used to support the findings of the study are approved and included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Cooccurrence of NDM-1, ESBL, RmtC, AAC(6′)-Ib, and QnrB in Clonally Related *Klebsiella pneumoniae* Isolates Together with Coexistence of CMY-4 and AAC(6′)-Ib in *Enterobacter cloacae* Isolates from Saudi Arabia

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The aim of this study was to investigate the mechanisms responsible for resistance to antimicrobials in a collection of enterobacterial isolates recovered from two hospitals in Saudi Arabia. A total of six strains isolated from different patients showing high resistance to carbapenems was recovered in 2015 from two different hospitals, with four being *Klebsiella pneumoniae* and two *Enterobacter cloacae*. All isolates except one *K. pneumoniae* were resistant to tigecycline, but only one *K. pneumoniae* was resistant to colistin. All produced a carbapenemase according to the Carba NP test, and all were positive for the EDTA-disk synergy test for detection of MBL. Using PCR followed by sequencing, the four *K. pneumoniae* isolates produced the carbapenemase NDM-1, while the two *E. cloacae* isolates produced the carbapenemase VIM-1. Genotyping analysis by Multilocus Sequence Typing (MLST) showed that three out of the four *K. pneumoniae* isolates were clonally related. They had been recovered from the same hospital and belonged to Sequence Type (ST) ST152. In contrast, the fourth *K. pneumoniae* isolate belonged to ST572. Noticeably, the NDM-1-producing *K. pneumoniae* additionally produced an extended-spectrum ß-lactamase (ESBL) of the CTX-M type, together with OXA-1 and TEM-1. Surprisingly, the three clonally related isolates produced different CTX-M variants, namely, CTX-M-3, CTX-M-57, and CTX-M-82, and coproduced QnrB, which confers quinolone resistance, and the 16S rRNA methylase RmtC, which confers high resistance to all aminoglycosides. The AAC(6′)-Ib acetyltransferase was detected in both *K. pneumoniae* and *E. cloacae*. Mating-out assays using *Escherichia coli* as recipient were successful for all isolates. The $bla_{\text{NDM-1}}$ gene was always identified on a 70-kb plasmid, whereas the $bla_{\text{VIM-1}}$ gene was located on either a 60-kb or a 150-kb plasmid the two *E. cloacae* isolates, respectively. To the best of our knowledge, this is the first report of the coexistence of an MBL (NDM-1), an ESBL (CTX-M), a 16S rRNA methylase (RmtC), an acetyltransferase (AAC[6′]-Ib), and a quinolone resistance enzyme (QnrB) in *K. pneumoniae* isolates recovered from different patients during an outbreak in a Saudi Arabian hospital.

1. Introduction

Metallo-β-lactamases (MBLs) are enzymes that hydrolyze most β-lactams including carbapenems, the most potent β-lactams. Therefore, resistance mediated by MBLs is considered the most serious mechanism for inactivating β-lactams [1]. Of the clinically relevant MBLs, NDM, VIM, and IMP are the most common worldwide [2, 3]. *Klebsiella pneumoniae* and *Enterobacter cloacae* are *Enterobacteriaceae* that are leading causes of nosocomial infections and can

Table 1: Isolate case histories and characteristics.

Isolate	Hospital/City	Patient age (years)	Patient sex	Specimen source	Diagnosis
KP-Q1	A/ Al-Gouf	65	Male	Blood	Septic encephalopathy
KP-Q2	A/ Al-Gouf	95	Male	Sputum	Pulmonary fibrosis
KP-Q3	A/ Al-Gouf	70	Female	Wound	Gangrene of diabetic foot with sepsis
KP-Q4	B/ Riyadh	50	Male	Wound	Urogenital infection
EN.C.Q5	B/ Riyadh	64	Female	Blood	ND
EN.C.Q6	B/ Riyadh	78	Male	Blood	Cystic fibrosis

KP: *Klebsiella pneumoniae*
EN.C: *Enterobacter cloacae*
ND: Not determined

frequently acquire antibiotic resistance [4]. *K. pneumoniae* and *E. cloacae* can acquire resistance to third-generation cephalosporins through plasmid-mediated AmpC β-lactamases, such as CMY, FOX, ACC, LAT, MIR, MOX, DHA, and ACT [5], as well as extended-spectrum β-lactamases (ESBL), such as CTX-M, TEM-, and SHV-derivatives. Acquisition of carbapenem resistance in those bacterial species may be related to several carbapenem-hydrolyzing ß-lactamases, such as KPC, NDM, VIM, and OXA-48 [6]. Several mechanisms may confer aminoglycoside resistance to *Enterobacteriaceae*, including production of aminoglycoside-modifying enzymes, such as aminoglycoside acetyltransferases (AACs), phosphotransferases (APHs), and adenylyltransferases (ANTs), but also target modifications by mutations in ribosomal proteins, or 16S rRNA methylation through the action of 16S rRNA methylases [7]. AACs are the most common aminoglycoside-modifying enzymes and are subdivided into four groups [AAC(1), AAC(2′), AAC(3), and AAC(6′)]. AAC(6′) comprises the most common enzymes in Gram-negative bacteria and can be subdivided into two groups: AAC(6′)-I and AAC(6′)-II [8, 9]. *Enterobacteriaceae* may also produce 16S rRNA methylases, including ArmA, RmtA to RmtH, and NpmA, which confer resistance to aminoglycosides [10]. The plasmid-mediated quinolone resistance genes *qnrA, qnrB, qnrC, qnrD, qnrS,* and *qnrVC* code for Qnr proteins that protect DNA gyrase and topoisomerase IV from the action of quinolones, conferring quinolone resistance [11]. In this study, we aimed to investigate the mechanisms responsible for carbapenem resistance in a collection of enterobacterial isolates recovered from two hospitals in Saudi Arabia. Carbapenemase-producing isolates were also further investigated for additional antibiotic resistance traits.

2. Materials and Methods

2.1. Bacterial Isolates. Four *K. pneumoniae* and two *E. cloacae* isolates from two hospitals in Riyadh, the capital of Saudi Arabia and Al-Gouf, the north region of Saudi Arabia were highly resistant to carbapenems. Three *K. pneumoniae* isolates (KP-Q1, KP-Q2, and KP-Q3) were obtained from hospital A while a single *K. pneumoniae* (KP-Q4) and two *E. cloacae* (EN.C.Q5 and EN.C.Q6) isolates were collected from hospital B in 2015 (Table 1). Isolates were identified in the clinical laboratory using the VITEK 2 system (bioMérieux, Marcy l'Etoile, France) and confirmed by conventional morphological and biochemical tests.

2.2. Antimicrobial Susceptibility Testing and Phenotypic Carbapenemase Detection. Etest strips (bioMérieux,Marcy l'Etoile, France) and disk diffusion methods (Oxoid, UK) were used to determine susceptibility to antimicrobials other than colistin, whose susceptibility was tested using a microbroth dilution method. The guidelines of the Clinical Laboratory Standards Institute (CLSI, 2016) [12] were used to interpret MICs except for tigecycline and colistin, whose data were interpreted according to the 2019 guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [13]. The Carba NP test [14] was carried out to detect carbapenemase activity. Production of MBL was screened by an EDTA-disk synergy test [15].

2.3. PCR Amplification of Resistance Genes. Carbapenem-resistant enterobacterial isolates were collected from 200 μL volumes of overnight Luria Bertani broth (Thermo Fischer Scientific, Waltham, MA, USA) cultures and resuspended in equal volumes of sterile distilled water, heated at 99°C for 10 min on a heating block, and then centrifuged at 15,000 rpm for two minutes. The supernatant, which contained released DNA, was used directly in PCR amplification of the resistance genes on a Techne Flexigene Thermocycler (Techne, Duxford, Cambridge, UK). Positive and negative controls were included in all PCR runs. All PCR amplicons were electrophoresed on 0.8% agarose gels containing 0.5 mg/L ethidium bromide and then analyzed under UV light (Pharmacia LKB; Biotechnology AB, Gothenburg, Sweden) and photographed using a documentation system.

The multiplex PCR protocol described by Dallenne et al. [16] was used to amplify four types of class A β-lactamase genes (TEM, SHV, five CTX-M families, and an OXA-1-like). Multiplex PCR was used to seek plasmid-mediated AmpC β-lactamase genes, including ACC, FOX, MOX, CMY, LAT, and MIR, as previously described [17] using six pairs of primers. Ten carbapenemase genes, including IMP, VIM, NDM, SIM, SPM, GIM, AIM, DIM, OXA-48, and KPC, were tested via three multiplex reactions according to the method of Poirel et al. [18]. Moreover, the isolates were screened by multiplex PCR for *qnrA*, *qnrB*, and *qnrS* plasmid-mediated quinolone resistance genes [19], whereas a simplex PCR was performed to detect AAC(6′)-Ib [7]. Additionally, six

Table 2: Antimicrobial resistance profile of six resistant *Klebsiella pneumoniae* and *Enterobacter cloacae* isolates.

Antibiotics	MIC (mg/L)					
	KP-Q1	KP-Q2	KP-Q3	KP-Q4	EN.C.Q5	EN.C.Q6
Amoxicillin	>256	>256	>256	>256	>256	>256
Piperacillin	>256	>256	>256	>256	>256	>256
Ticarcillin	>256	>256	>256	>256	>256	>256
Cefoperazone	>256	>256	>256	>256	>256	>256
Cefotaxime	>256	>256	>256	>256	>256	>256
Ceftazidime	>256	>256	>256	>256	>256	>256
Cefepime	>256	>256	>256	>256	>256	>256
Aztreonam	>256	>256	>256	>256	>256	>256
Cefoxitin	>256	>256	>256	>256	>256	>256
Cefotetan	>256	>256	>256	>256	>256	>256
Imipenem	>256	>256	>256	>256	>256	>256
Meropenem	>32	>32	>32	>32	>32	>32
Doripenem	>32	>32	>32	>32	>32	>32
Amikacin	>256	>256	>256	>256	24 (S)	>256
Gentamicin	>256	>256	>256	>256	64	>256
Nalidixic acid	>256	>256	>256	>256	>256	>256
Ciprofloxacin	>32	>32	>32	>32	2 (S)	8
Ofloxacin	>32	>32	>32	>32	2 (S)	8
Tigecycline	0.75	0.75	0.75	0.19 (S)	2	2
Colistin	0.75 (S)	3	1.5 (S)	2 (S)	0.75 (S)	0.75 (S)
	Inhibition zone diameter (mm) by disk diffusion					
Sulfamethoxazole/trimethoprim 23.75 / 1.25 μg	6	6	6	6	6	6
Tetracycline 30 μg	6	6	8	15 (S)	6	12 (I)
Chloramphenicol 30 μg	22 (S)	23 (S)	21 (S)	16 (S)	6	6

KP: *Klebsiella pneumoniae*, EN.C: *Enterobacter cloacae*
S: susceptible, I: intermediate
Disk diameter is 6 mm
MIC: Minimum Inhibitory Concentration
Resistance interpretation for all antimicrobials unless labeled S or I
Interpretation according to CLSI guidelines (2016) except for tigecycline and colistin, which were interpreted according to EUCAST guidelines (2019).

16S rRNA methylase genes were tested using two multiplex reactions, with the first detecting *armA*, *rmtB*, and *npmA* and the second *rmtA*, *rmtC*, and *rmtD* according to the previously published method [20]. The PCR products were sequenced and analyzed using the NCBI database with the BLAST program (http://www.ncbi.nlm.nih.gov).

2.4. Multilocus Sequence Typing (MLST) Analysis. Clonal relatedness was determined by MLST according to the Pasteur Institute scheme for the *K. pneumoniae* isolates (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) and according to the PubMLST protocol and database for the *E. cloacae* isolates (https://pubmlst.org/ecloacae/).

2.5. Plasmid Extraction and Conjugation Experiment. A plasmid extraction protocol [21] was used to investigate the presence of plasmids in all isolates and was followed by direct agarose gel electrophoresis of extracted DNA. Bac-tracker (Epicentre, Madison, WI, USA) was used as a plasmid size marker.

Transfer of imipenem resistance genes by conjugation from our isolates as donors was attempted by filter mating [22] with *Escherichia coli* J53 sodium azide resistant as the recipient strain. The initial donor/recipient ratio was 1:5 or 1:10. Transconjugants were selected on MacConkey agar containing sodium azide (100 mg/L) and ceftazidime (4 mg/L).

3. Results

MICs of different antibiotics for the six carbapenem-resistant *K. pneumoniae* and *E. cloacae* isolates are shown in Table 2. All isolates were highly resistant to all tested β-lactams. They all remained susceptible to tigecycline (MIC $\leq$ 2 mg/L) according to the EUCAST 2016 breakpoint, although the cut-off tigecycline in EUCAST 2019 guidelines is 0.5 mg/L. Consequently, all our isolates, except one *K. pneumonia*, were resistant to tigecycline using the new EUCAST definition. A single *K. pneumoniae* isolate (KP-Q2) was resistant to colistin according to the EUCAST definition (MIC > 2 mg/L is considered resistant). Moreover, the four *K. pneumonia*e isolates were determined to be susceptible to chloramphenicol with inhibition zone diameters > 12 mm according to the 2016 CLSI guidelines, whereas the two

E. cloacae isolates were resistant to this compound. Resistance to sulfamethoxazole/trimethoprim was also observed in all isolates. Four isolates (three *K. pneumoniae* and a single *E. cloacae*) were resistant to tetracycline. Resistance profiles of aminoglycosides (amikacin and gentamicin) and quinolones/fluoroquinolones (nalidixic acid, ciprofloxacin, and ofloxacin) are presented in Table 2.

All isolates exhibited a carbapenemase activity as detected by the Carba NP test. MBL production was detected by EDTA-disk synergy tests in all isolates. This was confirmed by PCR, with the $bla_{\text{NDM-1}}$ carbapenemase gene being amplified in the four *K. pneumoniae* isolates and the $bla_{\text{VIM-1}}$ gene being amplified in the two *E. cloacae* isolates (Table 3). No additional carbapenemase gene was identified in those six isolates.

Additionally, three *K. pneumoniae* isolates harbored a 16S rRNA methylase encoding gene, *rmtC*, and the quinolone resistance gene, *qnrB*. Moreover, a single *E. cloacae* isolate harbored the plasmid-mediated AmpC-encoding genes, $bla_{\text{CMY-4}}$. Additionally, all isolates were found to harbor CTX-M-ESBL-encoding genes. All *K. pneumoniae* isolates were found to harbor OXA-1 broad spectrum beta-lactamases (BSBL). TEM-1-BSBL was found in all isolates except *E. cloacae* isolate EN.C.Q6. The aminoglycoside-modifying enzyme AAC(6′)-Ib was found in all isolates except *E. cloacae* isolate EN.C.Q5 (Table 3).

Transfer of imipenem resistance from all isolates to *E. coli* J53 was attempted by filter mating. The conjugation experiment demonstrated conjugative transfer of $bla_{\text{NDM-1}}$ from *K. pneumoniae* and $bla_{\text{VIM-1}}$ from *E. cloacae* to *E. coli* J53, confirming carriage of both genes on transferable plasmids. In *K. pneumoniae*, a single 70-kb plasmid was identified in all four isolates, onto which the $bla_{\text{NDM-1}}$ gene was located. On the other hand, the $bla_{\text{VIM-1}}$ gene was located on a 60-kb plasmid in a single *E.* cloacae isolate and on a 150-kb plasmid in the other isolate (Figure 1).

MLST genotyping revealed that three *K. pneumoniae* isolates, which had actually been recovered from same hospital, belonged to ST152. The fourth *K. pneumoniae* isolate belonged to ST572 and had been recovered from the other hospital.

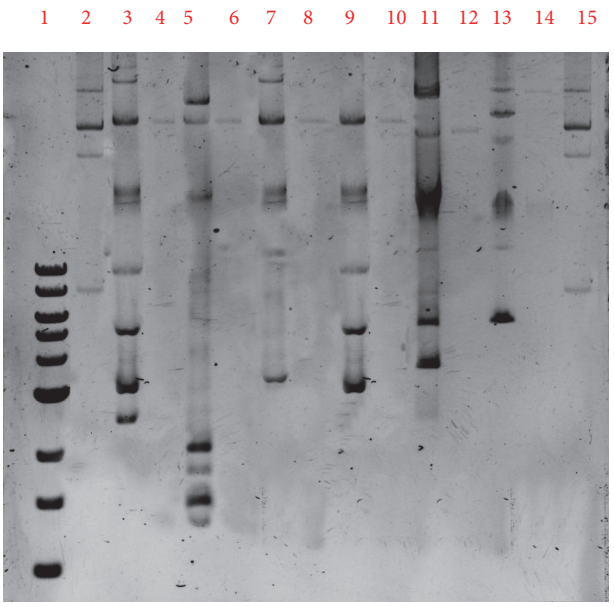

Figure 1: Electrophoretic profile of the plasmid DNA extracts from the strains and their transconjugants to confirm the results. 1. Ladder 1kb (10kb,8kb,6kb,5kb,4kb,3kb,2kb,1.5kb,1kb). 2. Ladder control strain 50192 (154kb, 66kb, 48kb, 7.8kb). 3. KP1 strain (70kb). 4. KP1 Transconjugant strains (70kb). 5. KP 2 (70kb). 6. KP2 Transconjugant strains (70kb). 7. KP 3 (70kb). 8. KP3 Transconjugant strains (70 kb). 9. KP 4 strains (70kb). 10. KP 4 Transconjugant strain 4 (70 kb). 11. EN.C 5 strains (60 kb). 12. EN.C 5 Transconjugant strain (60kb). 13. EN.C 6 strain (150kb). 14. EN.C6 Transconjugant strains (150kb). 15. Ladder control strain 50192 (154kb, 66kb, 48kb, 7.8kb).

4. Discussion

The overuse of carbapenems during the last decade has led to increasing levels of bacterial resistance toward these potent β-lactams. Enterobacterial isolates have shown high carbapenem resistance rates worldwide, making alternative antibiotics, such as colistin and tigecycline, urgently needed. In the current study, we investigated by using phenotypic and genotypic methods six carbapenem-resistant enterobacterial isolates for their multiresistance determinants. In a recent study from Saudi Arabia [23], tigecycline resistance was not detected among 31 *Enterobacteriaceae* clinical isolates (21 *K. pneumoniae* and 10 *E. coli*) whereas colistin resistance was seen in 10% (one isolate) and 4.8% (one isolate) of their *E. coli* and *K. pneumonia* isolates, respectively. Similarly, colistin and tigecycline susceptibility were 100 and 87.5%, respectively, in 16 extensively drug-resistant *K. pneumoniae* strains from Saudi Arabia [24]. According to several reports [25–27], colistin and tigecycline remain the most effective antibiotics against carbapenem-resistant Gram-negative pathogens. Interestingly, the 2019 EUCAST guidelines (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf) [13] changed the tigecycline resistance breakpoint to MIC > 0.5 mg/L. According to this new definition, all our isolates except one *K. pneumoniae* isolate would be considered resistant, suggesting an increasing challenge to public health. In the current study, resistance to colistin is not determined in our isolates, except one *K. pneumonia* isolate which has low-level colistin resistance (MIC 3 mg/L).

Two members of MBLs were detected among our isolates, namely, NDM-1-producing *K. pneumoniae* isolates and VIM-1-producing *E. cloacae* isolates. Although OXA-48 is commonly identified among *K. pneumoniae* isolates in some parts of Saudi Arabia [28–30], this resistance determinant was not detected in the present study. Moreover, a multicenter study in Saudi Arabia, Memish et al. [28], reported that OXA-48 and NDM-1 are the dominant carbapenemases among 124 *Enterobacteriaceae* (*E. coli*, *Klebsiella* spp., and *Enterobacter* spp.) isolated from 12 cities across the Kingdom of Saudi Arabia with low incidence of VIM and complete absence of KPC and IMP.

Table 3: Antibiotic resistance enzymes and genotypic screening of six resistant *Klebsiella pneumonia* and *Enterobacter cloacae* isolates.

Strain	Carbapenemase	Size of plasmids harboring carbapenemase-encoding genes	ESBL	BSBL	16S rRNA methylase	AmpC variant	Aminoglycoside-modifying enzyme (acetyltransferase)	Quinolone resistance enzymes	MLST
KP-Q1	NDM-1	70 kb	CTX-M-3	TEM-1, OXA-1	RmtC	-	AAC(6′)-Ib	QnrB	ST152
KP-Q2	NDM-1	70 kb	CTX-M-57	TEM-1, OXA-1	RmtC	-	AAC(6′)-Ib	QnrB	ST152
KP-Q3	NDM-1	70 kb	CTX-M-82	TEM-1, OXA-1	RmtC	-	AAC(6′)-Ib	QnrB	ST152
KP-Q4	NDM-1	70 kb	CTX-M-15	TEM-1, OXA-1	-	-	AAC(6′)-Ib	-	ST572
EN.C.Q5	VIM-1	60 kb	-	TEM-1	-	CMY-4	-	-	ST171
EN.C.Q6	VIM-1	150 kb	-	-	-	-	AAC(6′)-Ib	-	ST73

KP: *Klebsiella pneumoniae*
EN.C: *Enterobacter cloacae*
ESBL: Extended-spectrum β-lactamase
MLST: Multilocus sequence typing

The coexistence of 16S rRNA methylase genes with β-lactamase genes was previously observed in Saudi Arabia. ESBLs were reported along with *armA, rmtB, rmtC,* and *npmA* in *Enterobacteriaceae* [31]. OXA-48 and NDM were detected along with *armA* and *rmtB* in *K. pneumoniae* [24]. The current study also revealed that the three clonally related *K. pneumoniae* isolates harbored the *rmtC* gene along with $bla_{\text{NDM-1}}$. In addition, the plasmid mediated AmpC β-lactamase variant, CMY-4, was determined in our study in one *E. cloacae* which coproduces VIM-1 as well. This finding is the first report describing CMY carriage by a clinical *E. cloacae* isolate from Saudi Arabia.

The gene encoding the aminoglycoside-modifying enzyme AAC(6′)-Ib was detected in all but one isolates in our study. This enzyme was previously detected among extensively drug-resistant *K. pneumonia* isolates in Saudi Arabia [24]. Furthermore, the gene encoding the quinolone resistance protein QnrB was detected in the *K. pneumoniae* isolates from our study, in accordance with previous reports from Saudi Arabia [23, 24].

Noteworthy, strain EN.C.Q5 of our *E. cloacae* isolates was resistant to gentamicin although it did not have any of aminoglycoside resistance determinant examined (16S rRNA methylase: *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, and *npmA, or acetyltransferase* AAC(6′)-Ib), suggesting other resistance mechanism involved. *Similarly*, *K. pneumonia strain* KP-Q4 and *E. cloacae* strain EN.C.Q6 were resistant to ciprofloxacin and ofloxacin without *qnrA*, *qnrB*, and *qnrS* plasmid mediated quinolone resistance genes. Quinolone resistance in these isolates may be due to other plasmid mediated genes or mutations in quinolone resistance–determining regions of DNA gyrase (*gyrA* and *gyrB*) or DNA topoisomerase IV (*parC* and *parE*) [32].

5. Conclusions

Our study reports on a series of threatening resistance determinants responsible for the multidrug resistance pattern observed among clinical isolates. The identification of MBL-encoding genes (bla_{VIM} in *E. cloacae* and bla_{NDM} in *K. pneumoniae*) being identified onto conjugative plasmids raises concerns about the real extend of diffusion of those resistance genes in Saudi Arabia. Cooccurrence of multiple resistance determinants in clonally related *K. pneumoniae* isolates highlights the importance of controlling the dissemination of such isolates by early detection in hospital settings.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Snapshot of Phylogenetic Groups, Virulence, and Resistance Markers in *Escherichia coli* Uropathogenic Strains Isolated from Outpatients with Urinary Tract Infections in Bucharest, Romania

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Background. Urinary tract infections (UTIs) caused by Uropathogenic *Escherichia coli* (UPEC) are among the most common infections worldwide, including Romania. To the best of our knowledge, this is the first study performed on a significant number of community-acquired (CA) UPEC strains isolated from Romanian outpatients, aiming to evaluate and establish potential correlations among the phylogenetic groups (PG), resistance profiles, and the virulence factors (VF) genes of the CA-UPEC isolates. *Materials/Methods*. The present study was performed on a total of 787 UPEC nonrepetitive isolates consecutively isolated during one month from outpatients with CA-UTIs, visiting one of the biggest laboratories in Bucharest, Romania, receiving patients from all over the country. The strains identification was performed by MALDI TOF and the susceptibility patterns were tested using Microscan according to CLSI guidelines. PCR assays were performed to detect the presence of different VFs (*fimH* gene encoding for type 1 fimbriae, *afaBC* for A fimbriae, *sfaDE* for S fimbriae, *KpsMTII* for capsule, *hlyA* for haemolysin A, *hlyD* for haemolysin D, and *cnf-1* for tumor necrosis factor), the phylogenetic groups (PG) A, B1, B2, and D, and the extended spectrum beta-lactamases (ESBLs) genes. *Results*. The 787 CA-UPEC strains were isolated predominantly from female patients (90.95%) of >30 years (~74%). The resistance rates were 47.52% for ampicillin, 41.16% for tetracycline, 24.39% for cotrimoxazole, 19.18% for amoxicillin-clavulanic acid, 15.50% for cefazolin, 14.99% for ciprofloxacin, and 14.86% for levofloxacin; 35.19% of the investigated strains were MDR and 9.03% ESBL producers (from which 42.25% were positive for *bla*CTX-M, 38.02% for *bla*TEM, and 19.71% for *bla*SHV). *FimH* was the most frequent virulence gene (93.90%) followed by *hlyD* (44.34%); *afaBC* (38.24%); *KpsMTII* (32.65%); sfaDE (23.88%); *hlyA* (12.45%); and *cnf-1* (7.75%). The distribution of the analyzed UPEC strains in phylogenetic groups was different for non-MDR and MDR strains. Overall, 35% of the strains belonged to the phylogenetic group B2 (harboring the *yjaA* gene); 27% to group B1 (confirmed by the presence of the *TspE4C2* fragment); 16% to group D; and 22% to group A. The CA-UPEC strains included in PG B1 and PG B2 proved to be the most virulent ones, the number of strains carrying multiple VFs (>3) being significantly larger as compared to strains belonging to PG A and PG D) (p<0,0001). The presence of one or two ESBL genes was significantly associated (p =0.0024) with PGs A and D. *Conclusions*. Our findings showed that the community UPEC strains circulating in Bucharest, Romania, belong predominantly to group B2 and >90% harbored the *fimH* gene. High MDR resistance rates were observed, as well

as extended VF profiles, highlighting the importance of this type of studies for improving the epidemiological surveillance and the therapeutic or prophylactic management of the respective infections, in the context of antibiotic resistance emergence.

1. Background

Urinary tract infection (UTI) caused by Uropathogenic *Escherichia coli* (UPEC) is the most common community-acquired (CA) and nosocomial infection [1, 2] and represents an important worldwide health problem, leading to considerable morbidity costs [3–5]. *E. coli* is the leading cause of UTIs, being responsible for 75-90% of UTIs in ambulatory patients [6]. These isolates encode different virulence factors (VFs), like toxins, capsules, invasins, and adhesins, which are contributing to the UPEC strains pathogenicity and consequently, to the severity of the produced UTI [7]. Moreover, the emergence of multidrug-resistant (MDR) UPEC strains is currently leading to major difficulties in treating the infected patients [8–10].

UPEC strains have been classified into several main phylogenetic groups (PG) (A, B1, B2, C, D, E, and F) and one *Escherichia* cryptic clade I, based on the combination of four genetic markers: *arpA, chuA, yjaA*, and the DNA fragment *TspE4C2* [11]. UPEC strains usually belong to group B2 and to a lesser extent, to group D, whereas commensal strains belong to groups A and B1 [12]. Among B2 strains, *E. coli* sequence type 131 (ST131) is considered an important emerging pathogen, harboring numerous resistance and VF genes [13]. Strains belonging to this group are resistant to most β-lactam antibiotics, mediated by the production of extended spectrum β-lactamases (ESBLs). ESBLs are plasmid-encoded enzymes which confer resistance to penicillins, broad-spectrum cephalosporins, and monobactams, but not to cephamycins and carbapenems. Moreover it has been revealed that ESBL-producing isolates show coresistance to aminoglycosides, quinolones, tetracyclines, nitrofurantoin, and trimethoprim-sulfamethoxazole [14]. The MDR phenotype is due to the presence of large plasmids, which commonly carry resistance genes for β-lactams, quinolones, aminoglycosides, and cotrimoxazole. The most common β-lactamases in *E. coli* strains are TEM, SHV, and CTX-M types [14]. Most ST131 strains belong to the O25:H4 serotype, with the specific O25b type. However, ST131 strains with serotype O16:H5 have been recently identified, as well as some others that are nontypeable for O and H antigens [15]. UPEC clones ST69, ST95, and ST73 are also frequent causes of UTIs and bloodstream infections. A study performed in Brazil showed that UTIs in men were more frequently caused by PG B2 isolates, harboring an extended VFs genes profile [12]. A study performed in Iran on 232 UPEC strains revealed that the most frequent PG was D (both for hospital and CA infections (58%), exhibiting the highest number of VF and resistance markers [16].

The aim of the study was to characterize the resistance and virulence profiles of recently isolated UPEC strains from outpatients visiting Synevo Central Laboratory, Medicover, in Bucharest, south Romania, and to establish potential correlations among PG, resistance, and VF genes profiles of the analyzed strains. To the best of our knowledge, this is the first study performed on a significant number of UPEC strains isolated from CA-UTIs in Romania.

2. Results and Discussion

The 787 UPEC strains were isolated predominantly from female patients (90.95%) and the distribution on age groups was the following: 0-16 years (8.66%); 16-30 years (16.94%); 30-50 years (35.79%), and 50-90 years (38.29%).

2.1. Phylogenetic Group Distribution of UPEC Strains. The PG analysis of the UPEC strains [17, 18] showed that 35% of the strains belonged to B2 (harboring the *yjaA* gene); 27% to B1 (confirmed by the presence of *TspE4 C2* gene); 16% to D and 22% to A. The obtained results are very close to those reported from Nüesch-Inderbinen et al., in UPEC strains isolated from community-acquired UTI in Switzerland [19].

2.2. Antibiotic Resistance Profiles of UPEC Strains. In this study, the prevalence of antimicrobial resistance markers was relatively high for drugs commonly used as emergency therapy in the treatment of UTIs, such as ampicillin (47.52%), tetracycline (41.16%), trimethoprim-sulfamethoxazole (cotrimoxazole) (24.39%), amoxicillin-clavulanic acid (19.18%), cefazolin (15.50%), and fluoroquinolones (14.99% for ciprofloxacin and 14.86% for levofloxacin). Moreover, 35.19% of the UPEC strains were MDR, according to Magiorakos et al., 2012 [20] criteria. Lower resistance percentages were recorded for aztreonam, cefepime, ceftriaxone, piperacillin-tazobactam, and gentamicin (Table 1), in contrast with the study performed by Lavigne et al., in 2016, which reported higher resistance percentages for amoxicillin, amoxicillin-clavulanic acid, nalidixic acid, ciprofloxacin, and nitrofurantoin and for cotrimoxazole in *E. coli* strains from CA-UTI.

Out of the total of the analyzed strains, 71 isolates (9.03%) were resistant to third-generation cephalosporins, all of them being positive for the investigated ESBL genes, as follows: *bla*CTX-M (42.25%) and *bla*TEM (38.02%) and for *bla*SHV (19.71%) (Table 2). Regarding the resistance profiles of the ESBL strains, they were highly resistant to ampicillin (100%), aztreonam (98.59%), cefepime (98.59%), ceftriaxone (91.59%), cefazolin (87.32%), tetracycline (73.23%), ciprofloxacin (71.83%), and levofloxacin (70.42%) (Table 2).

A study performed in Algeria, aiming to investigate antibiotic resistance and VF in 150 nonrepetitive CA-UPEC isolates has revealed a MDR rate of 46.7% [21]. The detected *bla* genes were *bla*TEM (96.8% of amoxicillin-resistant isolates), *bla*CTX-M-15 (4%), *bla*AmpC (4%), *bla*SHV-2a, *bla*TEM-4, *bla*TEM-31, and *bla*TEM-35 (0.7%) [21].

Our study has revealed that the MDR strains were classified in PG D (44.71%), followed by PG A (40.58%), PG B1 (32.71%), and PG B2 (29.71%), while the non-MDR strains were predominantly associated with PG B2, followed by B1, and to a lesser extent with the other two PGs (Figure 1). A

Table 1: Antibiotic resistance phenotypes of *E. coli* strains isolated from CA-UTIs in Romania.

Antibiotic	GR A	GR B1	GR B2	GR D	Number (percentage)
AMP	90 (52.94%)	98 (45.16%)	125 (45.28%)	66 (53.65%)	374 (47.52%)
TET	75 (44.11%)	91 (41.93%)	98 (35.50%)	61 (49.59%)	324 (41.16%)
SXT	43 (25.29%)	52 (23.96%)	60 (21.73%)	41 (33.33%)	192 (24.39%)
AMC	38 (22.35%)	41 (18.89%)	55 (19.92%)	24 (19.51%)	151 (19.18%)
CFZ	35 (20.58%)	29 (13.36%)	42 (15.21%)	17 (13.82%)	122 (15.50%)
CIP	34 (20%)	38 (17.51%)	28 (10.14%)	18 (14.63%)	118 (14.99%)
LEV	34 (20%)	38 (17.51%)	28 (10.14%)	18 (14.63%)	117 (14.86%)
ATM	16 (9.41%)	21 (9.67%)	27 (9.78%)	11 (8.94%)	79 (10.03%)
FEP	16 (9.41%)	21 (9.67%)	26 (9.42%)	10 (8.13%)	73 (9.27%)
CRO	16 (9.41%)	21 (9.67%)	26 (9.42%)	10 (8.13%)	72 (9.14%)
TZP	17 (10%)	11 (5.06%)	16 (5.79%)	3 (2.43%)	47 (5.97%)
CN	12 (7.05)	6 (2.76%)	10 (3.62%)	7 (5.69%)	34 (4.32%)
NIT	5 (2.94%)	8 (3.68%)	7 (2.53%)	4 (3.25%)	24 (3.04%)
IMP	0	0	0	1 (0.81%)	1 (0.12%)
MEM	0	0	0	0	0
ETP	0	0	0	0	0

AMP, ampicillin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; AMC, amoxicillin-clavulanic acid; CFZ, cefazolin; CIP, ciprofloxacin; LEV, levofloxacin; ATM, aztreonam; FEP, cefepime; CRO, ceftriaxone; TZP, piperacillin-tazobactam; CN, gentamicin; NIT, nitrofurantoin; IMP, imipenem; MEM, meropenem; ETP, ertapenem.

Table 2: Antibiotic resistance phenotypes of ESBL strains.

Antibiotic	Number (percentage)
AMP	71 (100%)
ATM	70 (98.59%)
FEP	70 (98.59%)
CRO	65 (91.59%)
CFZ	62 (87.32%)
TET	52 (73.23%)
CIP	51 (71.83%)
LEV	50 (70.42%)
SXT	34 (47.89%)
AMC	32 (45.07%)
TZP	17 (23.94%)
NIT	6 (8.45%)

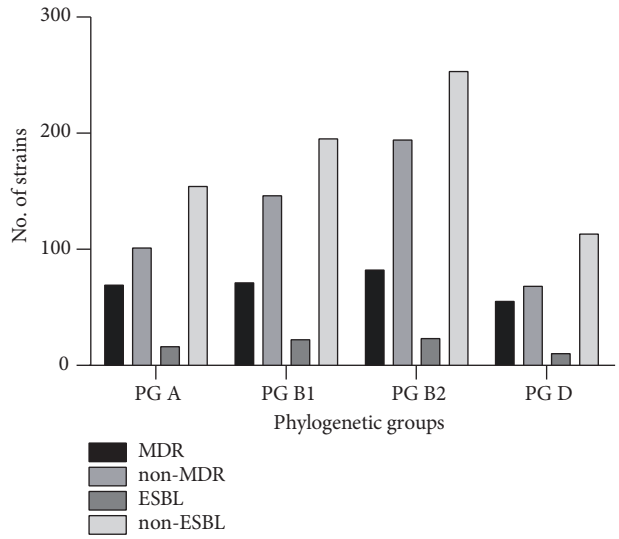

Figure 1: The distribution of ESBL and MDR isolates by phylogenetic groups.

different distribution was recorded for the ESBL-producing strains, which belonged to PG B1 (10.13%); PG A (9.41%); PG B2 (8.33%), and PG D (8.13%). However, the statistical analyses did not reveal any statistical significance of the correlation between the antibiotic resistance and *E. coli* phylogenetic groups.

In other studies, the CA-UPEC isolates belonged to phylogroups B2+D (50%), A+B1 (36%), and F+C+Clade I (13%). Most of D (72.2%) and 38.6% of B2 isolates were MDR and harbored the most extended VFs profiles [21].

2.3. Virulence Profiles of UPEC Strains. Regarding the virulence markers detected in the analyzed UPEC strains, the fimH gene was the most encountered VF (93.90%) followed by *hlyD* (44.34%); *afaBC* (38.24%); *KpsMTII* (32.65%); *sfaDE* (23.88%); *hlyA* (12.45%); and *cnf-1* (7.75%) (Table 3).

Distribution of the analyzed strains harboring different VF in PGs revealed a relatively equal distribution among the PG for fimH, while others (*hlyA, afaBC, kpsMTII, sfaDE,* and *cnf-1*) were significantly associated with certain PGs (Figure 2 and Table 3).

Regarding the correlation between the pathogenicity level (the number of VF genes) and the phylogenetic groups, the strains belonging to PG D (n=123 strains) revealed the following VF genes profiles: 5 VFs in 4.06% of the investigated strains; 4 VFs (*fimH, hlyD, sfaDE cnf-1,* and *hlyA*) in 5.69% of the isolates; 3 VFs (*fimH* was present in all the combinations

Table 3: The association of different VF genes with PGs.

	GR A	GR B1	GR B2	GR D	p-value
fimH	155 (91.17%)	204 (94%)	264 (95.65%)	116 (94.30%)	0.2873
hlyD	51 (30%)	72 (33.17%)	118 (42.75%)	51 (41.46%)	0.0196
hlyA	1 (0.58%)	3 (1.38%)	7 (2.53%)	25 (20.32%)	0.0693
afaBC	21 (12.35%)	43 (19.81%)	69 (25%)	14 (11.38%)	0.0010
KpsMTII	12 (7.05%)	72 (33.17%)	96 (34.78%)	13 (10.56%)	<0.0001
sfaDE	13 (7.64%)	42 (19.35%)	127 (46.01%)	9 (7.31%)	<0.0001
cnf-1	2 (1.17%)	13 (5.99%)	32 (11.59%)	6 (4.87%)	0.0005

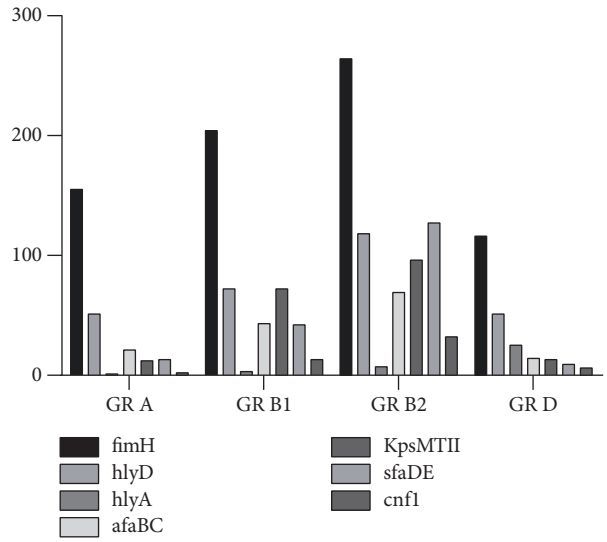

Figure 2: The distribution of VFs by PGs.

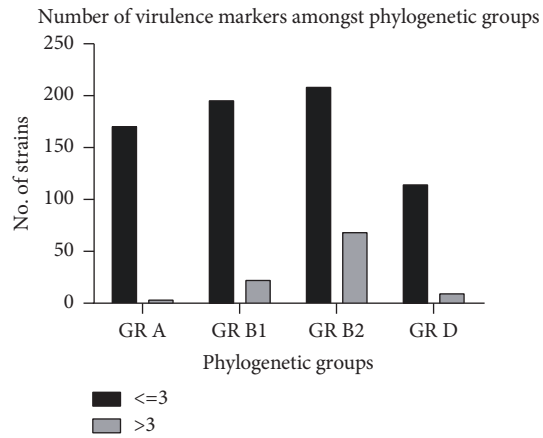

Figure 3: The distribution of the number of VFs among PGs.

also) in 16.26%; 2 VFs in 36.58%; and one VFs (37.39% of the isolates). The strains belonging to the PG A (n=170 strains) revealed 4 VFs (*fimH, hlyD, sfaDE,* and *cnf*-1) in 1.76% of the isolates; 3 VFs (fimH in all the combinations and *hlyD/afaBC/kpsMTII/sfaDE/cnf-1*) in 7.64%; 2 VFs in 32.94%; and one VFs (54.11% from which *fimH* was revealed by 96.73% and *hlyD* from 3.26% of the isolates). In case of PG B1 strains there were detected up to 5 VFs (*fimH, hlyD, kpsMTII, sfaDE, cnf-1* or*hlyA*) in 1.84% of the isolates; 4 VFs (with *fimH* being revealed in all of them excepting one) in 8.29% of the isolates; 3 VFs in 20.27%; 2 VFs in 35.48%; and one VFs (32.71% of the isolates). The strains classified in PG B2 demonstrated the presence of 6 VFs in 0.36% of the UPEC isolates; 5 VFs in 5.79%; 4 VFs (*fimH* being revealed in all of them) in 18.47% of the isolates; 3 VFs in 25.36%; 2 VFs in 28.62%; and one VF in 19.92% of the isolates.

The strains classified in PG B1 and PG B2 were the most virulent ones, the number of strains carrying >3VFs being significantly larger than that of strains belonging to PG A and PG D, where predominating the strains carrying less VFs (≤ 3) ($p<0.0001$) (Figure 3).

Only few studies are reported in literature for CA-UPEC isolates. A study performed on Uruguayan children with UTIs revealed that 48.2% of the *E. coli* isolates belonged to PG D and 35.5% to PG B2, with the most frequent VFs being kpsMTII and fimH [22]. Among 59 isolates of UPEC isolated in Pakistan from CA-UTIs, the PG B2 was the most frequent (50%), followed by PG A, B1 (19% each), and D (12%). Isolates present in group D showed the highest number of VFs, among which the most frequent were hlyA (37%), sfaDE (27%), papC (24%), cnf1 (20%), eaeA (19%), and afaBC3 (14%) [23].

Of the UPEC adhesins, fimH, a type 1 fimbriae, has a crucial role in UPEC colonization in the bladder, which is required for the initiation of UTI [24]. *E. coli* afimbrial adhesin (Afa) encoded by *afa* gene has been reported in cases of pyelonephritis and recurring cystitis; another adhesin that acts as a virulence factor is S fimbrial adhesin, which is encoded by *sfa* genes [25]. Other very important virulence factors in UPEC strains, like toxins, mediating invasion, dissemination, and persistence of bacteria in host cells have been demonstrated [26]. The most important soluble virulence factor is α-hemolysin (HlyA), which is encoded by the *hly* gene. Also, the cytotoxic necrotizing factor 1 (CNF1) is one of the most important virulence factors of *E. coli* involved in the development of an UTI. It has been revealed that α-hemolysin and CNF1 mediate the release of iron from red blood cells, induce dysfunction of phagocytic cells, and exhibit direct cytotoxicity to the tissues [27]. Some authors report that the prevalence ratios of *sfa*, *hlyA,* and iron uptake genes were 2.2 to 3.5 times more prevalent among outpatients compared with inpatients UTI isolates [28].

According to the present observations, *fimH* gene had the highest frequency (93.90%) among the tested VF genes, while

Table 4: Virulence genes profiles and PGs in the investigated *E. coli* strains.

VF	Number (percentage)
fimH	739 (93.90%)
hlyD	349 (44.34%)
afaBC	301 (38.24%)
kpsMTII	257 (32.65%)
sfaDE	188 (23.88%)
hlyA	98 (12.45%)
cnf-1	61 (7.75%)
PGs	
B2	276 (35%)
B1	216 (27%)
A	173 (22%)
D	122 (16%)

cnf-1 had the lowest one (7.75%) (Table 4). Similar results have been revealed in Romania by Grosu et al. in 2017 [29] in *E. coli* strains isolated from the ambulatory sector of Central Laboratory Regina Maria hospital in Bucharest. The *fimH* gene was also reported to have a high prevalence (>90%-100%) among UPEC strains isolated in other countries, and some authors are stating that FimH could be used as a possible diagnostic marker and/or vaccine candidate [30–32]. The study of Tobasi et al. [28] performed on 156 UPEC isolated from symptomatic and asymptomatic UTI outpatients and inpatients revealed that *fimH* was present in all analyzed strains. Derakhshandeh et al., in 2015 [33], have reported that, from 85 UPEC clinical isolates, 65.9% belonged to phylogenetic group A, 17.6% belonged to B2, and 16.5% of the isolates were found to belong to group D; *fimH* has also been reported with the highest frequency among the tested VF genes, while *cnf-1* had the lowest one, similar to our results.

According to Rodriguez-Siek study in 2005 [34], most of UPEC causing UTI in human revealed capsule, the capsular antigen K1 being more often observed in UPEC. The capsule production in *E. coli* trains is mediated by*kpsMT* (encoding for K1 antigen) and *kpsMTII* genes [35]. A study performed on a total of 194 *E. coli* strains isolated in Mexico from CA-UTIs has shown that *kpsMT* was the most frequently occurring virulence gene among the UPEC strains (92.2% strains), the *fim* gene being also recorded with a high positivity rate (61.3%) [36]. Farajzadah et al., in 2018 [16], have reported that, among the 232 analyzed UPEC strains, the most frequently encountered PG was D (58%) responsible for majority of nosocomial (64.7%) and community (48.4%) acquired infections with the largest panel of VF genes, including *kpsMT* (23%) and *cnf* (29.6%). Ochoa et al., in 2016 [37], have found that, among 500 UPEC clinical strains, 103 were MDR-UPEC strains and mainly associated with the phylogenetic groups D (54.87%) and B2 (39.02%) with a high percentage of positivity for *fimH*, an iron uptake gene (*chuA*), and a toxin gene (*hlyA*).

We have also investigated the potential correlations between the PG and VF genes and the presence of different resistance phenotypes (multiple logistic regression) or ESBL genes (chi square). Our results pointed out that there is no statistically significant correlation between PG and VF and the presence of certain resistance phenotypes, which suggests that there are no particular clones associated with UTIs in Romania. On the other hand, the presence of one or two ESBL genes was significantly associated ($p = 0.0024$) with PGs A and D.

In France, UPEC isolated from UTIs belonged more frequently to phylotypes B2 and D, the strains susceptible to ciprofloxacin harboring specific VFs profiles, more extended in comparison with the ciprofloxacin-resistant strains [38]. Another study performed on 146 *E. coli* strains isolated from cystitis and pyelonephritis in Turkey investigated the relationship among PGs and various adhesion virulence genes. The *sfa/focDE* genes were more frequent in ampicillin, amikacin, gentamicin, nalidixic acid, norfloxacin, cefuroxime, ceftriaxone, cefazolin, cefotaxime, ciprofloxacin and cotrimoxazole susceptible and extended spectrum β-lactamase (ESBL), and multidrug resistance (MDR) negative isolates. *fimH* was more common in amoxicillin-clavulanic acid susceptible isolates. The *afa* gene was more frequent in resistant isolates than in susceptible ones [39].

3. Conclusions

To the best of our knowledge, this is the first study performed on a significant number of *E. coli* strains isolated from outpatients with community-acquired urinary tract infections in Bucharest, Romania, aiming to investigate the correlations among the phylogenetic group, resistance, and virulence profiles of CA-UPEC strains. The analyzed strains exhibited resistance rates ranging from 47.52% for ampicillin to 14.86% for levofloxacin, 35.19% were MDR phenotype, and 9.03% were ESBL producers. The *fimH* gene was the most frequent (93.90%), followed by *hlyD* (44.34%); *afaBC* (38.24%); *KpsMTII* (32.65%); *sfaDE* (23.88%); *hlyA*(12.45%); and *cnf-1* (7.75%). The phylogenetic group distribution was different, depending on the resistance phenotype. Overall, our findings showed that the CA-UPEC strains isolated from outpatients in Bucharest, Romania, belong predominantly to group B2 and >90% harbor the *fimH* gene. High MDR resistance rates were observed, the ESBL phenotype being associated with PGs A and D. The most extended VF profiles were encountered in CA-UPEC strains classified in the PGs B1 and B2. The obtained results highlight the importance of this type of studies for improving the epidemiological surveillance and the therapeutic or prophylactic management of the respective infections, in the context of antibiotic resistance emergence.

4. Methods

The study was conducted on a total of 787 strains isolated during one month in 2018 from outpatients visiting Synevo Central Laboratory, Medicover, in Bucharest, Romania. The

Table 5: Primers sequences used in simplex and multiplex PCR assays for genes encoding BLSE.

The gene	Primer	Nucleotide sequence	Amplification size	References
bla_{TEM}	TEM-F TEM-R	5'-ATGAGTTTTCAACATTTTCG-3' 5'-TTACCAATGCTTAATCAG TG-3'	861	Eftekar et al., 2005 [40]
bla_{SHV}	SHV-F SHV-R	5'-GCCCTCACTCAAGGATGTAT-3' 5'-TTAGCGTTGCCAGTGCTCGA-3'	888	Naas et al., 1999 [41]
bla_{CTX-M}	CTX-M-F CTX-M-R	5'-CGCTGTTGTTAGGAAGTGTG-3' 5'-GGCTGGGTGAAGTAAGTGAC-3'	730	Israil et al., 2013 [42]

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Table 6: Primers sequences used in simplex and multiplex PCR assays for virulence genes.

The gene	Primer	Amplification size and Tm	References
chuA	F: 5′-GACGAACCAACGGTCAGGAT-3′ R: 5′-TGCCGCCAGTACCAAAGACA-3′	279 bp (multiplex) 55°C	Clermont et al., 2000
yjaA	F: 5′-TGAAGTGTCAGGAGACGCTG-3′ R: 5′-ATGGAGAATGCGTTCCTCAAC-3′	211bp (multiplex) 55°C	Clermont et al., 2000
TspE4C2	F: 5′-GAGTAATGTCGGGGCATTCA-3′ R:5′-CGCGCCAACAAAGTATTACG-3′	152 bp (multiplex) 55°C	Clermont et al., 2000
hlyD	F: 5′- CTCCGGTACGTGAAAAGGAC-3′ R: 5′-GCCCTGATTACTGAAGCCTG-3′	904 bp 55°C	Rodrigues-Sike et al., 2005
kpsMTII	F: 5′- GCG CAT TTG CTG ATA CTG TTG-3′ R: 5′-CAT CAG ACG ATA AGC ATG AGC A-3′	272 bp 60°C	Johnson et al., 2005 [43]
hlyA	F:5′-AACAAGGATAAGCACTGT TCTGGC T-3′ R:5′-ACCATATAAGCGGTCATT CCC GTC A-3′	1,177 bp 60°C	Yamamoto et al., 1995 [44]
sfaD/E	F:5′-CGGAGGAGTAATTACAAACCTGGCA -3′ R: 5′- CTCCGGAGAACTGGGTG ATCTTA C-3′	408 bp 60°C	Blanco et al., 1997 [45]
fimH	F: 5′-TGC AGA ACG GAT AAG CCG TGG -3′ R: 5′- GCA GTC ACC TGC CCT CCG GTA -3′	508 bp 63°C	Rodrigues-Sike et al., 2005
afaBC	F: 5′-GCTGGGCAGCAAACTGATAACTCTC -3′ R:5′CATCAAGCTGTTTGTTCGTCCGCCG-3	793 bp 63°C	Blanco et al., 1997
cnf-1	F: 5′- GAA CTT ATT AAG GAT AGT-3′ R: 5′-CAT TAT TTA TAA CGC TG-3′	543kb 40°C	Blanco et al., 1997

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strains identification was performed using the MALDI TOF system and the susceptibility patterns were tested by Microscan according to CLSI 2018 guidelines.

4.1. DNA Extraction and Molecular Detection. The genetic support of the antibiotic resistance (ESBLs) and virulence markers was investigated by simplex and multiplex PCR, using a reaction mix of 20μl (PCR Master Mix 2x, Thermo Scientific containing $MgCl_2$ 1.2mM, dNTP 2μM DNA 0.2U Taq-pol 1x and Reaction buffer until the final volume) to which the primers at 0.5μM and and 1 μl of bacterial DNA extracted by an adapted alkaline extraction method. In this purpose, 1-5 colonies of bacterial cultures were suspended in 1.5 ml tubes containing 20 μl solution of 0.05M NaOH (sodium hydroxide) and 0.25% SDS (sodium dodecyl sulphate). The amplification program was conducted under the following conditions: 94°C,10 min; 94°C, 30s; 52°C, 40s, 36 cycles; 72°C 50s; 72°C 5 min.

Bacterial DNA were subjected to simplex PCR targeting the chuA gene, the yjaA gene, and an unspecified DNA fragment termed TspE4.C2, as described previously [17]. Isolates were classified as belonging to one of the four phylogenetic groups A, B1, B2, or D. The sequence of the primers used in PCR experiments, the amplicon size obtained and the sources are presented in Tables 5 and 6.

5. Statistical Analysis

Statistical analysis was carried out using chi square and chi square test for trend tests using Graph Pad Prism version 8.0.1 (244) and multiple logistic regression using Stats Direct version 3. For all statistical tests, p values <0.05 were considered significant.

Data Availability

All data analyzed or generated during this study are included in this published article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Violeta Corina Cristea, Ioan Mircea Popa, and Mariana Carmen Chifiriuc designed the study and corrected the manuscript, Violeta Corina Cristea performed the isolation and identification, Irina Gheorghe, Laura Ioana Popa, Ilda Czobor Barbu, Bogdan Ispas, Georgiana Alexandra Grigore, Alexandra Velican, Luminita Marutescu, Marcela Popa, and Ilda Czobor Barbu performed the molecular analyses, Gabriela Loredana Popa has contributed to the design of the study, analysis of the results, drafting and the correction of the manuscript, Irina Gheorghe, and Ilda Czobor Barbu performed the statistical analysis and drafted the paper. Violeta Corina Cristea, Irina Gheorghe, Ilda Czobor Barbu, Gabriela Loredana Popa, Alexandra Velican, Luminita Marutescu, Marcela Popa, Mariana Carmen Chifiriuc, and Ioan Mircea Popa have equally contributed to this paper as main authors.

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Research Article

Laboratory and Clinical Evaluation of DNA Microarray for the Detection of Carbapenemase Genes in Gram-Negative Bacteria from Hospitalized Patients

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Background. The prevalence of a variety of carbapenemases in Gram-negative bacteria (GNB) has posed a global threat on clinical control and management. Monitoring and controlling the carbapenemase-producing GNB became imperative tasks for many healthcare centers. The aim of this study was to develop a high-throughput, specific, sensitive, and rapid DNA microarray-based method for the diagnosis, phenotypic confirmation, and molecular epidemiological study of carbapenemase genes. *Methods*. We targeted a panel of eight carbapenemase genes, including bla_{KPC}, $bla_{\mathrm{NDM\text{-}1}}$, $bla_{\mathrm{OXA\text{-}23}}$, $bla_{\mathrm{OXA\text{-}48}}$, $bla_{\mathrm{OXA\text{-}51}}$, bla_{IMP}, bla_{VIM}, and bla_{DIM} for detection. Ultrasensitive chemiluminescence (CL) detection method was developed and used to simultaneously detect eight carbapenemase genes, and plasmids were established as positive or limit of detection (LOD) reference materials. Antibiotic susceptibility was determined by disk diffusion according to Clinical and Laboratory Standards Institute (CLSI) guidelines in order to screen clinical isolates resistant to carbapenem antibiotics as well as Sanger sequencing which was used to confirm the reliability of the results presented by DNA microarray. *Results*. Eight carbapenemase genes could be detected with high sensitivity and specificity. The absolute LOD of this strategy to detect serially diluted plasmids of eight carbapenemase genes was 10^2- 10^3 copies/μL. Then, 416 specimens collected from hospital were detected and the results showed 96.6% concordance between the phenotypic and microarray tests. Compared with Sanger sequencing, a specificity and sensitivity of 100% were recorded for $bla_{\mathrm{NDM\text{-}1}}$, bla_{IMP}, bla_{VIM}, and bla_{DIM} genes. The specificity for bla_{KPC}, $bla_{\mathrm{OXA\text{-}23}}$, $bla_{\mathrm{OXA\text{-}48}}$, and $bla_{\mathrm{OXA\text{-}51}}$ genes was 100% and the sensitivity was 98.5%, 97.6%, 95.7%, and 97.9%, respectively. The overall consistency rate between the sequencing and microarray is 97.8%. *Conclusions*. The proposed ultrasensitive CL imaging DNA hybridization has high specificity, sensitivity, and reproducibility and could detect and differentiate clinical specimens that carried various carbapenemase genes, suggesting that the method can conveniently be customized for high-throughput detection of the carbapenemase-producing GNB and can be easily adapted for various clinical applications.

1. Introduction

Carbapenems are a class of β-lactam antibiotics with a broad spectrum and served as the last line against ESBLs efficiently and stably [1]. With wide and heavy use of various antibiotics, carbapenem-resistant isolates have become worldwide public-health issue with a widespread distribution, broad range of activities against β-lactams, and increased patient morbidity, mortality, and lengths of hospital stay [2], particularly among elderly patients, infants, and patients with severe underlying disease. In the clinic, it is imperative to develop a rapid, simple, and accurate test to detect and identify the clinical strains that produce carbapenemase, and this is critical for the management and control of the increasing prevalence of carbapenemase-producing strains worldwide [3].

The detection of carbapenemase producers in clinical specimens is based first on the analysis of susceptibility testing according to CLSI updated in 2017 [4], followed by confirmatory genotypic tests with methods like PCR and mass spectrometry [3, 5]. However, these tests are not the

best fit for detecting the carbapenemase-producing strains with desirable specificity and sensitivity [6]. Even the recommended CLSI methods are limited by their inherent disadvantages: the phenotypic tests for carbapenemase-producing strains suffer problems of false positives and false negatives [5, 7]. Mass spectrometry cannot provide molecular epidemiological information and is cost-ineffective. Addressing these deficiencies, we herein propose a novel DNA microarray-based method for rapid, sensitive, and specific detection of clinical carbapenamase-producing samples. The proposed assay may have important implications in the diagnosis, phenotypic confirmation, and the molecular epidemiology studies of carbapenemase-producing bacteria.

The Ambler molecular classification system, based on protein homology, categorizes β-lactamases into four classes (A to D). The most extensive distribution class A enzyme with carbapenemase activity is KPC. The class B enzymes are metalloenzymes of the most IMP or VIM series. Besides, NDM and DIM belong to class B enzymes which are widely concerned in Asia [8]. Many OXA enzymes (OXA-23-like, OXA-48-like, class D) are considered to be responsible for the worldwide resistance epidemics as well as their detailed properties which have been extensively reported [9, 10]. The presence of $bla_{\text{DIM-1}}$ has been considered lower once before, for a *Pseudomonas stutzeri* isolate from the Netherlands [11]. However, in a recent report, $bla_{\text{DIM-1}}$ was found in hospital isolates belonging to the families Enterobacteriaceae, Pseudomonadaceae, Burkholderiaceae, and Comamonadaceae and forty percent of the isolates were found to contain $bla_{\text{DIM-1}}$ among the tested isolates [12]. The $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-23}}$ genes are mainly responsible for the resistance in *Acinetobacter baumannii* [13], whereas the $bla_{\text{OXA-23-like}}$ genes are the most prevalent carbapenem-resistant genes identified in China [13, 14]. The $bla_{\text{OXA-51-like}}$ genes have been considered exclusively chromosomally encoded, intrinsic oxacillinase genes of *Acinetobacter baumannii* and are used by many investigators for species identification and strain typing [15]. However, a number of recent reports indicate that the $bla_{\text{OXA-51-like}}$ genes have been mobilized and are spreading to other *Acinetobacter* spp. by conjugative plasmids [16, 17]. While further work is required to determine carbapenem resistance of OXA-51-like group, the enzymes of this group remain a major concern, as they present the possibility that all *A. baumannii* isolates may be capable of becoming resistant to the carbapenems [18]. Therefore, in the current study, we included bla_{KPC}, $bla_{\text{NDM-1}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-51}}$, bla_{IMP}, bla_{VIM}, and bla_{DIM} in the detection panel for the DNA microarray assays.

DNA microarrays have had wide applications, including gene expression analysis [19], disease diagnosis [20], and pathogenic microorganism detection [21]. This technique is characterized by miniaturization, high-throughput, manageability, and easiness of automatization. The purpose of this work is to develop a rapid, reliable, and high-throughput DNA microarray method for detection of clinically relevant carbapenemase-encoding genes. In this assay, a reliable and portable, ultrasensitive chemiluminescence (CL) imaging DNA hybridization was developed to simultaneously detect eight genes. Plasmids were established as positive or limit of detection (LOD) reference materials. The specificity and sensitivity of the method was validated in 416 actual samples.

2. Materials and Methods

2.1. Ethics Statement. All patients provided informed consent in accordance with requirements of the Declaration of Helsinki, and the research project was approved by the Ethical Committee of Chinese People's Liberation Army (PLA) General Hospital.

2.2. Specimen Collection and Processing. The samples collected from PLA General Hospital of China were sputum, urine, and bacteria isolates. Sputum sample was liquefied by 4% NaOH for 30 min at room temperature with shaking and then centrifuged at 13,000 rpm for 2 min. Pellets were collected and washed. The DNA in sputum pellets and urine samples were isolated by QIAamp DNA Mini Kit according to the manufacturer's instructions. Bacteria isolates were prepared by modified boiling method. Briefly, colonies of each isolate were picked from Luria-Bertani plates and suspended in 100 μL of sterilized H_2O, followed by boiling at 95°C for 15 min and centrifugation at 12,000 rpm for 5 min. Supernatants were harvested and transferred to new tube and served as templates for PCR and microarray assays [13].

2.3. Primer and Probe Design. The DNA sequences of the carbapenemase (i.e., bla_{KPC}, $bla_{\text{NDM-1}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-51}}$, bla_{IMP}, bla_{VIM}, and bla_{DIM}) were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genomes/). Primers and probes were designed by DNAMAN and Primer Premier. Specific primers were designed for $bla_{\text{NDM-1}}$, $bla_{\text{OXA-48}}$, and $bla_{\text{DIM-1}}$. For bla_{KPC}, bla_{IMP}, bla_{VIM}, $bla_{\text{OXA-23-like}}$, and $bla_{\text{OXA-51-like}}$, primers were chosen in the conserved upstream or downstream regions. Microarray probes ranging from 33 to 42 nucleotides were synthesized for these genes. These genes were amplified by multiplex PCR in separate tubes, and an internal standard probe was included for each tube for process monitoring. Finally, ten primers and seventeen probes with favorable specificities were selected (Tables 1 and 2). All the primers and probes were verified by BLAST (http://blast.ncbi.nlm.nih.gov/).

2.4. Construction of Reference Plasmids. Oligonucleotides of bla_{DIM} and bla_{VIM} in this study were spliced by big primer amplification method in which 40 bp fragments were concatenated into final sequences of about 280 bp. Carbapenemase-producing samples of OXA-23, OXA-48, and OXA-51 were collected from Chinese PLA General Hospital in Beijing and samples of KPC, IMP, and NDM-1 were collected from Chinese PLA Academy of Military Medical Sciences. These eight well-characterized reference strains, each carrying bla_{KPC}, $bla_{\text{NDM-1}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-51}}$, bla_{IMP}, bla_{VIM}, or bla_{DIM}, were used for the design and validation of the microarray probes and primers. DNA fragments of KPC, NDM-1, OXA-23, OXA-48, OXA-51, IMP, VIM, DIM, mitochondrial DNA (mtDNA), and 16S rRNA were amplified by PCR, followed by digestion with PGM-T and cloning into DH5α. The cloned fragments were confirmed by sequencing the entire regions. Mitochondrial DNA

Table 1: The primer sequences for microarray.

Primer [a]	Sequence (5′-3′)	Positions [b]	Targeted gene/ GenBank accession
KPC-F	CTGGGCAGTCGGAGACAAAA	681-700	KPC/ KX619622.1
KPC-R	AGACGGCCAACACAATAGGT	765-784	
NDM1-F	GAATGTCTGGCAGCACACT	168-186	NDM-1/ KX249707
NDM1-R	TGGCATAAGTCGCAATCC	407-424	
OXA23-F	GCAGTCCCAGTCTATCAGGA	379-398	OXA-23/NG_049726
OXA23-R	CCCAACCAGTCTTTCCAA	641-658	
OXA48-F	TCGGGCAATGTAGACAGTT	548-556	OXA-48/ NG_049762
OXA48-R	CACCAGCCAATCTTAGGTTC	746-765	
OXA51-F	GCTCGTCGTATTGGACTTGA	406-425	OXA-51/ KX609247
OXA51-R	TGTGCCTCTTGCTGAGG	523-539	
IMP-F	GTAATTGACACTCCATTTAC	291-309	IMP /NG_049172
IMP-R	GCGGACTTTGGCCAAGCTTC	674-693	
VIM-F	TGGTGAGTATCCGACAG	190-206	VIM/NG_050336
VIM-R	ATGAAAGTGCGTGGAG	433-448	
DIM-F	GCTTGTCTTCGCTTGCTAA	38-56	DIM/NG_049077
DIM-R	ATTCCTGCGGTTCTATCCT	293-311	
mtDNA-F	GTCGAAGGTGGATTTAGCAGTAA	1413-1435	mtDNA/MG182040
mtDNA-R	GTAAGGTGGAGTGGGTTTGGG	1684-1704	
182-F	AGAGTTTGATCMTGGCTCAG	1-20	16S rRNA/ LN612729
756-R	CGTATTACCGCGGCTGCTG	518-530	

[a] F, forward primer; R, reverse primer; all reverse primers have biotin conjugated at 5′-ends.
[b] Positions refer to the nucleotide numberings of the corresponding GenBank genes.

Table 2: The probe sequences for microarray.

Probe	Sequences (5′-3′) [a]	Targeted gene
KPC-P	CAAATGACTATGCCGTCGTCTGGCC	KPC
NDM1-P1	ACCGATGACCAGACCGCCCAGATCCTCAAC	NDM-1
NDM1-P2	TCAGGACAAGATGGGCGGTATGGAC	NDM-1
OXA23-P1	TTTTAGAAGAGAGTAATGGCTACAAAA	OXA-23
OXA23-P2	ATTGGACAGCAGGTTGATAATTTCTGG	OXA-23
OXA48-P1	CGAATTTCGGCCACGGAGCAAATCAGCTT	OXA-48
OXA48-P2	CAGCGTATTGTCAAACAAGCCATGC	OXA-48
OXA51-P1	GAAGTGAAGCGTGTTGGTTATG	OXA-51
OXA51-P2	ATATCGGTACCCAAGTCGATAATTTTTGGC	OXA-51
IMP-P1	GGCTAGTTAAAAATAAAATTGAAG	IMP
IMP-P2	CCCACGTATGCRTCTGAATTAAC	IMP
VIM-P1	TGGTGTTTGGTCGCATATCGCAACG	VIM
VIM-P2	CTCATTGTCCGTGATGGTGATGAG	VIM
DIM-P1	GTCAGTTCAAACGGCCTTGTTGTCATAGATT	DIM
DIM-P2	CTTGGTCAGACCGAGATACAGAAACGCTCG	DIM
mtDNA-P	ATGTCCTTTGAAGTATACTTGAGGAGTT	mitochondria
551-P	ACTCCTACGGGAGGCAGCAGTT	16S rRNA
Quality control [b]	TTTTTTTTTTTTTTTTTTTT	Oligo dT_{20}

[a] An oligonucleotide of 12 T's with an amino-labeled 3′-end was conjugated to the 3′-ends of all probes. [b] An oligonucleotide of 20 T's with an amino-labeled 3′-end, biotin-labeled 5′-end was used as microarray quality control.

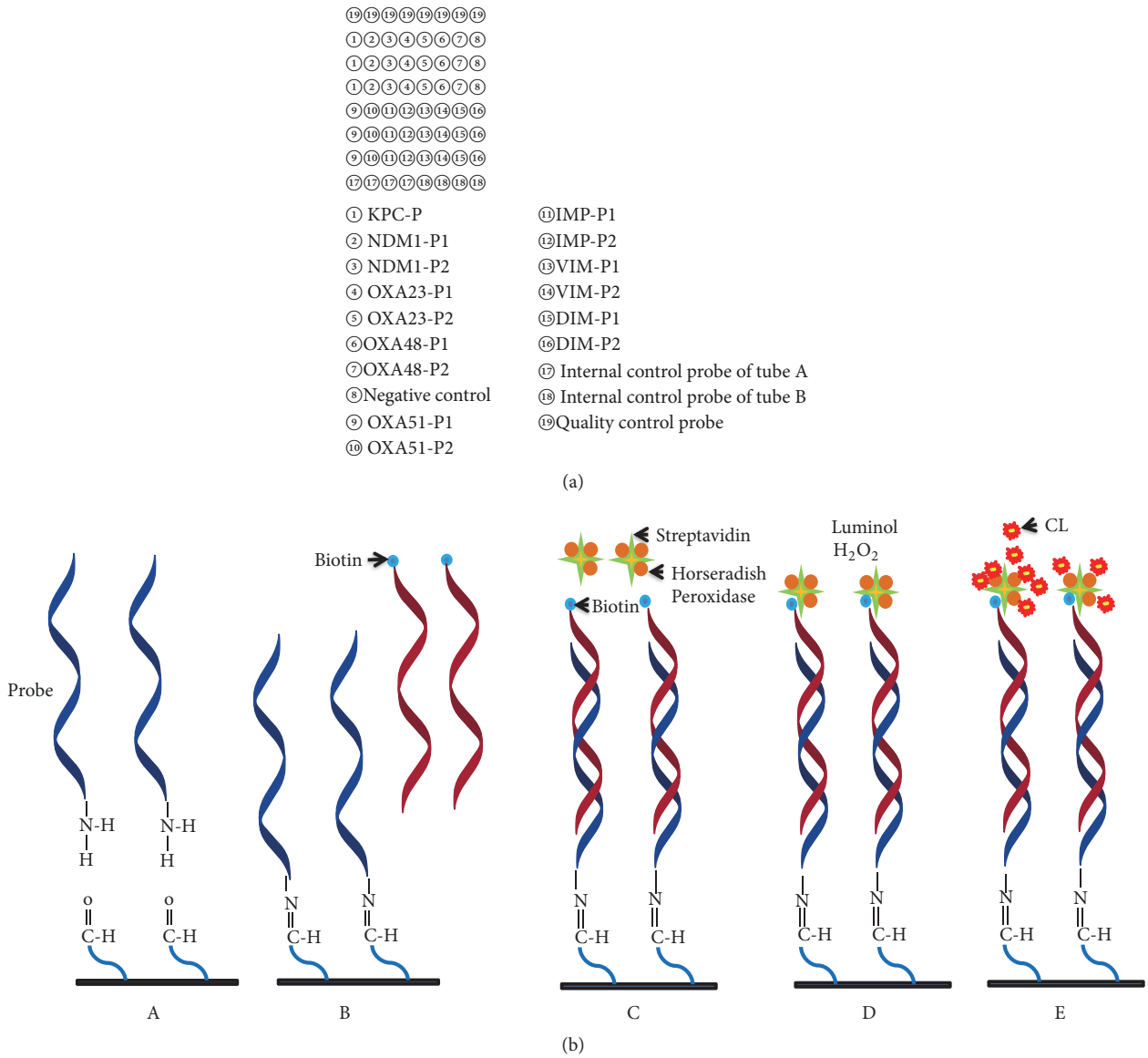

Figure 1: (a) The microarray layout of the target carbapenemase genes that are detected in this study. (b) Principle of hybridization and CL imaging of microarray. (A) Captured probes were fixed to the aldehyde-chip surface, (B) denatured PCR products were hybridized with capture probes, (C) horseradish peroxidase modified streptavidin was bond to biotin incorporated in hybridization, and (D) adding HRP substrates luminal and H_2O_2 and (E) CL signal was detected by catalyzed substrates.

and 16S rRNA were collected from cultured Hela cell and standard strains of *Escherichia coli* ATCC25922, respectively.

2.5. Microarray Fabrication. All microarray probes were synthesized by the Chinese PLA Academy of Military Medical Sciences. An oligo$(dT)_{12}$-with an amino-labeled $3'$-end was conjugated to the $3'$-end of all the probes, such that it served as a linker arm to be immobilized on the aldehyde modified glass surface (Baio Technology Shanghai Co., Ltd., Shanghai, China). An Oligo$(dT)_{20}$ with an amino-labeled $3'$-end and a biotin-labeled $5'$-end served as a quality control (QC) probe. Each probe (50 μM of final concentration) was spotted thrice repeatedly by using a noncontact inkjet Nano-plotter 2.1 (GeSim, Dresden, Germany) onto an aldehyde-chip after mixing with printing buffer [5% glycerol, 0.1% sodium dodecyl sulfate (SDS), 6× saline-sodium citrate buffer (SSC), and 2% (wt/vol) Ficoll 400]. Quality control (QC) probe was included to manage the standard of operation and used at 12.5 μM final concentration. It was spotted eight times repeatedly in the horizontal direction to calibrate the CL signal values. Each aldehyde slide was divided into 10 blocks (11 × 11 mm) by a waterproof film to detect 10 different samples. Microarrays were placed in a dryer for 24 h at room temperature. Unbound probes were washed off by 0.2% SDS and distilled water prior to use. The layout is shown in Figure 1(a).

2.6. Multiplex PCR. All reverse primers for target genes and internal controls (i.e., mtDNA and 16S rRNA) were labeled

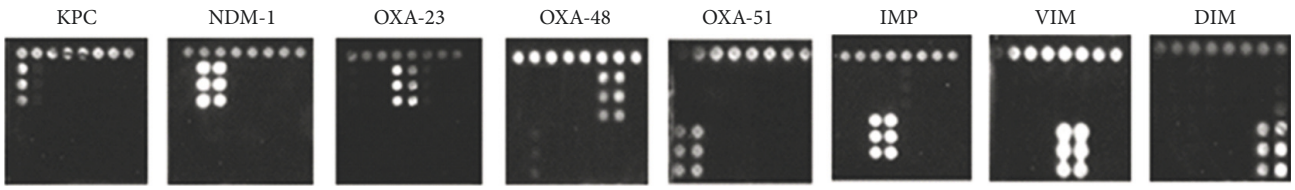

Figure 2: Representative microarray hybridization images with the reference carbapenemase plasmids (positive controls).

by biotin at the 5′-ends for acting a CL reaction. Multiplex PCRs were performed for bla_{KPC}, bla_{OXA-23}, bla_{VIM}, bla_{DIM}, and 16S rRNA in one tube (Tube A), and bla_{IMP}, bla_{NDM-1}, bla_{OXA-48}, bla_{OXA-51}, and mtDNA in another (Tube B). PCR reaction was in 30 μL and contained 15 μL of 2× Multiplex PCR Mix (cwBiotech, Beijing China) and 3 μL of mixed DNA templates. For Tube A, the concentrations of bla_{OXA-23}, bla_{VIM}, bla_{DIM}, and 16S rRNA forward and reverse primers all were 0.1 μM and 0.5 μM, and for bla_{KPC} the forward and reverse primer were 0.2 μM and 1 μM, respectively. For Tube B, the concentrations of the forward and reverse primers were 0.1 μM and 0.5 μM, respectively. PCR was performed on a Thermal Cycler PCR system (Applied Biosystems, Foster City, CA) using the following conditions: 10 min at 95°C; 37 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C; and a final extension of 5 min at 72°C.

2.7. Hybridization and Signal Detection. PCR products amplified by the two multiplex PCR reactions using the same template were mixed. The mixtures were denatured at 95°C for 5min and placed on ice immediately for 5 min. Then 5 μL of denatured PCR mixtures were blended with 5 μL of hybridization buffer [8× SSC, 0.6% SDS, 10% formaldehyde, and 10×Denhardt]. Hybridization reaction was proceeded in a hybrid-box by incubation for 1 h at 45°C. After hybridization, slide was washed successively in 1× SSC and 0.2% SDS, 0.2× SSC, and 0.1× SSC for 30 s. Slide was air dried at room temperature. To detect the CL signals, microarray was incubated in 37°C for 30 min with 10 μL of streptavidin horseradish peroxidase (Str-HRP, Sigma-Aldrich, St.Louis, USA), followed by wash with PBST (1×PBS, 0.05% Tween-20) for 30 s at room temperature. Dried microarray was covered in 10 μL of premixed CL HRP substrate luminal solution and H_2O_2 (Millipore Corporation, Boston, USA) (Figure 1(b)). Then immediately subject to scanning by Biochip Chemiluminescence Imager, a microlight level imaging system developed in our laboratory. Signal intensities were calculated by Array Vision 7.0.

2.8. Identification of Carbapenem-Resistant and Susceptible Strains. The strains isolated from samples were tested using the Kirby-Bauer (K-B) method of disk diffusion according to the recommendations of the CLSI to determine their susceptibilities to imipenem (10μg) and meropenem (10μg). *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as control strains for susceptibility testing. Isolates were considered to have a carbapenemase phenotype if they were resistant to at least one carbapenem (i.e., meropenem or imipenem) [22].

Four hundred sixteen (416) antibiotic-resistant bacterial samples were evaluated in this study. The initial samples were obtained from patients who had been hospitalized for a long time (>1 year).

2.9. Confirmation of the Resistance Genes by Sequencing. The resistance genes, including bla_{KPC}, bla_{NDM-1}, bla_{OXA-23}, bla_{OXA-48}, bla_{OXA-51}, bla_{IMP}, bla_{VIM}, and bla_{DIM}, that had been detected in antibiotic-resistant samples by microarray hybridization were validated by Sanger sequencing.

3. Results

3.1. Determination of Threshold Signal Intensity. To determine the threshold value for differentiating positive and negative microarray signal intensities, we have performed pilot microarray hybridization experiments using the Gram-positive bacterial strain *S. aureus* 04018 as negative controls and carbapenemase plasmids of 3×10^3 copies/μL as positive controls under the conditions specified in Materials and methods. If the signal intensity value is 10 times of the background intensity value, the probe was considered to be positive.

3.2. Specificity and Sensitivity of Microarray Test. To evaluate the specificity of the microarray method, we performed microarray hybridization assays for the reference carbapenemase plasmids (i.e., positive controls) (Figure 2), clinical carbapenem-resistant samples (Figure 3), and ten negative controls that were from ATCC standard strains and were sensitive to carbapenem (Figure 4). As shown by the microarray images, our method could effectively distinguish between the carbapenem resistance and carbapenem-sensitive genotypes among the clinical bacterial specimens with high specificity.

To evaluate the sensitivity of the microarray assay, we diluted the reference carbapenemase plasmids into various concentrations (i.e., from 3×10^1 copies/μL to 3×10^5 copies/μL). The different copy numbers of DNA were hybridized to the microarrays. The detection images were shown in Figure 5(a). In general, the microarrays yielded satisfactory sensitivity. For most reference plasmids, the detection limit was as low as 30 copies/μL (Figure 5(a)). Diagnostic Kit for Bacterial Resistance Gene KPC (PCR-Fluorescence Probing) and Diagnostic Kit for Bacterial Resistance Gene NDM-1 (PCR-Fluorescence Probing) (Puruikang Biotech, Shenzhen, China) were also used to detect KPC and NDM-1 reference plasmids (3×10^1 to 3×10^5 copies/μL), respectively. Real-time PCR amplified by ABI Prism 7500 real-time PCR apparatus (Applied Biosystems, Foster City,

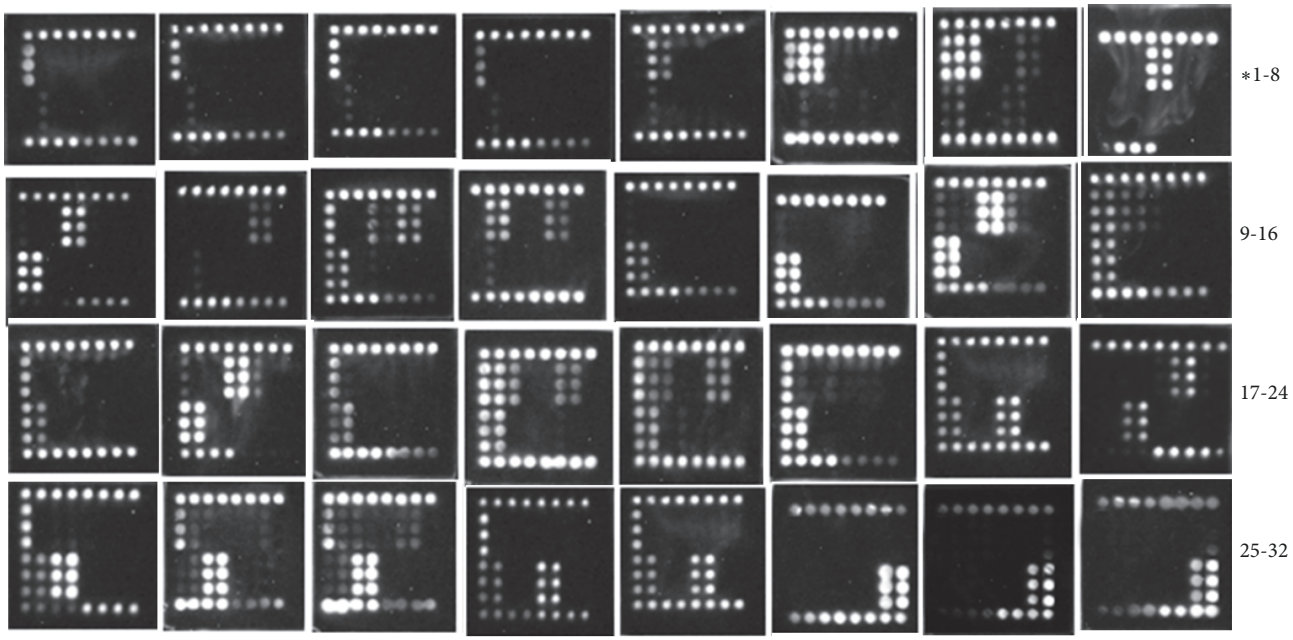

Figure 3: Representative images of microarray assays for clinical carbapenemase-producing specimens. ∗, the number of clinical carbapenemase-producing specimens (Supplementary Table).

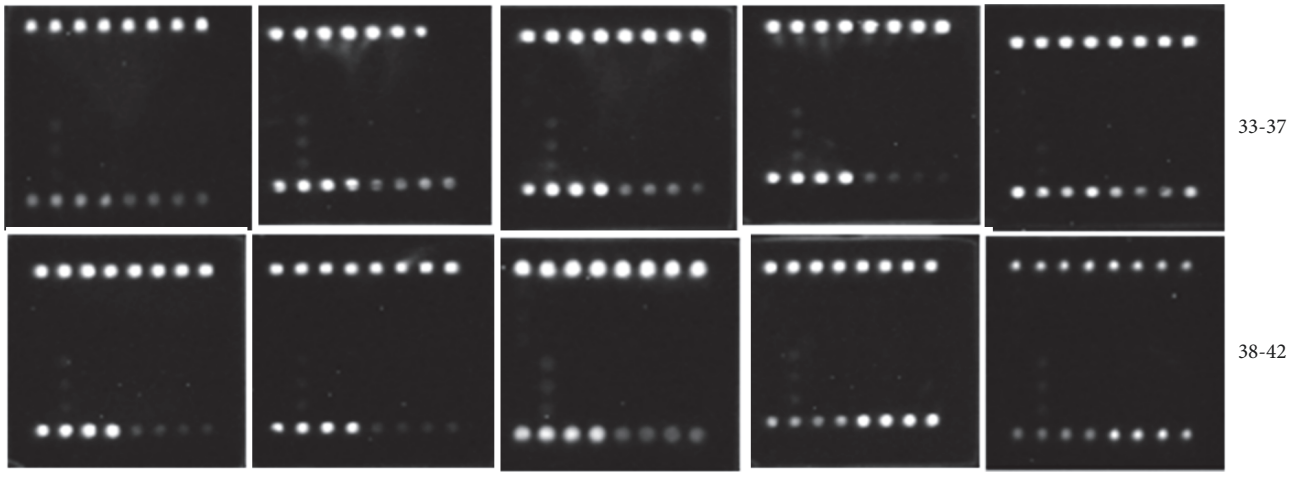

Figure 4: Images of microarray assays for carbapenemase-sensitive strains (negative control strains). 33, *Escherichia coli* (ATCC 25922); 34, *Enterococcus faecium* (ATCC 35667); 35, *Enterobacter cloacae* (ATCC 13047); 36, *Enterococcus faecalis* (ATCC 29212); 37, *Pseudomonas aeruginosa* (ATCC 27853); 38, *Acinetobacter baumannii* (ATCC 19606); 39, *Staphylococcus aureus* (ATCC25923); 40, *Streptococcus pneumoniae* (ATCC 49619); 41, *Klebsiella pneumonia* (ATCC700603); 42, *Streptococcus pneumonia* (ATCC 9007(serotype C)). ATCC, American Type Culture Collection.

US) and the results of sensitivity comparison between microarray assay and Real-time PCR showed they had similar sensitivities (Figures 5(b) and 5(c)), indicating that our DNA microarrays could be applied to the clinical detection of the carbapenemase-producing samples.

3.3. Stability of Microarray Assay. Diluted carbapenemase plasmids (3×10^5 copies/μL) and negative controls *S. aureus* 04018 were used to evaluate how the microarray assays performed as far as repeatability is concerned. The 3×10^3 copy/μL plasmid of each target gene was selected as the template to detect for the determination of interchip and intrachip variation, and a negative control was set up without template. The experiment was repeated three times, and the repeatability of interchip and intrachip variation were evaluated. Coefficient of variation (CV) = SD/mean of signal intensities×100%.

Statistical analysis showed that, for all target gene probes, the intrachip and interchip CV values of 8 probes ranged from 3.58% to 11.02% (below 15%), suggesting a favorable repeatability of the DNA microarray detection method (Table 3).

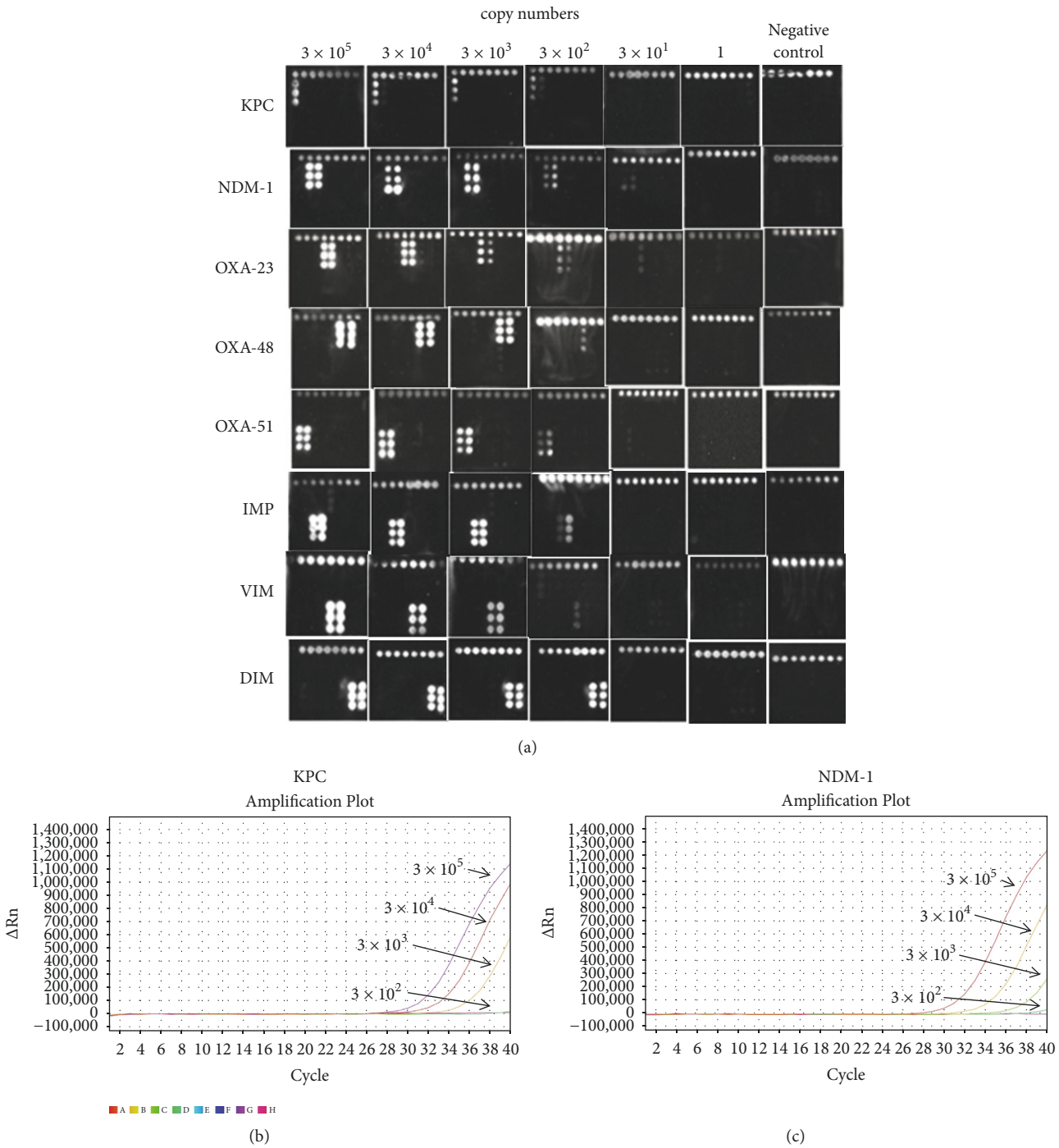

Figure 5: (a) Sensitivity of the microarray assays. The indicated carbapenemase reference plasmids were subject to series dilutions and hybridized to the DNA microarrays. The plasmids copy numbers are diluted from 3×10^1 to 3×10^5 copies/μL. (b) KPC reference plasmids (3×10^1 to 3×10^5 copies/μL) were detected by real-time PCR, (c) NDM-1 reference plasmids (3×10^1 to 3×10^5 copies/μL) were detected by real-time PCR.

3.4. Phenotypic Resistance. About 78% clinical specimens (326/416) were resistant to both imipenem and meropenem, 77 samples were susceptible to both imipenem and meropenem, three samples were resistant to imipenem but sensitive to meropenem, and five samples were reversely sensitive to imipenem but resistant to meropenem as well as five samples were intermediary to imipenem but sensitive to meropenem. Most carbapenem-resistant samples were nonsusceptible to diverse antibiotics containing cephalosporins, fluoroquinolone, aminoglycosides, etc. The rates of nonsusceptibility to different antimicrobial agents were commonly >90%. Clinical information and phenotypic

Table 3: Statistics of the microarray repeatability.

	Repeatability (CV%)			
	Intra-chip experiments			Inter-chip experiment
Repeat times (n)	1	2	3	3
KPC	5.23	4.75	9.85	10.16
NDM-1	3.67	4.12	6.44	6.71
OXA-23	4.47	5.03	7.82	7.54
OXA-48	3.58	3.87	6.83	6.42
OXA-51	4.45	5.31	8.09	7.88
IMP	6.84	6.95	10.24	11.02
VIM	5.87	6.04	9.97	10.19
DIM	4.02	3.89	6.52	6.67

results of all clinical samples were shown in Supplementary Table (available here).

3.5. Detection of Carbapenemase-Producing Strains by Microarray in Clinical Samples. A total of 416 clinical samples collected from Chinese PLA General Hospital were tested. The majority of the samples had previously been well-characterized as carbapenem resistance using K-B method. The microarrays for these bacteria revealed that 78% (325) of the samples carried one or more carbapenemase genes and that in some samples more than one *bla* gene had been identified (i.e., 496 genes were found in 325 specimens). The genotyping results of the clinical samples are listed as follows: 256 (62%) carried bla_{KPC}, 137 (33%) carried $bla_{OXA\text{-}51}$, 40 (9.6%) carried $bla_{OXA\text{-}23}$, 22 (5.3%) carried $bla_{OXA\text{-}48}$, 27 (6.5%) carried $bla_{NDM\text{-}1}$, 5 (1.2%) carried bla_{IMP}, 3 (0.7%) carried bla_{VIM}, and 6 (1.4%) carried bla_{DIM} (Table 4). Therefore, bla_{KPC} was the most frequent carbapenemase gene found in the antibiotic-resistant sample. Most interestingly, we found that $bla_{OXA\text{-}51}$ frequently coexisted with other *bla* genes. For example, 89 samples that carried bla_{KPC} also carried $bla_{OXA\text{-}51}$; all 40 samples of $bla_{OXA\text{-}23}$ carried $bla_{OXA\text{-}51}$; 9 samples carried all three *bla* genes: bla_{KPC}, $bla_{OXA\text{-}51}$, and $bla_{OXA\text{-}23}$; and 5 samples carrying $bla_{OXA\text{-}48}$ also carried both $bla_{OXA\text{-}51}$ and bla_{KPC}. It is worth noting that all carbapenemase genes in the 325 antibiotic-resistant samples had been verified by sequencing. In parallel, we also amplified the isolates that were susceptible to carbapenems performed in phenotypic tests as well as recruited in our microarray assays. The overall concordance between the microarray-based assay and the reference methods (standard DNA sequencing) was 97.8%, suggesting that our microarray is a highly reliable method for detecting the carbapenemase-producing GNB in the clinic.

Fourteen samples which failed to detect any genes by microarray but resistant to either imipenem or meropenem in phenotypic test could not be amplified, determining 96.6% concordance between the phenotypic and microarray tests. Taken together, the newly developed microarray detection method is comparable to the conventional antibiotic susceptibility test and therefore may be suitable for clinical applications.

4. Discussion

In this study, we developed a novel microarray method to detect carbapenemase genes that could be applied to clinical diagnosis and identification of carbapenemase-producing GNB. We included eight carbapenemase genes that have been shown or potential display to be most clinically relevant, bla_{KPC}, $bla_{NDM\text{-}1}$, $bla_{OXA\text{-}23}$, $bla_{OXA\text{-}48}$, $bla_{OXA\text{-}51}$, bla_{IMP}, bla_{VIM}, and bla_{DIM}, in the design of the microarray chips. MtDNA and 16S rRNA were chosen as internal controls because our samples were taken from human and the detected target genes were from bacteria. The high copy number sequence of mtDNA was used to monitor and control all PCR reactions and hybrids operations, and 16S rRNA was used to prove that the DNA microarray system could detect bacterial genes from all samples. The specificity, sensitivity, and reproducibility of the proposed method were highly favorable for clinical applications. Most importantly, the microarray results of the 416 clinical samples showed highly consistent agreement with results obtained from direct sequencing or antibiotic susceptibility tests.

It was intriguing for us to identify a number of resistant strains coharboring two or more carbapenemase genes; for instance, bla_{KPC} frequently coexisted with other genes and $bla_{OXA\text{-}51}$ always coexisted with $bla_{OXA\text{-}23}$ in *Acinetobacter baumannii*, indicating a more serious threat than before, when it came to the control and management of the extremely drug-resistant bacterial infections [23]. Of the transferable molecular class B metallo-β-lactamases, IMP, VIM, and NDM were common, while DIM was endemic [10]. However, in our study, bla_{IMP} and bla_{VIM} were detected in 5 and 3 samples, respectively, yet bla_{DIM} was in 6 specimens, which indicated that it might be necessary to improve the attention for DIM in the later study (Table 4 and Supplementary Table).

The DNA microarray could detect bacterial carbapenemase genes from not only clinical sputum, urine samples, and colony or bacterial culture in this study, but also specimens of pleural effusion, cerebrospinal fluid, oral swab, and throat swab even environmental swab (data not shown) directly. This remarkable capability of compatibility to detect of several original samples made it faster to obtain result than similar microarrays Check-MDR CT102 [24, 25] and VITEK2 [26] that should detect cultured bacterial isolates.

Table 4: Microarray results on various clinical samples harboring carbapenemase genes.

Species		*P. aeruginosa*	*A. baumann*	*E. coli*	*K. pneumoniae*	*S. marcescens*	*S. maltophilia*	*Flavobacterium meningosepticum*	*R. mannitolilytica*	*Achromobacter xylosoxidans*	*Burkholderia cepacia*	*Klebsiella oxytoca*	*Enterobacter cloacae*	*E. aerogenes*	Total
No[a]. of samples		263	42	36	55	5	4	3	1	1	1	2	2	1	416
No. with bla_{KPC} gene	PCR[b]	193	15	7	38	0	2	3	0	1	1	0	0	0	260
	Array[c]	190	15	7	37	0	2	3	0	1	1	0	0	0	256
	% Concordance of bla_{KPC} results	98.4	100	100	97.4	100	100	100	100	100	100	100	100	100	98.5[d]
No. with bla_{NDM-1} gene	PCR	19	2	0	5	0	1	0	0	0	0	0	0	0	27
	Array	19	2	0	5	0	1	0	0	0	0	0	0	0	27
	% Concordance of bla_{NDM-1} results	100	100	100	100	100	100	100	100	100	100	100	100	100	100[e]
No. with bla_{OXA-23} gene	PCR	8	31	1	0	0	1	0	0	0	0	0	0	0	41
	Array	8	30	1	0	0	1	0	0	0	0	0	0	0	40
	% Concordance of bla_{OXA-23} results	100	96.8	100	100	100	100	100	100	100	100	100	100	100	97.6[f]
No. with bla_{OXA-48} gene	PCR	20	1	1	1	0	0	0	0	0	0	0	0	0	23
	Array	19	1	1	1	0	0	0	0	0	0	0	0	0	22
	% Concordance of bla_{OXA-48} results	95	100	100	100	100	100	100	100	100	100	100	100	100	95.7[g]
No. with bla_{OXA-51} gene	PCR	84	40	5	7	0	2	1	0	1	0	0	0	0	140
	Array	82	39	5	7	0	2	1	0	1	0	0	0	0	137
	% Concordance of bla_{OXA-51} results	97.6	100	100	100	100	100	100	100	100	100	100	100	100	97.9[h]
No. with bla_{IMP} gene	PCR	4	1	0	0	0	0	0	0	0	0	0	0	0	5
	Array	4	1	0	0	0	0	0	0	0	0	0	0	0	5
	% Concordance of bla_{IMP} results	100	100	100	100	100	100	100	100	100	100	100	100	100	100[i]
No. with bla_{VIM} gene	PCR	3	0	0	0	0	0	0	0	0	0	0	0	0	3
	Array	3	0	0	0	0	0	0	0	0	0	0	0	0	3
	% Concordance of bla_{VIM} results	100	100	100	100	100	100	100	100	100	100	100	100	100	100[j]

Table 4: Continued.

Species		*P. aeruginosa*	*A. baumann*	*E. coli*	*K. pneumoniae*	*S. marcescens*	*S. maltophilia*	*Flavobacterium meningosepticum*	*R. mannitolilytica*	*Achromobacter xylosoxidans*	*Burkholderia cepacia*	*Klebsiella oxytoca*	*Enterobacter cloacae*	*E. aerogenes*	Total
No. with bla_{DIM} gene	PCR	4	1	0	0	0	0	0	1	0	0	0	0	0	6
	Array	4	1	0	0	0	0	0	1	0	0	0	0	0	6
	% Concordance of bla_{DIM} results	100	100	100	100	100	100	100	100	100	100	100	100	100	100[k]
Total no. of strains that agree/total no. of strains		257/263	38/40	36/36	54/55	5/5	4/4	3/3	1/1	1/1	1/1	2/2	2/2	1/1	407/416
% agreement for all strains		97.7	95	100	98.2	100	100	100	100	100	100	100	100	100	97.8

[a] All tested samples include sputum, urine, and cultured isolates; the specific clinical information and test results of each sample are shown in the Supplementary Table.

[b] Results obtained with classical PCR/sequencing.

[c] Microarray results were obtained using carbapenemase-array designed in this study.

[d] A sensitivity of 98.5%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 97.5% for KPC detection.

[e] A sensitivity of 100%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 100%f or NDM-1 detection.

[f] A sensitivity of 97.6%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 99.7% for OXA-23 detection.

[g] A sensitivity of 95.7%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 99.7% for OXA-48 detection.

[h] A sensitivity of 97.9%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 98.9% for OXA-51 detection.

[I] A sensitivity of 100%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 100% for IMP detection.

[j] A sensitivity of 100%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 100% for VIM detection.

[k] A sensitivity of 100%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 100% for DIM detection.

The microarray was rapid and portable, when starting from the clinical sample, less than 7 hours with overall 2 hours of hands-on time, enabling one day analysis. Whole detection operation of the DNA microarray consisted of 5 steps and costs 4-5 h including PCR amplification, and whole detection did not need sophisticated instrument, which was far simpler than Check-MDR CT102. The DNA microarray could detect 9 specimens at one chip one time and the cost per sample was below five dollars, which was far cheaper than Check-MDR CT102 and VITEK2. In microarray assay, a proprietary CL imaging system was developed in our laboratory. The Biochip Chemiluminescence Imager relied on charge-coupled device (CCD) camera imaging technology and equipped with a power supply unit for portable use. The new CL imager had a lower cost ($3000) than other commercial CCD imaging technology CL imagers (e.g., Amersham Imager 600, GE Healthcare Life Sciences) and much faster than other visual microarray system which was based on quantum dot-catalyzed silver deposition. The newly designed DNA microarray system had yielded high specificity, sensitivity, and reproducibility in detecting the eight carbapenemase genes among the clinical specimens. For $bla_{\text{NDM-1}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-51}}$, bla_{IMP}, bla_{VIM}, and bla_{DIM}, there were two different probes (22-30 nt) designed to hybridize, respectively, which could effectively minimize the false hybridization signals. Our study has demonstrated that the microarrays with greatly simplified the protocol to determine the cabapenemase-encoding genes in the clinical samples and can offer an efficient means for the molecular epidemiological studies of cabapenemase genes: the emergence of the resistant genes may potentially be traced back to their origins where community- and hospital-based bacterial infections were frequently happening. On the other hand, microarray hybridization is highly sensitive. In this study, they showed similar sensitivities as the real-time PCR kit. We could detect as low as 30 copies/μL of DNA targets. Furthermore, the DNA microarrays can generally detect target DNAs with much larger dynamic ranges. Finally, the microarray method is also highly reproducible: we have shown that the averaged coefficient of variations (CV %) for interchip and intrachip experiments were low, and most of them were less than 10%. Therefore, we propose that the new microarray method has a great potential to be applied to clinical studies.

The microarray based on multi-PCR, which made it possible to detect multiple resistant genes at the same time in a single tube. But it could not effectively identify a variety of fragments by electrophoretic separation. Direct sequencing of DNA is not suitable for identification of multiple PCR products. The microarray is used to confirm the results of multiple PCR. The concordance of microarray to PCR of detection 8 carbapenemase genes ranges from 95.7%-100% (Table 4).

The microarray had some limitations, because resistance to carbapenems in particular was produced through several mechanisms: such as synthesis of carbapenem β-lactamases, efflux pumps [27], loss of membrane permeability [28], and penicillin-binding proteins variants. The DNA microarray could not cover genes form all the mechanisms. Fourteen samples in our study which failed to detect any genes by microarray but resistant to either imipenem or meropenem in phenotypic test could not be amplified, indicating resistant genes from other mechanisms were beyond of detection reach of the microarray. Whole genome sequencing (WGS) has potential to detect many different molecular mechanisms leading to resistance. Our lab has taken WGS as new powerful technology to find novel resistant genes located in bacterial plasmid or genome DNA, as well as new resistant mechanisms. However, WGS-based antimicrobial susceptibility testing in clinical laboratories remain the current high-cost and taking more time [29]. In clinical testing, using PCR-based microarray to detect some specific resistance genes simultaneously was more economical and faster. In this study, multi-PCR amplification was divided into two tubes, and reducing PCR amplification systems (amplifying 8 carbapenemase genes in one tube) might increase sensitivity [30].

In conclusion, we developed a new microarray detection system that could directly detect eight carbapenemase genes from several kinds of clinical specimens. It was convenient, readily to be customized for high-throughput detection, and could be easily adapted for clinical applications.

Data Availability

The date generated and analyzed during this study are available from the corresponding author on reasonable request.

Disclosure

The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Authors' Contributions

Yi Song and Fengna Dou contributed equally as first authors. Qiqi Liu and Yu Zhou conceived and designed the study. Yi Song, Fengna Dou, Sha He, and Qiqi Liu were responsible for the experimental analysis. Yi Song wrote the paper and other coauthors contributed to the final draft. All authors read and approved the final manuscript.

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Supplementary Materials

Additional file 1: Supplementary Table. Clinical information and detection results of carbapenemase-producing samples. *(Supplementary Materials)*

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