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Carbapenem resistance in bacteria isolated from soil and water environments in Algeria

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Highlights

- High levels of carbapenem resistance were detected in natural environments
- Host species included a number of opportunistic Gram negative pathogens
- A novel member of the DHA β-lactamase family was discovered
- The natural environment is a reservoir of clinically relevant resistance mechanisms

Abstract

Objectives: Recent research has demonstrated that natural populations of bacteria carry large numbers of mobile genetic elements which may harbour antibiotic resistance determinants. The aim of this study was to investigate carbapenem resistance in Gram negative bacteria isolated from natural environments in Bejaia (Algeria), and determine the horizontal gene transfer potential of a subset of these resistance genes.

Methods: Resistant bacteria were isolated and host identified with MALDI-TOF/16S rRNA sequencing. Resistance gene carriage was investigated using double disc synergy, metallo-β-lactamase (MBL) production tests and PCR screening for carbapenemase resistance genes. To determine potential mobility, conjugation experiments were performed. To identify resistance genes, genomic libraries were constructed, functionally screened; then inserts were sequenced.

Results: From soil and water samples, 62 resistant strains were classified as belonging to the *Enterobacteriaceae, Pseudomonadaceae, Xanthomonadaceae* and *Aeromonadaceae* families. Four highly imipenem and cefotaxime resistant (MICs >64 µg/ml and >8 µg/ml, respectively), clinically relevant strains were selected for further characterization. All four strains produced extended spectrum β-lactamases, but MBL production was not confirmed. Imipenem and cefotaxime resistance was transferable to *E. coli* strains but was not conferred by $\mathrm{bla}_{\mathrm{AMPc}}$, $\mathrm{bla}_{\mathrm{IMP}}$, $\mathrm{bla}_{\mathrm{NDM}}$, $\mathrm{bla}_{\mathrm{KPC}}$, $\mathrm{bla}_{\mathrm{OXA\text{-}48}}$ or $\mathrm{bla}_{\mathrm{GES}}$ genes. Novel putative resistance mechanisms were identified, including a novel DHA β-lactamase which conferred clinical resistance to cefotaxime.

Conclusions: The environment is a reservoir of carbapenem resistant bacteria. Further investigation of evolution and dissemination of antibiotic resistance in environmental bacteria is required, to understand and prevent the emergence of resistance in clinical environment.

Keywords: Carbapenem, gene transfer, resistance, soil, water.

1. Introduction

The emergence of antibiotic resistance within Gram negative bacteria is a significant clinical and economic threat to human and animal medicine. *Pseudomonas, Stenotrophomonas, Aeromonas* and *Enterobacteriaceae* spp. are environmental species but are also multi-resistant, opportunistic pathogens associated with serious infections in humans and animals. *Enterobacteriaceae* are ubiquitous Gram negative microorganisms, usually colonizing the gut environment. They are a major cause of contamination in food and water and are one of the most important opportunistic pathogenic organisms in the clinical environment. They are implicated in large numbers of community and hospitals acquired infections and can exchange and acquire genetic information, including antibiotic resistance genes, *via* horizontal gene transfer (HGT).

β-lactam antibiotics are widely used in clinical therapy to treat such enteric bacterial infections. Third generation cephalosporins (3GCs) are β -lactams that are currently widely used; and carbapenems are one of the antibiotics of last resort to treat nosocomial and severe urinary tract and skin infections [1]. However, their overuse contributes to development of multidrug resistance among enteric bacteria [2]. Carbapenem resistance can result from production of β-lactamase enzymes such as extended spectrum β-lactamases (ESBLs) combined with porin alteration, hyper production of the AmpC enzyme coupled with loss of porin functionality; or production of β-lactam hydrolyzing carbapenemases [3]. Using this enzymatic arsenal, multidrug resistant Gram negative bacteria have become a serious problem in the clinical environment [4].

Outside of the clinic, antibiotic molecules can be detected in aquatic and soil environments where they are produced by environmental microorganisms or are excreted by humans and animals. Heavy metals used in animal farming and aquaculture are also known to drive the evolution of antibiotic resistance within bacteria through co-selection [5]. Numerous investigations have revealed that the environmental metagenome contains highly diverse antibiotic resistance genes termed the environmental "resistome" which can be horizontally transferred to new hosts, and that the natural environment contains reservoirs of multidrug resistant bacteria [6].

This motivated the study of carbapenem resistant bacteria from different sites in the natural environment from North Africa in the Bejaia (Algeria) region. Aquatic and soil environments were screened for the presence of Gram negative bacteria resistant to carbapenem antibiotics. The host species of the resistant isolates were identified and the phenotypic resistance profiles were characterized. The HGT potential of this resistance was also determined for a subset of isolates.

2. Materials and methods

2.1 Sampling

Soil and water samples were collected in the province of Bejaia located in North Africa. During the period of October to November 2013, soil samples (n=95) were collected from the rhizosphere of wild legumes (*Genista numidica, Calycotum spinosa and Spartium junceum*) growing in a coastal area, an arid region and in a heavy metal contaminated soil. The water samples (n=90) were obtained from natural (forest) and bore hole (80m deep) water sources, animal drinking trough water and municipal wastewater. We highlight that the natural environment samples had not been exposed to anthropogenic antibiotics or human manipulation such as agricultural activity or hospital waste contamination. Samples were transported to the laboratory at 4°C for bacterial isolation.

2.2 Isolation and bacterial characterization

Serial dilutions in saline were prepared from each sample. One ml of bacterial suspension was inoculated in nutrient broth (Himedia, USA) and incubated at 37°C for 24 hours. In order to reduce the bacterial density before isolation, the cultures were diluted to one tenth (10^{-1}) in saline water. Then, 100µl of each dilution was streaked onto MacConkey agar (Himedia, USA) supplemented with 2µg/ml of imipenem [7]. After an overnight incubation at 37°C, the isolates were purified and stored on agar slants and in 20% glycerol at -20°C.

Identification of isolates was based on amplification of the 16S rRNA gene (n=22) and/or MALDI-TOF spectroscopy (n=40). PCR amplification was carried out using the primers 27F and 1492R and following PCR conditions presented in Mao et al [8]. The 16S r RNA sequences were analyzed using MEGA6 software [9], then compared within sequences in GenBank (NCBI).

2.3 Antimicrobial susceptibility testing

To examine the phenotypic resistance profiles of bacterial isolates, discs containing cefotaxime (CTX 30 µg), ceftazidime (CAZ 30 µg), aztreonam (ATM 30 µg), ertapenem (ETP 10 µg), meropenem (MEM 10µg) and imipenem (IMP 10µg) (Oxoid, UK) were used in disc diffusion assays. To achieve high turbidity, isolates were grown on nutrient agar (Himedia, USA) then incubated overnight at 37°C. Colonies were resuspended in 0.9% of saline solution to the turbidity equivalent of a 0.5 Macfarland standard. Bacterial suspensions were inoculated by streaking the cotton swab over the surface of Muller Hinton agar plates, then antibiotic discs were placed on the agar surface. After 24 hours incubation at 37°C, resistance and sensitivity was estimated by measuring the inhibition zones and comparing to EUCAST guidelines [10].

2.4 Antibiotic minimal inhibitory concentration determination

Minimal inhibitory concentrations (MICs) were determined for imipenem and cefotaxime, following the agar dilution method: after 24 hours incubation at 37°C, the MICs of the strains were defined as the lowest concentration of antibiotic that inhibited the visible growth of bacteria [11]. Isolates possessing clinically significant MICs were selected for ESBLs and MBL production tests. ESBL production was screened with the double disc synergy test assay (DDST) [12]. Detection of MBL production was performed following the EDTA test; combined disc [13] and synergy test [14]. Finally, the Carba NP test was carried out as reported in Bakour et al [15].

2.5 Resistance gene transfer

In order to determinate whether the putative resistance genes were mobile and transferable; conjugation experiments were performed. Conjugal transfer of the resistance gene from the isolates to a rifampicin resistant *E coli* (CV 601) recipient was performed following the broth and filter assay [16]. *E. coli* (CV601) was used as the recipient strain and *Klebsiella pneumonia* KX159722 ('KP1'), *Morganella morganii* BHWSO8 ('MM1'), *Morganella morganii* KX159721 ('MM2') and *Klebsiella oxytoca* KX15970 ('KO1'), isolated in this study, were the donor strains. Each strain pellet was resuspended in 1 ml of LB broth, then incubated for two hours. Cells were harvested from the mixed suspension of 100 µl of donor and 200 µl of recipient by centrifugation for 5 minutes at 8K rpm. The pellet was resuspended in 100 µl in Luria Bertani broth (LB) and applied on Millipore filter (0.22 µm) which was placed on Luria Bertani agar (LB) without antibiotic. After overnight incubation, filters were resuspended in 10 ml of sterile saline (0.85%) by vigorous vortexing. In order to determine the numbers of recipient cells, 50µl of 10-fold serial dilutions were streaked on three PCA plates supplemented with the following antibiotic concentrations, respectively: rifampicin (50 µg/ml), cefotaxime (2 µg/ml) and mixed rifampicin (50 µg/ml) and cefotaxime (2µg/ml). PCA plates were then incubated for two days at 28°C. In addition, purified plasmid (GENE JET Plasmid miniprep Kit (Thermo Scientific)) extracted from isolates was transferred into competent *E. coli* EC-100 (Epicentre) cells by electroporation (1.4KV, 4.3 ms). Transformants were selected on Luria Bertani agar (LB) supplemented with ampicillin (100µg/ml), twice. Transconjugants and transformants were selected on Luria Bertani agar (LB) containing rifampicin (125 µg/ml) and imipenem (2µg/ml). MICs were determined for imipenem (0.1 - 8µg/ml), cefotaxime (0.1 – 4 µg/ml) and ampicillin (1 – 125 µg/ml). Note that for all experiments, *E. coli* CV6001 and EC-100 strains were used as negative controls and that all the analyses were performed in triplicate.

2.6 Screening for known resistances genes

Plasmid DNA was extracted from transconjugants using the GENE JET Plasmid miniprep Kit (Thermo Scientific), and underwent PCR amplification for resistance genes bla_{AMPc}, bla_{IMP}, bla_{NDM}, bla_{KPC}, $bla_{OXA\text{-}48}$ and bla_{GES} using the primers (Table S1) and cycling programmes cited in Endimiani et al [17] for AMPc and IMP primers and Bogaerts et al., [18] for NDM, KPC, OXA-48 and GES primers (Table S1).

2.7 Construction of genomic libraries

Genomic libraries were constructed by using Electrocompetent *E. coli* EC-100 (Epicentre, Madison, WI) and fragmented DNA (≈3000-5000bp) extracted from the following resistant strains: KP1, MM1, MM2 and KO1. Whole genome coverage was equivalent to 10^3 cells. Transposon mutagenesis was carried out using CloneSmart Blunt Cloning Kits (Lucigen, WI, USA). Briefly, the extracted DNA was blunt ended at the 5'phosphorylated site, then ligated into the pSMART LcKan Vector. This was then transferred into *E. coli* EC-100 by electroporation (2.2-2.5KV) and inoculated onto LB plates. Plate 01 contained imipenem (0.5 µg/ml) plus kanamycin (0.5 µg/ml), plate 02 contained cefotaxime (0.5 µg/ml) plus kanamycin (0.5 µg/ml) and plate 03 contained ampicillin (25 µg/ml) plus kanamycin (0.5 µg/ml). In order to study the clone sensitivity, imipenem (0.2 – 8 µg/ml), cefotaxime (0.2 - 8µg/ml), and ampicillin (1 – 125 µg/ml) MICs were carried out.

2.8 Quality control of the genomic library

Colonies growing on selective medium were grown in 10 mL LB medium (kanamycin 10 µg/ml). The plasmid was extracted according to the manufacturer's protocol (Plasmid miniprep Kit GENE JET, Thermo Scientific, UK), then restricted using restriction enzymes: *EcorI* and *Xbaz* to excise the inserts of clones. The restriction product was visualized by electrophoresis.

2.9 Insert sequence analysis

Using One Shot LA PCR Mix Ver 2.0 (TAKARA Bio INC, Japan) the insert sequences were amplified, then sequenced (Macrogen, Europe). Sequenced inserts were analyzed with MEGA6 and compared with other *Enterobacteriaceae* strains by using BLASTp. *The ResFinder* web server (www.genomicepidemiology.org) was used to identify acquired antimicrobial resistance genes [19]. Alignment was performed with MegAlign from the Lasergene software package from DNASTAR (Madison, WI). Relevant sequences deposited in GenBank are available under the following accession numbers: **MF186235** (*Klebsiella pneumoniae* KX159722, 'KP1'), **MF186233** (*Morganella morganii* BHWSO8, 'MM1'), **MG701058** (*Morganella morganii* KX159721, 'MM2'), and **MF186234** (*Klebsiella oxytoca* KX159720, 'KO1').

3. Results

3.1 Diversity of carbapenem resistant isolates

In the current study of antibiotic resistant bacteria from the natural environment in Algeria, a high diversity of Gram negative isolates were characterized as being resistant to carbapenem antibiotics. Using MALDI-TOF mass spectrometry, 16S RNA PCR and sequencing, thirteen (13) out of 62 isolates were classified within the *Enterobacteriacaea* family including: *Serratia marcescens* (n=3), *Enterobacter cloacae* (n=3), *Morganella morganii* (n=2), *Klebsiella pneumoniae* (n=3) and *Klebsiella oxytoca* (n=2). Most of these isolates were from water, rhizosphere and saline soil. From natural water sources, two (2) isolates were identified as *Aeromonas veronii*. Eighteen (18) isolates were attributed to the genus *Pseudomonas*, six (6) characterized as *Pseudomonas aeruginosa*. The majority of the *Pseudomonas spp.* was isolated from rhizosphere soil samples. Twenty seven (27) isolates were identified as *Stenotrophomonas maltophilia,* with the majority isolated from water samples. Two strains isolated from soil were identified as *Ochrobactrum intermedium* (Table S2). For the isolates characterized by 16S rRNA sequencing, nucleotide sequences were deposited in GenBank (Table S2). Antimicrobial susceptibility and MIC tests revealed that most of the 62 isolates were resistant to imipenem (62.90%), ertapenem (79.03%), cefotaxime (64.51%), ceftazidime (43.54%) and aztreonam (80.64%), whilst most were sensitive to meropenem (30.64%) (Table S2). The highest MICs were for imipenem (>64 µg/ml) and cefotaxime (>8 µg/ml), equating to 8x and 4x the clinical breakpoint concentrations defined by EUCAST for *Enterobacteriacaea* [20].

3.2 Characterization of resistance mechanisms

Phenotypic resistance profile characterization for four multidrug resistant *Enterobacteriaceae* spp. (KP1, MM1, MM2 and KO1) was carried out due to their significance in causing clinical infections (Table 1). The first assay for detection of ESBLs (DD test) showed synergy results by formation of ghost inhibition zones between the central discs, suggesting that these isolates may possess ESBL genes. However, the MBL production tests showed negative results. For the EDTA test, no synergy was observed and no difference was observed between the inhibition zone of imipenem + EDTA and the inhibition zone of imipenem alone. For the Carba NP test, MM1, MM2 and KO1 isolates showed negative results; whereas for KP1, the color changed from red to yellow as early as 10 to 15min after incubation, indicating possible carbapenemase carriage.

3.3 Transfer of resistance mechanisms to an *E. coli* recipient

In this study, the transfer of carbapenem resistance from donor isolates (KP1, MM1, MM2 and KO1) to the recipient strain *E. coli* (CV601) was observed. The transconjugants grew at a concentration of cefotaxime ≥ 4µg/ml and showed an imipenem MIC of ≥8 µg/ml, as did EC100 cells with plasmids inserted by transformation (Table 1). However, both transconjugants and transformants were negative for PCR detection of bla_{AMPC}, bla_{IMP}, bla_{NDM}, bla_{KPC}, bla_{OXA} and bla_{GES} genes.

3.3 Genomic library analyses

Genomic libraries were constructed from isolates expressing high levels of resistance to imipenem (MIC ~64 µg/ml). Libraries contained clones with phenotypic resistance to imipenem (≥1 µg/ml and ≥ 4 µg/ml) and cefotaxime (≥ 4 µg/ml) – see Table 1. The analysis of DNA insert sequences was performed using BLAST. This revealed that KP1, MM1, MM2 and KO1 insert lengths were respectively: 3003, 2592, 1199 and 2979 base pairs.

ORF Finder and BLASTn were used to identify potential genes conferring resistance within the genomic libraries. A KP1 clone bearing the insert MF186235 contained a novel *bla*$_{DHA}$ like gene. This ORF had 99% amino acid identity to bla_{DHA-16} (accession **WP_063860099.1**), but with 4 amino acid substitutions. The gene was flanked by a gene encoding aLDT IgD-like hypothetical protein (226 amino acid) on one side and by an ampR like regulatory protein gene as well as a helicase gene (*armA*) and a periplasmic binding protein on the other side. ORFs identified within the insert sequence MF186233 from MM1 included the transcription protein DDETnp1 (402 amino acids) which was flanked between two sequences encoding galactose-1-phosphate uridyl-transferase-like proteins, and an iron uptake protein. The resistant clone from MM2 isolate library bearing the MG701058 insert contained a *bla*$_{TEM-116}$ (100% amino acid identity). The insert sequence isolated from the KO1 library contained a protein with 99% amino acid identity to an ATP dependent helicase (897 amino acids) and an iron uptake protein (221 amino acids), both from *Pseudomonas aeruginosa* (WP_031629204.1).

All inserts described had their MICs redetermined in the EC100 host (Table 1). All inserts conferred clinical resistance to cefotaxime with MICs ≥4 µg/ml (EUCAST breakpoint 2 µg/ml). All inserts conferred reduced susceptibility to imipenem, relative to the EC100 control (Table 1) and conferred intermediate clinical resistance to imipenem with MICs of ≥4 µg/ml (EUCAST breakpoint for resistance > 2 µg/ml and < 8 µg/ml.

4. Discussion

Carbapenemases and ESBLs are important resistance determinants in Gram negative pathogens, including: *Enterobacteriaceae, Pseudomonas, Acinetobacter* and *Aeromonas* spp [21]. Outside the clinical environment, soil is described as harboring the most diverse microbial populations on earth and is considered a large reservoir of antibiotic resistance genes [22] and multidrug resistant bacteria [7]. Previous findings from soil samples report resistance to β-lactams (penicillin, carbapenicillin, dicloxacillin, ampicilin) and first and second generation cephalosporins; these resistance profiles are mainly associated with ɣ Proteobacteria [21, 22, 23]. Resistance to third generation cephalosporins and carbapenems is rarely observed in natural environments, though Gudeta et al., have isolated carbapenem resistant bacteria from soils samples from Algeria, United Kingdom, Germany, Denmark, Norway, and Spain including carbapenemase producing *Pedobacter*, *Epilithonimonas*, *Sphingomonas, Massilia, Chryseobacterium, Janthinobacterium,* and *Stenotrophomonas* [24]. Metallo-β-lactamases have also been reported from non-pathogenic marine organisms (*Novosphingobium pentaromativorans* and *Simiduia agarivorans*) [25] and from a remote Alaskan permafrost metagenome [26]. In the current study, high levels of resistance to the 3GC cefotaxime and the carbapenem antibiotic imipenem were detected in a range of opportunistic pathogenic, Gram negative species isolated from soil and aquatic environments in North Algeria. These have the potential to cause infections in humans and animals, and included *Serratia marcescens, Enterobacter cloacae, Morganella morganii, Klebsiella pneumoniae, Aeromonas veronii, Pseudomonas* spp. and *Stenotrophomonas maltophilia.*

Multidrug resistance in *Stenotrophomonas maltophilia* mainly involves low membrane permeability, chromosomally encoded multidrug resistance efflux pumps, and β-lactamase and antibiotic modifying enzymes [23]. Carbapenem resistance among pseudomonads, specifically *P. aeruginosa,* can be mediated by plasmid or integron-borne carbapenemases; or reduced porin expression and increased chromosomal cephalosporinase activity [24]. Carbapenem resistance in *Enterobacteriaceae* may be explained by: β-lactamase production, by enzymes such as ESBLs combined with porin alteration that affect antibiotic uptake, hyper production of an AmpC coupled with loss of porin functionality and production of β-lactamase hydrolyzing carbapenemases [3]. Here, high levels of resistance to imipenem and cefotaxime were observed in *Klebsiella* and *Morganella* spp. but common β-lactamase genes were undetectable by PCR.

Klebsiella pneumoniae (KP1) was positive for the Carba NP test but no known carbapenemase resistance genes were detected with PCR (like all isolates characterized in this study). KP1 did contain a gene with high homology to the bla_{DHA} gene class, namely the cephalosporinase $bla_{\mathrm{DHA-16}}$ gene but with 4 amino acid replacements, which conferred reduced susceptibility to imipenem and clinical resistance to cefotaxime. One amino acid substitution makes a new variant, indicating the gene in this isolate is a novel gene member of *bla*$_{\mathrm{DHA}}$ family. To our knowledge this is the 26th member of this family [27]. Carbapenem resistance in this isolate may be mediated by the expression of this *bla*$_{\mathrm{DHA}}$ like gene, regulated by the ampR regulatory protein. The higher MICs for imipenem in the KP1 transconjugants and transformants relative to the clone insert host suggests the presence of multiple resistance mechanisms in the accessory genome of this strain. Indeed, carbapenem resistance from *Klebsiella* species is often attributed to an innate resistance such as loss of outer membrane production; or acquired resistance through harboring ESBLs genes such as CTX-M, SHV-2, AmpC enzymes such as ACT-1, CMY-4 or DHA-1; or OXA-48 [28]. Gupta *et al* and Erdemli *et al* in their recent investigations also report L-D transpeptidase activity as the major contributor of β-lactam resistance in Mycobacterium tuberculosis [29], which was flanking the novel *DHA-16* like gene. This isolate was isolated from water in a forest site unimpacted by human activities, which suggests a possible environmental origin of the clinically important resistance *bla*$_{\mathrm{DHA}}$ genes. The environmental resistome has been shown to be a source for problematic clinical resistance previously; for example, the ESBL CTX-M is believed to have originated from the chromosome of an environmental *Kluyvera* spp. [30].

Morganella morganii (MM1) clone insert contained two copies of galactose-1-phosphate uridyltransferase (galT) -like proteins. GalT is a key enzyme in the LeLoir pathway which converts galactose-1-phosphate into UDP-galactose. UDP-galactose is required for exopolysaccharide synthesis and so may confer antibiotic resistance by reduced compound uptake into the bacterial cell. In addition, UDP-galactose-4-epimerases encoded by *galE* genes perform the final step in the LeLoir pathway, by converting UDP-galactose into UDP-glucose [31]. *GalE* genes have been shown previously to have a role in resistance to a range of antibiotics and some biocides [32, 33], again presumably by reduced drug uptake due to production of exopolysaccharide or lipopolysaccharide [34]. There is therefore increasing evidence genes involved in the LeLoir pathway can have a secondary role of conferring reduced susceptibility to antibiotics and other antimicrobials, and in this case, two copies of this gene resulted in clinical resistance to cefotaxime and intermediate resistance to imipenem. Previous work has shown that other mechanisms including HU DNA-binding proteins, the GroEL/GrOES chaperonin complex and GrpE proteins may contribute to carbapenem resistance [35].

The *Morganella morganii* (MM2) isolate was positive for the β-lactamase assay and was shown to harbor a bla$_{TEM\text{-}116}$ gene. A previous study which characterized the phenotype of this resistance gene in clinically-isolated *E. coli,* found bla$_{TEM-116}$ conferred only reduced susceptibility to imipenem [36]; unlike this study which showed it can confer intermediate clinical resistance. This could be due to the genetic context of the gene.

The predicted proteins responsible for resistance in the *Klebsiella oxytoca* (KO1) clone had highest hits for ATP-dependent helicase and iron uptake proteins. Mutations in key bacterial enzymes involved in DNA processing such as gyrase and topoisomerase IV can result in fluoroquinolone resistance [37]. However, the iron uptake protein is a transporter protein and may confer resistance through efflux of β-lactam antibiotics.

In Gram negative bacteria, dissemination of antimicrobial resistance is often attributed to HGT of resistance genes encoded on plasmids. This phenomenon is considered the principal reason for the acquisition of resistance in bacterial pathogens causing community or hospital acquired infections [38]. HGT may occur between bacteria of different species and genera, occupying natural environments or colonizing different areas of a host species [39]. A subset of highly resistant *Enterobacteriaceae* isolates in the present study, were shown to harbor transferable mobile resistance mechanisms conferring clinical resistance to cefotaxime and, in some cases, resistance or intermediate resistance to imipenem depending on host background. Though the MICs to imipenem reduced when strains were subcloned and expressed in *E. coli*, this is common and can be attributed to a range of factors including lower gene expression compared to the original host and improper protein folding, amongst other reasons. As carbapenem resistance was transferable but not detectable by PCR for well-known resistance genes, this suggests the isolates identified in this study possess novel mechanisms, including those characterized in this study, that have the potential to be mobilized into bacteria in the human microbiome. Woodford et al state that "active surveillance and monitoring for carbapenem-resistant bacteria in the food chain and other non-human sources is urgently needed, with an enhanced and rigorous follow-up of all positive results" [40]. These results, combined with detection of a novel member of the AmpC DHA β-lactamase family and bla$_{TEM-116}$, suggest environmental monitoring could be valuable for identifying resistance genes which could become clinically significant in the future.

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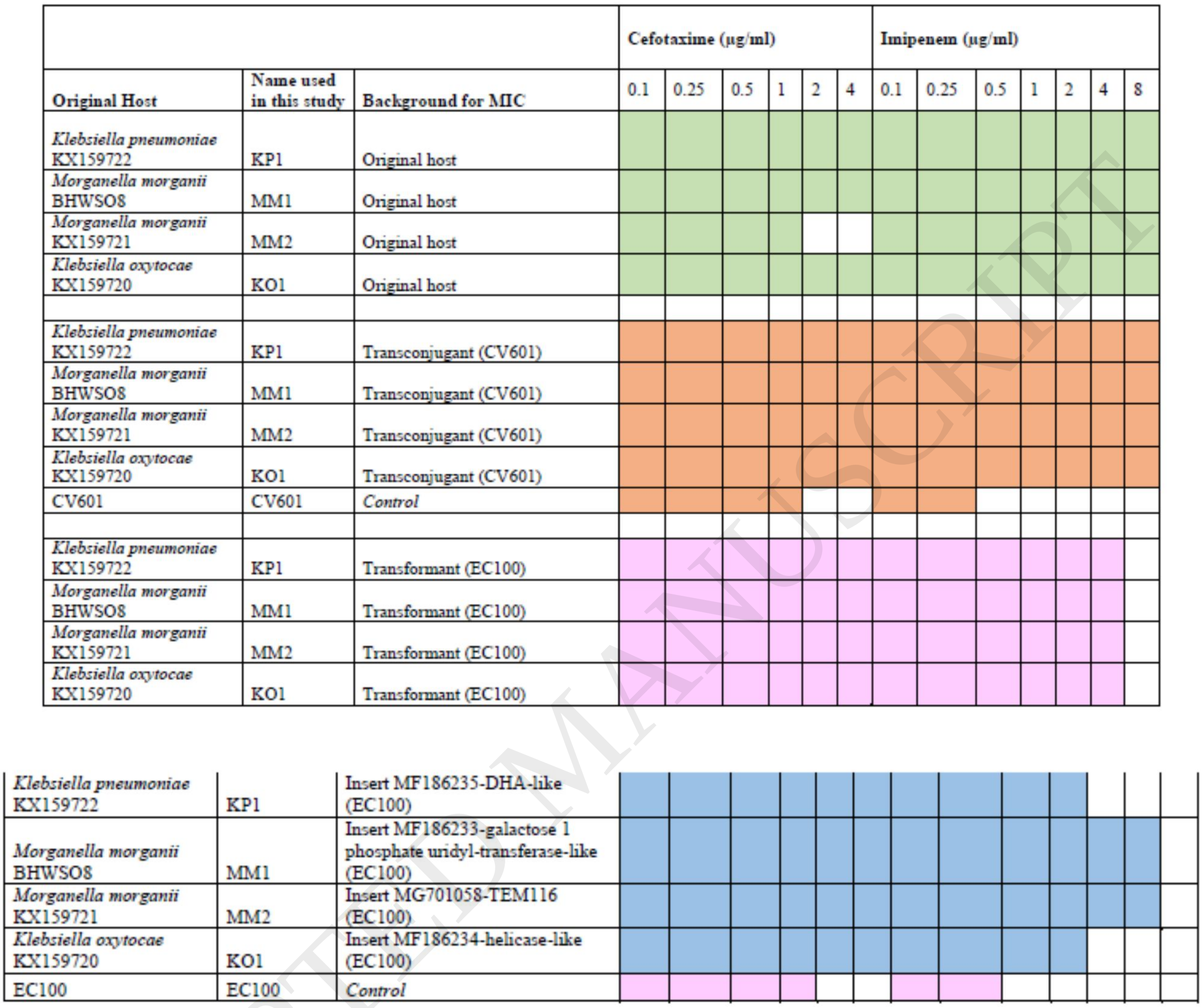
Table 1. Cefotaxime (TAX) and Imipenem (IMP) MICs for the original host species, lab strains CV601 and EC100 bearing conjugated and transformed plasmid DNA respectively, and resistant clones from the EC100 genomic libraries. Colour indicates growth, no colour indicates no growth.



			Cefotaxime (μg/ml)						Imipenem (μg/ml)						
Original Host	Name used in this study	Background for MIC	0.1	0.25	0.5	1	2	4	0.1	0.25	0.5	1	2	4	8
Klebsiella pneumoniae KX159722	KP1	Original host	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Morganella morganii BHWSO8	MM1	Original host	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Morganella morganii KX159721	MM2	Original host	🟩	🟩	🟩	🟩			🟩	🟩	🟩	🟩	🟩	🟩	🟩
Klebsiella oxytocae KX159720	KO1	Original host	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Klebsiella pneumoniae KX159722	KP1	Transconjugant (CV601)	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧
Morganella morganii BHWSO8	MM1	Transconjugant (CV601)	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧
Morganella morganii KX159721	MM2	Transconjugant (CV601)	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧
Klebsiella oxytocae KX159720	KO1	Transconjugant (CV601)	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧
CV601	CV601	*Control*	🟧	🟧	🟧	🟧			🟧	🟧					
Klebsiella pneumoniae KX159722	KP1	Transformant (EC100)	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	
Morganella morganii BHWSO8	MM1	Transformant (EC100)	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	
Morganella morganii KX159721	MM2	Transformant (EC100)	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	
Klebsiella oxytocae KX159720	KO1	Transformant (EC100)	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	🟪	
Klebsiella pneumoniae KX159722	KP1	Insert MF186235-DHA-like (EC100)	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦			
Morganella morganii BHWSO8	MM1	Insert MF186233-galactose 1 phosphate uridyl-transferase-like (EC100)	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	
Morganella morganii KX159721	MM2	Insert MG701058-TEM116 (EC100)	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	
Klebsiella oxytocae KX159720	KO1	Insert MF186234-helicase-like (EC100)	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦			
EC100	EC100	*Control*	🟪	🟪	🟪	🟪			🟪	🟪					