Original Article

An unequivocal superbug: PDR *Klebsiella pneumoniae* with an arsenal of resistance and virulence factor genes

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Abstract

Introduction: Infections caused by extensively-drug resistant (XDR) and pan-drug resistant (PDR) *Klebsiella pneumoniae* represent an emerging threat due to the high associated mortality. This study aimed to characterize two carbapenem resistant *K. pneumoniae* strains from the same patient, the first being PDR (referred to as IMP 1078b) and the second being XDR (referred to as IMP 1078s) isolated from the same patient.

Methodology: Antimicrobial susceptibility testing was done for the 2 *K. pneumoniae* isolates, followed by carbapenem/β-lactamase inhibitor combination assay, and fitness cost against cefepime and meropenem. Then, whole-genome sequence analysis was performed to decipher the molecular mechanisms behind the high level of resistance recorded in both isolates. Finally, qRT-PCR was done for β-lactam resistant genes.

Results: This is the first report about a *K. pneumoniae* isolate harboring 47 antimicrobial resistance genes and having type IV pilli (*Yersinia*) and the fimbrial adherence determinant Stb (*Salmonella*) as virulence factors. Further analysis on both isolates are discussed within the article.

Conclusions: The co-existence of a high number of antimicrobial resistant (AMR) genes and virulence factor genes may lead to a life threatening invasive and untreatable infection.

Key words: *K. pneumoniae*; XDR; PDR; AMR; NDM; OXA.

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Introduction

Healthcare associated infections, such as pneumonia, catheter associated blood stream infections, surgical site infections, and catheter associated urinary tract infections caused by resistant Gram-negative bacteria such as *Klebsiella pneumoniae* are increasing worldwide [1]. Their burden is particularly heavy in the critically ill patients where there is an association between infection with such multidrug-resistant (MDR) organisms and poor outcomes [2,3]. Carbapenems are the mainstay of treatment for infections with extended-spectrum beta-lactamase producing (ESBL) *K. pneumoniae* [4]. However, some strains have acquired resistance against these antibiotics, leaving colistin as the only treatment option [5].

Infections caused by antibiotic resistant bacteria are increasing worldwide. Each year the U.S. reports more than 2.8 million infections with antibiotic resistant bacteria. This led the Centers for Disease Control and Prevention (CDC) to publish an antibiotic resistance threat report in 2019, classifying carbapenem-resistant Enterobacteriaceae (CRE) as an urgent threat [6]. In Lebanon and the Middle East North Africa (MENA) region, the rates of Gram-negative resistance are very high [7–9]. With the raging conflicts in the MENA region, especially the Syrian conflict and due to the
transfer of patients from field hospitals at the Lebanese-Syrian border to hospitals within Lebanon, there has been a noticeable increase in the rates of Gram-negative resistant organisms in peripheral towns and later in central Lebanese hospitals. Here we report a case of two K. pneumoniae strains that were recovered from a patient who was initially hospitalized at a peripheral hospital at the Lebanese-Syrian border. We therefore aimed at testing the susceptibility of the isolates against a battery of antibiotics used in clinical settings and determining phenotypic and genotypic mechanisms of resistance of these isolates using whole genome sequencing.

Following a motorcycle accident, a 22 year-old Lebanese man sustained severe trauma in his hometown close to the Lebanese-Syrian border. Unconscious, he was taken initially to a peripheral hospital where he was intubated for mechanical ventilation and a central line was inserted and admitted to the ICU. After 6 days in the other hospital, during which he received piperacillin-tazobactam, vancomycin and dexamethasone, he was transferred to a tertiary care hospital in Lebanon for continuity of care. Upon admission, he was afebrile and comatose. Workup revealed a subarachnoid hemorrhage and brain contusions with surrounding edema and multiple closed fractures of the extremities, chest subcutaneous emphysema, small pneumopericardium and pneumomediastinum. The old central line was discontinued and a new one inserted. Cultures from blood, urine and deep tracheal aspirates were taken in addition to skin screening as per standard screening protocols for ICU transfers at our institution. Those cultures later grew carbapenem-resistant Klebsiella pneumoniae from the deep tracheal aspirate (DTA) and the skin (sensitive to tigecycline, intermediate to colistin and fosfomycin). The following day the patient developed a high-grade fever. He was started on piperacillin-tazobactam and vancomycin after removing the newly inserted central line and sending appropriate cultures. He remained febrile for several days with evidence of a left lower lobe pneumonia prompting changes to the antibiotics he was receiving based on the initial screening cultures: the DTA culture grew carbapenem-resistant K. pneumoniae and E.coli both sensitive to tigecycline, with the K. pneumoniae being intermediate to colistin and resistant to fosfomycin. A week following admission, he was persistently febrile, therefore new blood cultures were taken and the sample from the central line grew K. pneumoniae after 16 hours (sensitive to tigecycline, resistant to colistin and fosfomycin). The peripheral blood cultures remained negative. The patient received inhaled colistin and later inhaled amikacin; carbapenems were discontinued as the minimum inhibitory concentrations (MICs) to these agents were all greater than 32. Despite the infection with pan-resistant organisms, our patient’s condition improved. The bacteremia was related to the central line and it resolved as soon as the line was discontinued, which is essential with Gram-negative rod (GNR) central line-associated bloodstream infections (CLABSIs). He became afebrile with marked neurologic and clinical recovery and was extubated, and transferred to the regular floor, with eventual discharge home. The Supplementary Table1 lists all the different cultures and results. The Supplementary Figure1 shows the timeline of different antibiotic administration.

Methodology

Ethical approval was not required as clinical isolates were collected and stored as part of routine clinical care. Clinical isolates and patient records/information were anonymous and de-identified prior to analysis.

Identification of the isolates

The recovered isolates in culture were identified using the Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) system (Bruker Daltonik, GmbH, Bremen, Germany) with a score of green flags.

Broth Micro-dilution assay

Broth microdilution was done against 19 different antibiotics from different families. Serial dilution took place between columns 1 and 11 to have concentration ranging from 2048 μg/mL to 2 μg/mL. Half of the wells in column 12 were used as a positive control and the other half as a negative control. For each isolate a bacterial suspension of 0.5 MacFarland was prepared, followed by dilution to reach a concentration of $5 \times 10^6$ CPU/mL. This was followed by adding 10 μL of the latter into all the well between columns 1-11, and in the positive control designated wells in column 12, ending with a final volume of 100 μL in all the wells. The plate was then placed in the incubator at 37 °C for 18 hours after which the negative control was checked to ensure the absence of contamination. The positive control was checked to ensure that the bacterial suspension was properly prepared, and growth took place. Wells 1-11 were checked for bacterial growth, the well preceding the first well with bacterial growth, was referred as the well containing the MIC. Experiments were run in
duplicates for each bacterial isolate. The results were interpreted according to the CLSI M100 guideline [10].

**Disk Diffusion**

The experiment was performed using the Kirby-Bauer technique. For each isolate, a bacterial suspension equivalent to 0.5 MacFarland was prepared. Then it was subcultured on a round Mueller-Hinton agar plate, in all the directions, to ensure that the bacterial suspension covered all the plate using a sterile swab. The plate was left for around 10 minutes closed on the bench, followed by the addition of the 24 tested antibiotics (8 per plate). The plate was then incubated at 37 °C for 18-24 hours after which the zone of inhibition diameters were measured and the results were interpreted according to the CLSI M100 guideline [10].

**Fitness Cost assay**

The tested isolates were first subcultured on MacConkey agar and incubated at 37 °C for 18-24 hours. The next day, a loop full of each bacterial isolate was transferred into 10 mL of sterile cation adjusted Mueller-Hinton broth and incubated at 37 °C for 18-24 hours. Then, the turbid inoculated broth of each isolate was diluted at a 1:1000 ratio. The latter was then transferred into 4 distinct wells (200 µL each) of a 96 well microtiter plate. The replication rate of each tested isolate was measured using a densitometer (OD 600 nm) for 16 hours with reads at 30 minutes intervals. The results were then averaged, normalized, and plotted against the *K. pneumoniae (DSM® 30104)* [11].

**Carbapenem/β-Lactamase Inhibitor Combination assay**

Following the MIC determination of both isolates against carbapenems, Meropenem/ β-lactamase inhibitor combinations experiment was performed by adding fixed concentrations of the inhibitors to the experimental wells of a standard antimicrobial broth microdilution assay. We followed CLSI guidelines in this assay. However, minor modifications to broth volumes were made in order to accommodate for the presence of the β-lactamase inhibitors (βLIs) while keeping the concentrations of the meropenem and bacterial suspensions in accordance with CLSI recommendations. For isolates harboring *bla*OXA*-type* carbapenemases, Avibactam (MedChem Express, Monmouth Junction, NJ, United States) was used as the βLI at a fixed concentration of 4 µg/mL. However, for isolates that harbored *bla*NDM, ethylenediaminetetraacetic acid calcium disodium salt (calcium-EDTA) (Sigma R, St. Louis, MO, United States) was used as the βLI at a fixed concentration of 32 µg/mL. In addition, both isolates were tested against both βLIs at their aforementioned fixed concentrations without the addition of meropenem in order to rule out any antibacterial activity exhibited by the inhibitors on the tested isolates. The MICs of the 4 tested isolates were interpreted according to the CLSI M100 guideline.

| Antibiotics                     | IMP 1078b | IMP 1078s |
|---------------------------------|-----------|-----------|
|                                 | MIC (µg/mL) | Int* | MIC (µg/mL) | Int* |
| Cefuroxime                      | > 2,048 | R | > 2,048 | R |
| Cefazidime                      | > 2,048 | R | > 2,048 | R |
| Cefepime                        | 512 | R | 512 | R |
| Ertapenem                       | 2,048 | R | 1,024 | R |
| Meropenem                       | 256 | R | 256 | R |
| Imipenem                        | 128 | R | 128 | R |
| Aztreonam                       | 256 | R | 512 | R |
| Nalidixic acid                  | 256 | R | 512 | R |
| Ciprofloxacin                   | 32 | R | 64 | R |
| Norfloxacin                     | 256 | R | 256 | R |
| Levofloxacin                    | 64 | R | 64 | R |
| Colistin                        | 32 | R | < 2 | S |
| Gentamicin                      | 1,024 | R | 2,048 | R |
| Amikacin                        | > 2,048 | R | > 2,048 | R |
| Fosfomycin                      | > 2,048 | R | 1,024 | R |
| Tigecycline                     | 16 | R | 8 | R |
| Trimethoprim Sulfamethoxazole   | 256 | R | 256 | R |
| Piperacillin Tazobactam         | 512 | R | 512 | R |
| Ceftolozane Tazobactam          | > 2,048 | R | > 2,048 | R |

*Int: Interpretation; S: Susceptible; R: Resistant.
[10]. *Escherichia coli* 1176 (harbors *bla*<sub>NDM-1</sub> only) and *E. coli* 57 (harbors *bla*<sub>OXA-48</sub> only) were used as a control in the experiment [12].

**Whole Genome Sequencing (WGS)**

To prepare whole-genome sequencing libraries, the cryopreserved stocks were grown on MacConkey agar. Genomic DNA was extracted using standard methods (Qiagen, Valencia, CA), and NexteraXT libraries were prepared using the manufacturer’s protocols (Illumina, San Diego, CA) and sequenced on an Illumina HiSeq 4000, 2 × 150 bp.

**Bioinformatics analysis of the isolates**

Assembly of the genome was performed using Unicycler on Galaxy (https://usegalaxy.org/). Antimicrobial resistant genes were acquired through ResFinder on Center of Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/) and CARD (https://card.mcmaster.ca/). Plasmids harbored by our isolates were determined by using PlasmidFinder on CGE (https://cge.cbs.dtu.dk/services/PlasmidFinder/). Virulence factors were identified using VFDB (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi). Genetic differences between the 2 isolates was determined using DNAdiff on Galaxy (https://usegalaxy.org/). Finally, the circular genome was drawn and annotated using CGView server (http://cgview.ca/).

**Results**

**Screening results**

Two *K. pneumoniae* isolates were recovered from the patient. The cultures led to the isolation of a *K. pneumoniae* isolate from the blood (IMP 1078b). Moreover, the skin screening led to the isolation of the second *K. pneumoniae* isolate (IMP 1078s). The 2 isolates were identified using MALDI-TOF mass spectrometry and later confirmed by WGS.

**Antibiotics Susceptibility Testing**

The antibiotic susceptibility testing results done by both broth micro-dilution assay (Table 1) and Kirby-Bauer technique (Table 2) showed that the IMP 1078s is XDR since it was resistant to all the tested antibiotics except for colistin. However, the IMP 1078b is PDR since the isolate was resistant to all the tested antibiotics.

**WGS**

The MLST typing results revealed that both clinical isolates were assigned to be ST383. A 99% similarity

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Table 2. Antibiotic disk test results of both IMP 1078b and IMP 1078s against 24 different antibiotics.

| Antibiotic                  | IMP 1078b |   | IMP 1078s |   |
|-----------------------------|-----------|---|-----------|---|
|                             | Diameter (mm) | Int* | Diameter (mm) | Int* |
| Ampicillin                  | 6         | R  | 6         | R  |
| Cefoxitin                   | 6         | R  | 6         | R  |
| Cefuroxime                  | 6         | R  | 6         | R  |
| Cefazidime                  | 6         | R  | 6         | R  |
| Ceftriaxone                 | 6         | R  | 6         | R  |
| Cefotaxime                  | 6         | R  | 6         | R  |
| Cefepime                    | 6         | R  | 6         | R  |
| Ertapenem                   | 6         | R  | 6         | R  |
| Meropenem                   | 6         | R  | 6         | R  |
| Imipenem                    | 7         | R  | 8         | R  |
| Doripenem                   | 6         | R  | 6         | R  |
| Aztreonam                   | 6         | R  | 6         | R  |
| Gentamicin                  | 6         | R  | 6         | R  |
| Amikacin                    | 6         | R  | 6         | R  |
| Kanamycin                   | 6         | R  | 6         | R  |
| Levofoxacin                 | 6         | R  | 6         | R  |
| Erythromycin                | 6         | R  | 6         | R  |
| Azithromycin                | 6         | R  | 6         | R  |
| Chloramphenicol             | 6         | R  | 6         | R  |
| Fosfomycin                  | 11        | R  | 12        | R  |
| Tetracycline                | 6         | R  | 6         | R  |
| Rifampicin                  | 6         | R  | 6         | R  |
| Trimethoprim Sulfoxanthoxazole | 6     | R  | 6         | R  |
| Piperacillin Tazobactam     | 6         | R  | 6         | R  |

*Int: Interpretation; R: Resistant.
was found between the 2 isolates (Figure 1 and Supplementary Table 2).

Both isolates harbored 47 antimicrobial resistant genes. The genes encoding resistance for each antibiotic class were distributed as the following: tetracycline (1), trimethoprim (1), phenicols (1), bleomycin (1), elfamycin (1), sulfonamides (2), fosfomycin (2), macrolides (3), fluoroquinolones (4), aminoglycosides (8), β-lactams (9), in addition to 14 genes encoding for efflux pumps with 12 being multidrug (Table 3). Moreover, the same 3 plasmids (IncFIB, IncHI1B, and IncL/M) were harbored by each isolate.

A plethora of virulence genes were detected in both isolates, including: Type I and III fimbriae, serum resistance loci, anti-phagocytic genes, iron acquisition system (Salmochelin and Yersiniabactin), rcsAB gene, and acrAB efflux pump gene (Table 4). Furthermore, we hereby report the first type IV pilli (Yersinia) pilW and the fimbrial adherence determinant Stb (Salmonella) in K. pneumoniae.

Fitness cost results
To assess the fitness cost of harboring AMR genes on the clinical K. pneumoniae isolates, growth kinetics assays was performed. K. pneumoniae DSM 30104 was used as a control strain, as it is the Wild Type. The growth rates did not vary significantly for IMP 1078b (p = 0.9946) nor IMP 1078s (p = 0.1860) when compared to K. pneumoniae DSM (Figure 2). These observations were made when each of the three isolates were grown in unmodified LB broth. To assess the effect of meropenem and cefepime on the fitness cost of both isolates, we evaluated the effect of exposure of the bacteria to antibiotics on the fitness cost of the clinical isolates and the wild type strain. We used the breakpoints of the selected antibiotics, 4 μg/mL for meropenem and 16 μg/mL for cefepime, as the concentration to grow the bacteria. We witnessed that for IMP 1078b (Figure 3A) and IMP 1078s (Figure 3B), the growth rates did not change significantly when comparing the division of the bacteria incubated with meropenem to that of the bacteria grown in broth alone (p = 0.2510 and p = 0.7728 respectively). Furthermore, as seen in Figure 4A and Figure 4B, similar results could be observed for the growth rates of these isolates when incubated with or without Cefepime (p = 0.3107 and p = 0.8985 for IMP 1078b and IMP 1078s respectively).
Table 3. Antimicrobial resistant genes harbored by both IMP 1078b and IMP 1078s.

| Gene        | Resistance Phenotype                              | IMP 1078b | IMP 1078s | Comments                                      |
|-------------|---------------------------------------------------|-----------|-----------|-----------------------------------------------|
| oqxB5       | Quinolone                                         | +         | +         |                                               |
| qnrS1       | Fluoroquinoline                                   | +         | +         |                                               |
| parC        | Fluoroquinoline                                   | +         | +         |                                               |
| gyrA        | Fluoroquinoline                                   | +         | +         |                                               |
| aac(6’)-Ib  | Fluoroquinoline and aminoglycoside                | +         | +         | Salmonella enterica gyrA conferring resistance to fluoroquinolones |
| aadA1       | Aminoglycoside                                    | +         | +         |                                               |
| aph(3’)-Ib  | Aminoglycoside                                    | +         | +         |                                               |
| aph(3’)-Ia  | Aminoglycoside                                    | +         | +         |                                               |
| aph(3’)-VI  | Aminoglycoside                                    | +         | +         |                                               |
| aph(3’)-Vb  | Aminoglycoside                                    | +         | +         |                                               |
| aph(6)-Id   | Aminoglycoside                                    | +         | +         |                                               |
| armA        | Aminoglycoside                                    | +         | +         |                                               |
| fosA        | Fosfomycin                                        | +         | +         |                                               |
| tet(A)      | Tetracycline                                      | +         | +         |                                               |
| dfrA5       | Trimethoprim                                      | +         | +         |                                               |
| sulI        | Sulfonamide                                       | +         | +         |                                               |
| sul2        | Sulfonamide                                       | +         | +         |                                               |
| catA1       | Phenicol                                           | +         | +         |                                               |
| mph(A)      | Macrolide                                         | +         | +         |                                               |
| mph(E)      | Macrolide                                         | +         | +         |                                               |
| msr(E)      | Macrolide, Lincosamide and Streptogramin B        | +         | +         |                                               |
| blaCTX-48   | Beta-lactam                                        | +         | +         |                                               |
| blaCTX-M-15 | Beta-lactam                                        | +         | +         |                                               |
| blaNDM-5    | Beta-lactam                                        | +         | +         |                                               |
| blaOXA-48   | Beta-lactam                                        | +         | +         |                                               |
| blaOXA-9    | Beta-lactam                                        | +         | +         |                                               |
| blaTEM-1    | Beta-lactam                                        | +         | +         |                                               |
| blampH      | Beta-lactam                                        | +         | +         |                                               |
| PBP3        | Cephalosporin, cephaprin, carbapenem              | +         | +         | Escherichia coli ampH beta-lactamase          |
| Ble         | Bleomycin resistant protein against glycopeptide  antibiotic | +         | +         | Haemophilus influenzae PBP3 conferring resistance to beta-lactam antibiotics |
| UhpT        | Fosfomycin                                        | +         | +         | Escherichia coli UhpT with mutation conferring resistance to fosfomycin |
| EF-Tu       | Elafamycin antibiotic                              | +         | +         | Escherichia coli EF-Tu mutants conferring resistance to Pulvomycin |
| KpnE        | Macrolide, aminoglycoside, cephaprin, cephaprin,  | +         | +         | Klebsiella pneumoniae KpnE (MFS antibiotic efflux pump) |
| KpnF        | tetracycline, peptide, and rifamycin              | +         | +         | Klebsiella pneumoniae KpnF (MFS antibiotic efflux pump) |
| KpnG        | Macrolide, fluoroquinoline, aminoglycoside,      | +         | +         | Klebsiella pneumoniae KpnG (MFS antibiotic efflux pump) |
|             | carbapenem, cephaprin, penam, peptide, and penem |           |           |                                               |
| KpnH        | Macrolide, fluoroquinoline, aminoglycoside,      | +         | +         | Klebsiella pneumoniae KpnH (MFS antibiotic efflux pump) |
|             | carbapenem, cephaprin, penam, peptide, and penem |           |           |                                               |
| emrD        | Fluoroquinolone                                   | +         | +         | MFS antibiotic efflux pump                    |
| OmpK37      | Monobactam, carbapenem, cephaprin, cephaprin,     | +         | +         | Klebsiella pneumoniae OmpK37 (General Bacterial Porin with reduced permeability to beta-lactams) |
|             | cephaprin, penam, and penem                       |           |           | RND antibiotic efflux pump                    |
| baeR        | Aminoglycoside and aminocoumarin                  | +         | +         | RND antibiotic efflux pump                    |
| CRP         | Macrolide, fluoroquinoline antibiotic, and penam  | +         | +         | ABC antibiotic efflux pump                    |
| adeF        | Fluoroquinolone and tetracycline                  | +         | +         |                                               |
| msbA        | Nitroimidazole                                    | +         | +         | Escherichia coli marR mutant conferring antibiotic resistance (RND antibiotic efflux pump) |
| marR        | Fluoroquinolone, glycylcycline, penam, tetracycline, rifamycin, phenicol, and triclosan | +         | +         | MFS antibiotic efflux pump and RND antibiotic efflux pump |
| H-NS        | Macrolide, fluoroquinolone, cephaprin, cephaprin, | +         | +         | RND antibiotic efflux pump, and General Bacterial Porin with reduced permeability to beta-lactams) |
| marA        | cephaprin, penam, and tetracycline                | +         | +         |                                               |
| oqxA        | Fluoroquinolone, glycylcycline, tetracycline,     | +         | +         | RND antibiotic efflux pump                    |
|             | diaminopyrimidine, and nitrofurans                |           |           |                                               |
| VFactor | Virulence factors            | Related genes | IMP 1078b | IMP 1078s |
|---------|-----------------------------|---------------|-----------|-----------|
| Adherence | Type 3 fimbriae | MrkA | orf01624 | orf01742 |
|         |                 | MrkB | orf01625 | orf01741 |
|         |                 | MrkC | orf01626 | orf01740 |
|         |                 | MrkD | orf01627 | orf01739 |
|         |                 | MrkF | orf01628 | orf01738 |
|         |                 | MrkH | orf01631 | orf01735 |
|         |                 | MrkI | orf01630 | orf01736 |
|         |                 | MrkJ | orf01629 | orf01737 |
|         | Type I fimbriae | FimA | orf01617 | orf01749 |
|         |                 | FimB | orf01619 | orf01747 |
|         |                 | FimC | orf01615 | orf01751 |
|         |                 | FimD | orf01614; orf01629 | orf01752; orf04626 |
|         |                 | FimE | orf01618 | orf01748 |
|         |                 | FimF | orf01613 | orf01753 |
|         |                 | FimG | orf01612 | orf01754 |
|         |                 | FimH | orf01611 | orf01755 |
|         |                 | FimL | orf01616 | orf01750 |
|         |                 | FimK | orf01610 | orf01756 |
|         | Type IV pili(Yersinia) | PilW | orf00174 | orf00260 |
|         | Antiphagocytosis | Capsule | orf01201; orf01202; orf01203; orf01204; orf01205; orf01206; orf01207; orf01208; orf01209; orf01210; orf01211; orf01212; orf01213; orf01214; orf01215; orf01216; orf01217; orf01218; orf04070 | orf00893; orf00894; orf00895; orf00896; orf00897; orf00898; orf00900; orf00901; orf00902; orf00903; orf00904; orf00905; orf00906; orf00907; orf00908; orf00909; orf00910; orf04187 |
|         | Efflux pump | AcrAB | orf00340 | orf00095 |
|         |                 | AcrB | orf00341; orf02038 | orf00094; orf02035 |
|         | Iron acquisition | Aerobactin | orf04473 | orf04505 |
|         |                 | IucA | orf04474 | orf04504 |
|         |                 | IucB | orf04475 | orf04503 |
|         |                 | IucC | orf04476 | orf04502 |
|         |                 | IucD | orf04445; orf04477 | orf02907; orf04501 |
|         |                 | EntA | orf00156 | orf04505 |
|         |                 | EntB | orf00157 | orf04504 |
|         |                 | EntC | orf00159 | orf04503 |
|         |                 | EntD | orf00169 | orf00275 |
|         |                 | EntE | orf00158 | orf00276 |
|         |                 | EntF | orf00165 | orf00269 |
|         |                 | EntS | orf00161 | orf00273 |
|         |                 | FepA | orf00168; orf01499 | orf00266; orf01498 |
|         |                 | FepB | orf00160 | orf00274 |
|         |                 | FepC | orf00164 | orf00270 |
|         |                 | FepD | orf00162 | orf00272 |
|         |                 | FepG | orf00163 | orf00271 |
|         |                 | Feb | orf00167 | orf00267 |
|         | Salmochelin | IroE | orf02963 | orf03244 |
|         |                 | IroN | orf03392 | orf03626 |
|         |                 | YbtU | orf02612 | orf02806 |
|         | Yersiniabactin | YbtU | orf02612 | orf02806 |
|         | Regulation | RcsA | orf03201 | orf02189 |
|         |                 | RcsB | orf03201 | orf02189 |
|         | Secretion system | T6SS-I | orf01085 | orf01026 |
|         |                 | ClpV | orf04519 | orf04568 |
|         |                 | DotU | orf04522 | orf04565 |
|         |                 | Hcp | orf04520 | orf04567 |
|         |                 | IcmF | orf02028 | orf02402 |
|         |                 | ImpA | orf02027 | orf02401 |
|         |                 | ScnA | orf02203 | orf02397 |
|         |                 | TssF | orf02205 | orf02399 |
|         |                 | TssG | orf02204 | orf02398 |
|         |                 | VasE | orf04523 | orf04564 |
|         |                 | VgrG | orf04518 | orf04569 |
|         |                 | VipA | orf04525 | orf04562 |
|         |                 | VipB | orf04524 | orf04563 |
| T6SS-II |                 | ClpV | orf04765 | orf04763 |
Additionally, the growth rate was slightly improved for both isolates when incubated with either antibiotics, when matched with its unchanged control. Moreover, the efficiency of both antibiotics was supported by visualizing the growth rates of *K. pneumoniae* DSM grown in intact broth or media containing either meropenem or cefepime. As noticed in Figures 3C and 4C, the growth rates of *K. pneumoniae* DSM decreased significantly when incubated with either meropenem or cefepime respectively (*p* < 0.0001).

**Inhibitors**

There are multiple mechanisms for resistance in CRE. Our aim is to understand the mechanisms by which our isolates escape the action of carbapenems. Both *K. pneumoniae* isolates expressed carbapenemases: Class B Metallo-β-lactamases (*bla*<sub>NDM-5</sub>) and class D β-lactamases (*bla*<sub>OXA-48</sub> and *bla*<sub>OXA-9</sub>). In order to show which enzyme plays the major role in carbapenem resistance in these isolates, each class of carbapenemase was inhibited and the effect on meropenem MICs was recorded. Calcium-EDTA inhibits class B Metallo-β-lactamases by chelating their zinc ions, while avibactam obstructs the action of class D β-lactamases via acylation of their serine. As seen in Table 5, the MIC of Meropenem for both isolates was 256 μg/mL. However, the combination of Meropenem with EDTA significantly dropped the MIC to 64 μg/mL for IMP 1078b and to 32 μg/mL for IMP 1078s. Interestingly, when adding

**Table 4 (continued).** Virulence genes harbored by both IMP 1078b and IMP 1078s.

| VFclass       | Virulence factors | Related genes                  | IMP 1078b | IMP 1078s |
|---------------|------------------|--------------------------------|-----------|-----------|
| Secretion system | T6SS-III       | orf00452                        | orf00452  |           |
|                | dotU             | orf04175                        | orf02039  |           |
|                | icmF             | orf00446                        | orf00446  |           |
|                | impA             | orf00451                        | orf00451  |           |
|                | impF             | orf00450                        | orf00450  |           |
|                | impG             | orf00447                        | orf00447  |           |
|                | impH             | orf00448                        | orf00448  |           |
|                | impJ             | orf04176                        | orf02040  |           |
|                | ompA             | orf04174                        | orf02038  |           |
|                | sciN             | orf00449                        | orf00449  |           |
|                | vgrG             | orf04173                        | orf02037  |           |
| Serum resistance | LPS rfb locus   | orf01221; orf01222; orf01223;   |           |           |
|                |                  | orf01224; orf01225              |           |           |
| Fimbrial adherence | Stb(Salmonella) | stbA                            | orf04623  | orf04620  |
|                |                  | stbB                            | orf04622  | orf04619  |
|                |                  | stbC                            | orf04621  | orf04618  |
|                |                  | stbD                            | orf04620  | orf04617  |

Figure 3. Fitness cost of *K. pneumonia* DSM, IMP 1078b and IMP 1078s against meropenem.

Figure 4. Fitness cost of *K. pneumonia* DSM, IMP 1078b and IMP 1078s against cefepime.
Table 5. Minimal inhibitory concentration variation of IMP 1078b and IMP 1078s after the addition of β-lactamase inhibitors.

| MIC (µg/mL)                  | Meropenem | Meropenem + Ca-EDTA | Meropenem + Avibactam | Meropenem + Ca-EDTA + Avibactam |
|------------------------------|-----------|----------------------|-----------------------|--------------------------------|
| IMP 1078b                    | 256       | 64                   | 256                   | 4                               |
| IMP 1078s                    | 256       | 32                   | 256                   | 16                              |
| E. coli 1176                 | 64        | < 1                  | NA                    | NA                             |
| E. coli 57                   | 32        | NA                   | < 1                   | NA                             |
expression of resistance genes showed no significant changes with or without antibiotics. While trying to determine the mechanisms of carbapenem resistance of these *K. pneumoniae* isolates, we used an inhibitor-based approach. We discovered that the main enzyme used by these isolates to hydrolyze carbapenems is the class B metallo-β-lactamase *bla*<sub>NDM-5</sub>. Once inhibited by Ca-EDTA, the bacteria utilize class D carbapenemases to disable the action of carbapenems. However, the use of a combination of inhibitors (Ca-EDTA + Avibactam) showed that even when both types of carbapenemases are inhibited, both isolates remain resistant to meropenem. This persistence of resistance could be attributed to 2 reasons. First, both isolates possess a variety of MDR efflux pumps capable of ejecting carbapenems to the outside of the cell. Second, the inability of avibactam to block the action of *bla*<sub>OXA-9</sub>-<sub>48</sub>. Although the action of avibactam on *bla*<sub>OXA-48</sub> has been repeatedly proven [30,31], no study has directly linked avibactam to an inhibitory activity on *bla*<sub>OXA-9</sub>. This hypothesis is also supported by the variation of effect of avibactam on Class D carbapenemases [32].

The current report focused on the phenotypic and molecular characterization of two clinical *Klebsiella pneumoniae* isolates recovered from a patient at a tertiary care Lebanese hospital. Both isolates demonstrated resistance to a wide range of antibiotics. This resistance is encoded by an overabundance of AMR genes. Additionally, the presence of the H-NS factor capable of reducing the burden imposed by the plasmid acquisition and facilitating its conjugatable transfer increases the risk of nosocomial outbreaks related to these isolates. Moreover, the co-existence of a high number of AMR genes and virulence factors may lead to a life-threatening invasive *K. pneumoniae* infection. Despite infection with highly resistant organisms, our patient recovered and did not succumb to the bloodstream infection. In fact, *in-vitro* observations do not correlate always with the real life experience and the most resistant organism may not always be the most virulent.

In addition, the initial bacterial screening revealed *K. pneumoniae* strains that we believe evolved under antibiotic pressure and multiple courses of antibiotics, and acquired resistance through different mechanisms.

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Authors’ contributions
Drs. Antoine Abou Fayad and Ghassan Matar designed the study. The clinical case was handled by Drs. Nesrine Rizk, Soha Kanj, and Michele Mocadie. Experiments were performed by Ahmad Sleiman, Bassel Awada, and Nour Sherri. The manuscript was written by Ahmad Sleiman, Drs. Antoine Abou Fayad and Louis-Patrick Harauoi.

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### Annex – Supplementary Items

**Supplementary Table 1. DNA difference between IMP 1078b and IMP 1078s.**

| Feature Estimates | IMP 1078b | IMP 1078s |
|-------------------|-----------|-----------|
| **Sequences**      |           |           |
| TotalSeqs         | 140       | 135       |
| AlignedSeqs       | 140 (100.00%) | 135 (100.00%) |
| UnalignedSeqs     | 0 (0.00%)   | 0 (0.00%)   |
| TotalBases        | 5722168    | 5721916    |
| **Bases**          |           |           |
| AlignedBases      | 5721714 (99.99%) | 5721908 (100.00%) |
| UnalignedBases    | 454 (0.01%) | 8 (0.00%)   |
| 1-to-1            | 143       | 143       |
| **Alignments**     |           |           |
| TotalLength       | 5740303    | 5740268    |
| AvgLength         | 39049.68   | 39049.44   |
| AvgIdentity       | 100        | 100        |
| Breakpoints       | 15         | 31         |
| Relocations       | 1          | 1          |
| Translocations    | 1          | 6          |
| Inversions        | 7          | 0          |
| Insertions        | 7          | 10         |
| **Feature Estimates** |       |           |
| InsertionSum      | 767        | 471        |
| InsertionAvg      | 109.57     | 47.1       |
| TandemIns         | 0          | 1          |
| TandemInsSum      | 0          | 16         |
| TandemInsAvg      | 0          | 16         |
| **TotalSNPs**     | 31         | 31         |
| AT                | 3 (9.68%)  | 4 (12.90%) |
| AC                | 0 (0.00%)  | 4 (12.90%) |
| AG                | 5 (16.13%) | 5 (16.13%) |
| TC                | 2 (6.45%)  | 4 (12.90%) |
| TG                | 1 (3.23%)  | 1 (3.23%)  |
| TA                | 4 (12.90%) | 3 (9.68%)  |
| GC                | 2 (6.45%)  | 0 (0.00%)  |
| GA                | 5 (16.13%) | 5 (16.13%) |
| GT                | 1 (3.23%)  | 1 (3.23%)  |
| CG                | 0 (0.00%)  | 2 (6.45%)  |
| CT                | 4 (12.90%) | 2 (6.45%)  |
| CA                | 4 (12.90%) | 0 (0.00%)  |
| **TotalGSNPs**    | 19         | 19         |
| TG                | 1 (5.26%)  | 1 (5.26%)  |
| TC                | 1 (5.26%)  | 2 (10.53%) |
| TA                | 2 (10.53%) | 1 (5.26%)  |
| AT                | 1 (5.26%)  | 2 (10.53%) |
| AG                | 5 (26.32%) | 5 (26.32%) |
| AC                | 0 (0.00%)  | 1 (5.26%)  |
| GC                | 0 (0.00%)  | 0 (0.00%)  |
| GT                | 1 (5.26%)  | 1 (5.26%)  |
| GA                | 5 (26.32%) | 5 (26.32%) |
| CG                | 0 (0.00%)  | 0 (0.00%)  |
| CA                | 1 (5.26%)  | 0 (0.00%)  |
| CT                | 2 (10.53%) | 1 (5.26%)  |
| **SNPs**          |            |            |
| TotalIndels       | 1          | 1          |
| A                 | 0 (0.00%)  | 0 (0.00%)  |
| T                 | 0 (0.00%)  | 0 (0.00%)  |
| G                 | 1 (100.00%)| 0 (0.00%)  |
| C                 | 0 (0.00%)  | 0 (0.00%)  |
| .C                | 0 (0.00%)  | 0 (0.00%)  |
| .G                | 0 (0.00%)  | 1 (100.00%)|
| .A                | 0 (0.00%)  | 0 (0.00%)  |
| .T                | 0 (0.00%)  | 0 (0.00%)  |
| **TotalGIndels**  | 0          | 0          |
| T                 | 0 (0.00%)  | 0 (0.00%)  |
| A                 | 0 (0.00%)  | 0 (0.00%)  |
| G                 | 0 (0.00%)  | 0 (0.00%)  |
| C                 | 0 (0.00%)  | 0 (0.00%)  |
| .G                | 0 (0.00%)  | 0 (0.00%)  |
| .C                | 0 (0.00%)  | 0 (0.00%)  |
| .A                | 0 (0.00%)  | 0 (0.00%)  |
| .T                | 0 (0.00%)  | 0 (0.00%)  |
### Supplementary Table 2. Timeline of infection.

| Date | Organism          | Skin screening | Urine culture | Blood culture | Miscellaneous | MIC vs: |
|------|-------------------|----------------|---------------|--------------|---------------|---------|
| 26/3 | Klebsiella pneumonia | CRE | Klebsiella pneumonia | CRE | negative |
|      | Pure mod. growth *CRE | Tigecycline: S; Colistin: I; Fosfomycin: I | | | | |
|      | Tigecycline: S; Colistin: I; Fosfomycin: I | | Negative | | Catheter Tip: Klebsiella pneumoniae >15 colonies | Cre same as above |
| 27/3 | | | | | | Ertapenem: 8 ug/ml |
|      | | | | | | Imipenem: 0.5 ug/ml |
| 28/3 | Escherichia coli - Heavy growth | | | | | Meropenem: 4 ug/ml |
|      | Klebsiella pneumonia - Heavy growth | | | From Central line: Klebsiella pneumonia 2:2 after 16 hrs *CRE | | |
|      | 1-2-Tigecycline: S | | Negative | One set, negative | | Ertapenem: >32 ug/ml |
|      | 2-Colistin: I; Fosfomycin: R | | | | Meropenem: >32 ug/ml |
|      | Escherichia coli - Heavy growth | | | | Imipenem: >32 ug/ml |
| 30/3 | Klebsiella pneumonia - Heavy growth | | | | From Central line: Klebsiella pneumonia 2:2 after 16 hrs *CRE | |
|      | Heavy growth*CRE | 1-2 Tigecycline: S | Negative | | | Ertapenem: >32 ug/ml |
|      | 2-Colistin: I; Fosfomycin: R | | | | Meropenem: >32 ug/ml |
|      | Klebsiella CRE Tigecycline: S; Fosfomycin: I | | | | Imipenem: >32 ug/ml |
| 01/4 | Klebsiella pneumonia - Heavy growth | | | Blood ex: 2 sets: negative | Catheter tip: negative | |
|      | Heavy growth*CRE | 1-2 Tigecycline: S | Negative | | | |
|      | 2-Colistin: I; Fosfomycin: R | | | | | |
| 2/4  | Klebsiella CRE Tigecycline: S; Fosfomycin: I | | | Blood Cxs: 2 sets: negative | | |
| 3/4  | Klebsiella pneumonia-Heavy growth | | | | Blood Cxs: 2 sets: negative | |
| 5/4  | Escherichia coli -Heavy growth | | | | | |
| 7/4  | Klebsiella pneumonia-Heavy growth | | | | | |
|      | ESBL | 1-2-Tigecycline: S | Negative | | | Ertapenem: >32 ug/ml |
|      | 1-Colistin: I; Fosfomycin: R | | | | Meropenem: >32 ug/ml |
|      | 2-Cefepime: S-DD | | | | Imipenem: >32 ug/ml |
| 8/4  | Klebsiella pneumonia-Moderate growth | | | | | |
|      | CRE Candida species not albicans | 1-Tigecycline: S; Fosfomycin: R; Colistin: I | Negative | One set negative | | |
| 9/4  | Klebsiella pneumonia CRE-Tigecycline: S; Fosfomycin: R | | | | | |
| 12/4 | Klebsiella pneumonia-Heavy growth | | | | | Ertapenem: >32 ug/ml |
|      | CRE Tigecycline: S; Fosfomycin: R | Negative | | | | Meropenem: >32 ug/ml |
|      | 1-Colistin: I; Fosfomycin: R | | | | Imipenem: >32 ug/ml |
| 15/4 | Klebsiella pneumonia-Heavy growth | | | | | |
| 17/4 | Proteus mirabilis -Heavy growth | | | | | |
| 23/4 | Candida species not albicans | | | | One set negative | |
| 29/4 | Proteus mirabilis-Heavy growth | | | | |
**Supplementary Figure 1.** Timeline of infection.

| Day of Admission | Culture from central line grew *K. pneumoniae* CRE | Persistently febrile throughout the day | Fever trend improving with defervescence on April 26 |
|------------------|-----------------------------------------------|----------------------------------------|---------------------------------------------------|
| 26-Mar | Intubated, ventilated, GCS:9 Afibrile On Dexamethasone | | |

**Timeline of events**

- **Vancomycin**
- **Tigocycline**
- **Colistin IH**
- **Amikacin IV**
- **Imipenem EI**
- **Rifampin**
- **Ceftazidime-avibactam**

27-March: High grade fever Central line removed
28-March: Dexamethasone stopped
05-May: Transferred to the floor, on Nasal Cannula, afebrile
Supplementary Figure 2. qRT-PCR results of IMP 1078b and IMP 1078s against \textit{blaNDM-5}, \textit{blaOXA-48}, and \textit{blaOXA-9}. 

A) IMP 1078s and NDM-5

B) IMP 1078b and NDM-5

C) IMP 1078s and OXA-48

D) IMP 1078b and OXA-48

E) IMP 1078s and OXA-9

F) IMP 1078b and OXA-9
Supplementary Figure 3. qRT-PCR results of IMP 1078b and IMP 1078s against bla_{CTX-M-14b} and bla_{CTX-M-15}.
Supplementary Figure 4. qRT-PCR results of IMP 1078b and IMP 1078s against bla<sub>SHV</sub> and bla<sub>TEM</sub>. 