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The Emergence of Carbapenem-Resistant Gram-Negative Bacteria in Mizoram, Northeast India

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Abstract: (Background) Numerous reports on carbapenem resistance in different parts of India have been published, yet there are insufficient studies on the prevalence of antibiotic resistance in the northeast region of the country. This study evaluated the emergence of carbapenem resistance in the clinical isolates collected in Mizoram. (Results) A total of 141 Gram-negative clinical isolates were collected from the two hospitals, including the Civil Hospital in Lunglei and the Synod Hospital in Aizawl. The isolates include *Escherichia coli* (*n* = 62, 43.9%), *Klebsiella* spp. (*n* = 43, 30.4%), *Pseudomonas aeruginosa* (*n* = 9, 6.3%), *Serratia marcescens* (*n* = 3, 2.1%), *Proteus mirabilis* (*n* = 2, 1.4%), *Shigella* spp. (*n* = 4, 2.8%), *Enterobacter* spp. (*n* = 6, 4.2%) and *Acinetobacter* spp. (*n* = 12, 8.5%). The isolates were found to be resistant to meropenem (11%), colistin (48%), tigecycline (25%) and cefotaxime (50%). A total of four *E. coli* and one *Shigella sonnei* encoded the $bla_{\text{OXA-48-like}}$ gene. The $bla_{\text{CTX-M-1}}$ gene was detected in 13 isolates, of which eight were *E. coli*, two *Shigella flexneri*, and one isolates each of *K. pneumoniae*, *K. oxytoca* and *Shigella sonnei*, respectively. (Conclusion) Carbapenem-resistant *Enterobacteriaceae* are common among other parts of India, despite limited access to antibiotics, the emergence of resistance in the northeastern region is worrying.

Keywords: carbapenem-resistance; Gram-negative bacteria; *Enterobacteriaceae*; meropenem; antibiotic resistance

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1. Introduction

Antimicrobial resistance in bacterial pathogens is a widespread and critical challenge in clinical treatment resulting in high morbidity and mortality rates globally [1]. Gram-negative bacteria can result in severe complications or even treatment failure when caused by multi-drug resistant (MDR) strains that are increasingly common around the globe. Carbapenems, a highly effective class of broad-spectrum antibiotics usually reserved for MDR infections, are increasingly deployed, and as a result, resistance among Gram-negative bacteria has now become a worldwide concern [2]. Carbapenems are transpeptidase inhibitors that inhibit the peptide crosslinking during cell wall synthesis leading to autolytic activity (cell death) [3]. The basis of Carbapenem resistance mechanisms is either acquired via horizontal gene transfer or is based on intrinsic mechanisms, which include mutations

of the target site, enzymatic degradation of the antimicrobial compound, and changes in the efflux pump mechanisms. Among these, enzymatic degradation is the most prevailing mechanism [4]. Molecular mechanisms of carbapenemase enzymes have been proposed and are being classified as class A, class B and class D. Class A types are more often found in *Enterobacteriaceae* but rarely encountered in *P. aeruginosa*, which covers SME, NMC, KPC, IMI, GES-type of enzymes. Class B is found in *Acinetobacter* species, *P. aeruginosa*, and *Enterobacteriaceae* and includes VIM, SPM, GIM, and IMP-type enzymes. Class D includes the OXA-type, which is again more common among *Acinetobacter* [5].

The distribution of carbapenem-resistant *Enterobacteriaceae* is increasing, but the availability of data is limited in many countries. For lactose non-fermenters, the recorded rate of carbapenem resistance appears to be substantially higher than for lactose fermenters globally [6]. The virulence, as well as the pathogenicity of the carbapenem-resistant bacterial strains, were found to vary in the country in which a person is infected [7,8]. The epidemiological distribution of carbapenemase producers varies vastly. In Asian sub-continents, $bla_{\text{OXA-48-like}}$ and $bla_{\text{NDM-types}}$ are more common, while bla_{KPC} and other $bla_{\text{OXA-types}}$ are recorded quite often in the European countries. In northern America, half of the carbapenem-resistant *Enterobacteriaceae* (CRE) is found to be carbapenemase-producing, while bla_{NDM}, bla_{VIM} and $bla_{\text{OXA-48-like}}$ are less prevalent. In comparison to other Asian countries, the rate of carbapenem resistance was found to be significantly lower in India. Yet, in hotspots in India, an average of 13% of cases were registered as CRE [9].

In the northeast regions, in the states of Mizoram, Tripura, Nagaland and Meghalaya, the prevalence of carbapenem resistance were found to be below 5% due to less developed healthcare and the comparably reduced amounts of carbapenems used in these regions [10]. Based on this information, this study was conducted to assess the emergence of Gram-negative bacteria in Mizoram that are carbapenem-resistant, as well as to detect the presence of carbapenemase genes in the clinical isolates collected from two hospitals in the region.

2. Materials and Methods

2.1. Collection of Bacterial Isolates

A total of 141 Gram-negative bacterial isolates were obtained from Civil Hospital Lunglei (Lunglei District) as well as Synod Hospital Aizawl (Aizawl District), Mizoram, between 2018 and 2019. The isolates were transported as cryoprotectant stocks to the Antibiotic Resistance and Phage therapy research Laboratory at VIT, Vellore. All the isolates were sub-cultured and stored at −20 °C. The bacterial identification was performed using the VITEK identification system and the isolates were further confirmed by 16S rRNA identification using the universal primers 27F and 1492R. PCR products were Sanger sequenced (Eurofins Genomics Ltd., India) and analyzed using the NCBI-BLAST tool. The blast hit was analyzed against the available data from the NCBI database and the sequences were deposited in NCBI-GenBank [11].

2.2. Minimal Inhibitory Concentration (MIC)

A test to determine the MIC was performed for all the isolates using meropenem, cefotaxime, colistin and tigecycline (Sigma Aldrich, India). Briefly, Muller Hinton No. 2 cation-controlled broth (Hi-Media, India) was prepared and 100 µL of the broth was added to the 96 well-microtiter plates. The antibiotic to be tested was diluted by adding 100 µL of the respective antibiotic ranging from 0.25 µg/mL to 256 µg/mL. The bacterial culture at 0.5 McFarland standards (approx. 1×10^7–10^8 CFU/mL) was prepared and 5 µL was added to each well. The plates were incubated at 37 °C for 18 h. The results were recorded and interpreted according to CLSI guidelines (CLSI, 2019) [11].

2.3. DNA Isolation

Bacterial DNA was isolated from the clinical isolates following the boiling lysis method as described in our previous study [11]. Briefly, 10 colonies were inoculated into sterile distilled water and boiled for 10 min (temp: 95 °C) to break open the cells. The mixture

was allowed to cool down to room temperature and centrifuged at 8000× *g* for 5 min and the supernatant containing the DNA was used as a template for PCR analysis.

2.4. Screening of Beta-Lactamase Genes

The resistance genes screening was performed on both chromosomal and plasmid DNA. Chromosomal DNA was isolated by the boiling lysis method (above), while plasmid DNA was isolated using the Plasmid DNA isolation kit (Hi-Media, India). Carbapenem resistance genes bla_{NDM}, $bla_{\text{OXA-48-like}}$, bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{DIM}, bla_{BIC}, bla_{GIM}, bla_{SIM} and bla_{AIM} and cefotaxime resistance genes, CTX-M group of genes: $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-26}}$ and $bla_{\text{CTX-M-8}}$ were screened using PCR [12,13]. The integrase genes *intI1*, *intI2* and *intI3* were amplified (n = 16) by multiplex PCR using the primers described in a previous study [14]. The clonality of the bacterial species was determined (n = 141) by ERIC-PCR (enterobacterial repetitive intergenic consensus) using ERIC2 primers as described by Versalovic et al. [15]. ERIC-PCR results were analyzed by PyElph software (Python-based gel images analyzer) [16]. In brief, a DNA gel image was generated by photographing it with UV gel Doc. The DNA banding patterns (gel image) were inserted into PyElph software in JPEG format. The ERIC-PCR patterns obtained were interpreted and compared as described by Bilung et al. [17]. For cluster studies by ERIC-PCR, a dendrogram was generated using the unweighted pair group average (UPGMA) against a 1 kb DNA marker (HiMedia, Mumbai, India). The dendrogram was drawn for the entire genus separately and when the band similarity was greater than 80%, the clones were treated as related. The strains with carbapenem resistance genes are highlighted in red (Supplementary Figures S1–S7).

2.5. Identification of Carbapenemase Producers

The Modified Hodge test (MHT) was performed to detect the carbapenemase production in the resistant isolates. Briefly, an indicator organism, *E. coli* ATCC 25922, and the test bacteria were inoculated in MH broth and the turbidity was adjusted to 0.5 McFarland standard. Then, the indicator organism was plated onto Mueller–Hinton agar plates and the plates were allowed to dry for 10 min. Meropenem antibiotic disks (10 mcg) were placed at the centre of the agar plate. The test bacteria were streaked as a straight line from the edge of the disk towards the edge of the plate. The plates were incubated at 37 °C for 18 h. The results were interpreted according to the CLSI guideline [18].

2.6. Synergy Testing

The synergistic effect of the combination of two antibiotics was analyzed by the checkerboard method. The synergy was assessed using the two last-resort antibiotics, colistin and meropenem. Briefly, the test bacteria were diluted to 0.5 McFarland standard. Both the antibiotics were diluted in separate microtiter plates and combined into a single plate. In brief, meropenem (A) was diluted vertically from column A to H (1 µg/mL to 256 µg/mL) in 96 well microtiter plates marked as A and colistin (B) was diluted horizontally in rows from 1 to 12 in 96 well microtiter plate marked as B (1 µg/mL to 256 µg/mL). The diluted antibiotic from plate A was transferred to plate B onto their respective wells. The selected test bacteria were inoculated and the plates were incubated at 37 °C for 18 h. The synergistic effect was measured by calculating the Fractional Inhibitory Concentration (FIC) index of the antibiotic alone and in combination [19]. The FIC was calculated by a universal mathematical expression, FIC = (FIC A/=FIC B), where FIC A = (MIC of the antibiotic A in combination/MIC of the antibiotic A alone) and FIC B = (MIC of the antibiotic B in combination/MIC of the antibiotic B alone). Based on the value obtained at FIC, the results are interpreted as synergy (FIC $\leq$ 0.5), indifference (2 > FIC $\geq$ 0.5), and antagonistic (FIC $\geq$ 2).

3. Results

3.1. Bacterial Isolates and Carbapenem Resistance

The collected 141 non-repetitive Gram-negative clinical strains of bacteria included *E. coli* (*n* = 62, 43.9%), *Klebsiella* spp. (*n* = 43, 30.4%), *P. aeruginosa* (*n* = 9, 6.3%), *Serratia marcescens* (*n* = 3, 2.1%), *Proteus mirabilis* (*n* = 2, 1.4%), *Shigella* spp. (*n* = 4, 2.8%), *Enterobacter* spp. (*n* = 6, 4.2%) and *Acinetobacter* spp. (*n* = 12, 8.5%). The MIC results showed that 16/141 (11.3%) were resistant to meropenem, 68/141 (48.2%) were resistant to colistin, 35/141 (24.8%) to tigecycline and 71/141 (50.3%) to cefotaxime. MIC_{50} and MIC_{90} values for meropenem were 0.12 µg/mL, for tigecycline MIC_{50} and MIC_{90} value were 0.5 µg/mL and 0.25 µg/mL, for cefotaxime MIC_{50} and MIC_{90} value were 128 µg/mL and 0.12 µg/mL, for colistin MIC_{50} and MIC_{90} value were 16 µg/mL and 8 µg/mL. For none of these isolates we detected carbapenemase production by MHT. The isolates that showed co-resistance to colistin and meropenem was 4 (2.8%), tigecycline and meropenem was 9 (6.3%) and cefotaxime and meropenem was 12 (8.5%) (Figure 1).

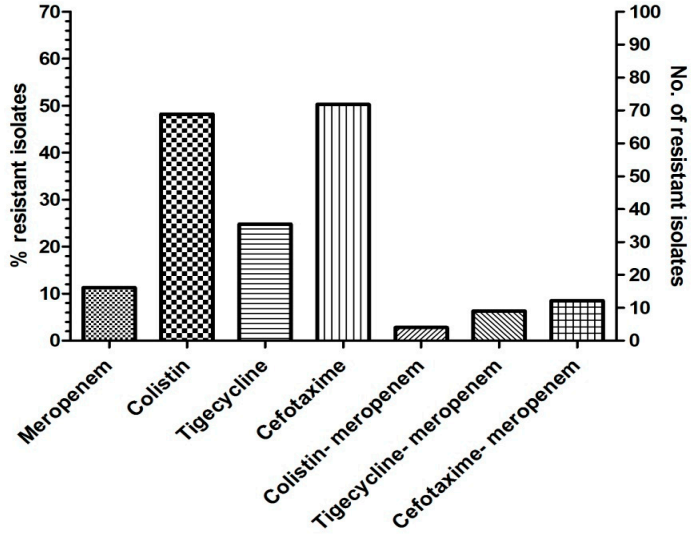

Figure 1. The distribution of antibiotic-resistant isolates among the Gram-negative bacterial isolates. A total of 141 Gram-negative bacteria were investigated in this study.

3.2. Molecular Studies

Of the 141 isolates, five carried $bla_{\text{OXA-48-like}}$ and 13 encoded the $bla_{\text{CTX-M-1}}$ gene. One isolate each of *E. coli* and *Shigella sonnei* was identified to carry both the $bla_{\text{OXA-48-like}}$ and the $bla_{\text{CTX-M-1}}$ gene. In total, 16/141 isolates carried resistance genes, $bla_{\text{OXA-48-like}}$ and $bla_{\text{CTX-M-1}}$ (Table 1). The $bla_{\text{OXA-48-like}}$ (5/141) carriers were *E. coli* (*n* = 4) and *Shigella sonnei* (*n* = 1). The isolates with $bla_{\text{CTX-M-1}}$ (13/141) were *E. coli* (*n* = 8), *Shigella flexneri* (*n* = 2), *K. pneumoniae* (*n* = 1), *K. oxytoca* (*n* = 1) and *Shigella sonnei* (*n* = 1). Other beta-lactamase genes such as bla_{KPC}, bla_{VIM}, bla_{BIC}, bla_{GIM}, bla_{DIM}, bla_{SIM} and bla_{AIM} were absent. None of the isolates had plasmid-borne resistance genes. A total of 16 isolates that carried resistance genes were identified at the species level and the sequences were deposited in GenBank.

Integron gene screening showed the presence of *intl1* in 13 isolates and interestingly, all the isolates were $bla_{\text{CTX-M-1}}$ positive (Table 1). The two isolates, *E. coli* and *S. sonnei*, that carried both $bla_{\text{CTX-M-1}}$ and $bla_{\text{OXA-48-like}}$ genes were positive for *intl1*. The ERIC-PCR dendrogram demonstrates that the $bla_{\text{CTX-M-1}}$ carrying carbapenem-resistant *E. coli* strains (EC46, EC48 and EC60) are closely related. Other strains of *E. coli* that carried $bla_{\text{OXA-48-like}}$ appear not related to each other. The ERIC-PCR results are shown as dendrograms in Supplementary Figures S1–S7.

Table 1. The distribution of beta-lactamase genes among clinical Gram-negative bacteria is investigated in this study.

S.no	Isolate ID	Bacteria	MIC				Resistance Gene	*Int* Gene
			Col	Tig	Mer	Cef		
1	EC 2	*E. coli*	1	**4**	**64**	**>128**	$bla_{\text{OXA-48-like}}$	-
2	EC 20	*E. coli*	2	<0.12	**32**	**>128**	$bla_{\text{OXA-48-like}}$	-
3	EC 15	*E. coli*	1	<0.12	**128**	**>128**	$bla_{\text{OXA-48-like}}$	-
4	SS 1	*Shigella sonnei*	2	<0.12	**128**	**>128**	$bla_{\text{OXA-48-like}}$ and CTX-M-1	*Intl1*
5	EC 17	*E. coli*	2	<0.12	**32**	**>128**	$bla_{\text{OXA-48-like}}$ and CTX-M-1	*Intl1*
6	SF 1	*Shigella flexneri*	2	**4**	**>128**	**>128**	CTX-M-1	*Intl1*
7	EC 4	*E. coli*	2	0.5	0.5	**>128**	CTX-M-1	*Intl1*
8	SF 2	*Shigella flexneri*	2	<0.12	<0.12	**>128**	CTX-M-1	*Intl1*
9	EC 8	*E. coli*	1	<0.12	<0.12	**>128**	CTX-M-1	*Intl1*
10	EC 12	*E. coli*	2	0.5	<0.12	4	CTX-M-1	*Intl1*
11	EC 13	*E. coli*	2	0.5	<0.12	**>128**	CTX-M-1	*Intl1*
12	KO 1	*K. oxytoca*	**16**	0.5	<0.12	**>128**	CTX-M-1	*Intl1*
13	KP 37	*K. pneumoniae*	**8**	<0.12	<0.12	**>128**	CTX-M-1	*Intl1*
14	EC 47	*E. coli*	**4**	<0.12	<0.12	**>128**	CTX-M-1	*Intl1*
15	EC 49	*E. coli*	**8**	0.5	<0.12	<0.12	CTX-M-1	*Intl1*
16	EC 61	*E. coli*	2	0.12	<0.12	**>128**	CTX-M-1	*Intl1*

Mer—Meropenem; Col—Colistin; Tig—Tigecycline; Cef—Cefotaxime; EC—*E. coli*; SF—*Shigella flexneri*; KO—*Klebsiella oxytoca*; SS—*Shigella sonnei*; KP—*Klebsiella pneumoniae*. The number in bold represents resistance by MIC. Bold numerals represent resistant isolates.

3.3. Combinational Activity of Colistin-Meropenem

The effect of antibiotic combinations on the bacterial cells was studied by the checkerboard method using colistin and meropenem. Isolates that showed resistance to both meropenem and colistin were chosen for this experiment. Four *K. pneumoniae* isolates (KP1, KP2, KP11 and KP12) were selected. The selected isolates had meropenem MIC of >128 μg/mL, while colistin MIC was 4 μg/mL (KP 1, KP2 and KP11) and 128 μg/mL (KP 12). All four isolates showed a reduced MIC during combinational studies. The FIC value for KP1, KP2, KP11 and KP12 were 1 (indifference), 0.5 (synergy), 1 (indifference) and 50 (antagonistic), respectively.

4. Discussion

Our findings illustrate the emergence of carbapenem resistance among Gram-negative bacteria in the northeastern region of India. The occurrence of 11.3% meropenem resistance, previously uncommon in the Mizoram region, is an indicator of the spread of resistance across India and possibly also globally. In addition, the higher prevalence of resistance to other last-resort antibiotics is highly concerning; we found resistance to colistin in more than 48% of the isolates, while almost a quarter of the strains are resistant to tigecycline.

In India, the prevalence of carbapenem-resistant bacterial infections is extensive, especially in the Southern and Northern regions, where the population density is high [20]. This is the first study to report the distribution of carbapenem resistance among Gram-negative bacteria from clinical samples collected from the two districts of Mizoram. The most common carbapenemase genes reported in the Indian isolates were bla_{NDM} and bla_{OXA} [20], though other beta-lactamase genes such as bla_{IMP}, bla_{SIM}, bla_{VIM} and bla_{AIM}, also contribute to carbapenem resistance. In this study, only the $bla_{\text{OXA-48-like}}$ carbapenemase gene was detected in the chromosomal DNA of *E. coli* and *Shigella sonnei*. To the best of our knowledge, this is the first report of a carbapenemase gene in *Shigella sonnei*. A recent report from

northeast India showed a low level of around 5% of carbapenem resistance, especially in the *Enterobacteriaceae* [21]. One of the previous studies in this region showed the presence of the $bla_{\text{NDM-1}}$ gene in *K. pneumoniae* and its association with insertion sequence (IS) elements [22]. In this study, integrase (*intl1*) genes were detected in *E. coli*, *Klebsiella* spp., and *Shigella* spp., which carried beta-lactamase genes. Their role in disseminating resistance remains to be investigated. Though plasmid-borne genes were reported to disseminate resistance among the bacterial community, none of the isolates had plasmid-borne resistance genes in this study. Possibly, the genetic information might be acquired by transduction/infection by temperate phages; recently, it was shown that around 5% of prophage sequences in the nosocomial pathogen *Acinetobacter baumannii* encode antimicrobial resistance genes, including bla_{OXA23} and $bla_{\text{NDM-1}}$ [23]. A recent study in the northeast region of Assam reported the presence of 18.9% of carbapenem-resistant *Enterobacteriaceae* [24]. The absence of carbapenemase production (detected by MHT) in the isolates carrying the $bla_{\text{OXA-48-like}}$ gene indicated that they were not efficient carbapenemase producers (or did not produce the enzyme at all). This implies that the strains are resistant to some other beta-lactamases.

Extended-spectrum cephalosporin-resistant isolates (n = 71) were also identified in this study. The common beta-lactamase genes found in the northeastern regions include $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-3}}$, SIM and VIM [22,25]. The presence of $bla_{\text{CTX-M-1}}$ in *E. coli* and *K. pneumoniae* in this study is one of the first reports in the Mizoram region. To add to this report, two *E. coli* isolates were found to carry both the $bla_{\text{CTX-M-1}}$ and $bla_{\text{OXA-48-like}}$ genes. This is a rather alarming finding for the northeastern region of India because it indicates faster dissemination of antibiotic resistance genes had occurred among the bacterial population. A study conducted by the state referral hospital in Falkawn, Mizoram, showed the emergence of a large number of MDR strains in this region [10]. Our study, which focused on two areas, Lunglei and Aizawl, found a high incidence of antibiotic resistance in Gram-negative bacteria to the last-resort antibiotics carbapenem, colistin and tigecycline. This indicates that resistance is disseminating to the northeastern parts of India, where the population is not dense. Possibly, the extensive use of colistin and other antibiotics in farming may contribute to the increasing numbers of resistant isolates as the resistance can spread from animal to human pathogens. In our study, carbapenem resistance was high among the *E. coli*, *K. pneumoniae* and *Shigella* spp. The discovery of Metallo-β-lactamases (MBL) and OXA-type carbapenemase in the clinical isolates of Gram-negatives are significant because the majority of the isolates are resistant to almost all -lactams and mostly involved in horizontal gene transfer [26,27]. A clonal similarity (ERIC-PCR) was not found between most of the resistant isolates, which is an indication that the resistance is currently just developing.

Clinical therapeutic options to treat carbapenem/colistin-resistant infections are mostly combinational therapies. Interestingly, the combination of two last-resort antibiotics, colistin and meropenem, showed a synergistic effect against four MDR *K. pneumoniae*. Although earlier studies showed synergy of the colistin-meropenem combination [28], this is one of the first against the clinical isolates collected from Mizoram. These results again support the combinational activity of two antibiotics against resistant bacterial pathogens. This study highlights the emergence of carbapenem-resistant bacteria in Mizoram, northeast India, and is a clear indication for immediate surveillance of AMR in this region. A high frequency of colistin, tigecycline, cefotaxime, and carbapenem resistance in association with beta-lactamase genes is a matter of concern to public health, not only in India but globally.

5. Conclusions

In this study, the emergence of carbapenem resistance and the high level of resistance developed towards cefotaxime, colistin and tigecycline are reported from the northeastern region of India, Mizoram, for the first time. A detailed investigation of the antibiotic susceptibility pattern and the exact cause of antibiotic resistance with the geographical location are some of the most crucial data to combat AMR. The present study showed the increased prevalence of carbapenem-resistant Gram-negative bacteria among clinical

isolates from Lunglei and Aizawl regions covering the disseminating resistance genes, $bla_{\text{OXA-48-like}}$ and $bla_{\text{CTX-M-1}}$ in northeast India. Similar to other studies from different parts of the world, this study also confirms the emergence of MDR bacteria against last-resort antibiotics with multiple resistance genes. This study contributes to the development of new strategies counteracting or limiting the spread of drug resistance in the northeastern region of India, the whole country and the world.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres13030027/s1, Figure S1: Dendrogram of *Enterobacter* spp. (EB) based on ERIC-PCR; Figure S2: Dendrogram of *Shigella* spp. based on ERIC-PCR. Ssp1—*Shigella* spp.; SS—*Shigella sonnei*; SF—*Shigella flexneri*; Figure S3: Dendrogram of *Klebsiella pneumoniae* (KP) based on ERIC-PCR; Figure S4: Dendrogram of *E. coli* (EC) based on ERIC-PCR; Figure S5: Dendrogram of *Acinetobacter* spp. based on ERIC-PCR; Figure S6: Dendrogram of *Pseudomonas aeruginosa* (PA) based on ERIC-PCR; Figure S7: Dendrogram of *Serratia marcescens* (SM) based on ERIC-PCR.

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Data Availability Statement: All the data that support the findings of this study have been deposited in GenBank under the accession numbers: MW600382, MW600383, MW600387, MW600385, MW600381, MW600380, MW60039, MW600384, MW600394, MW600392, MW600386, MW600389, MW588017, MW600393, MW600390 and MW600388.

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