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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 pneumonia*e 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 (CLABSI). 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 5×10^6 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

Table 1. Broth micro-dilution results of both IMP 1078b and IMP 1078s against 19 different antibiotics.

Antibiotics	IMP 1078b		IMP 1078s	
	MIC (µg/mL)	Int*	MIC (µg/mL)	Int*
Cefuroxime	> 2,048	R	> 2,048	R
Ceftazidime	> 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_{NDM-1} only) and *E. coli* 57 (harbors bla_{OXA-48} 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

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
Ceftazidime	6	R	6	R
Ceftriaxone	6	R	6	R
Ceftizoxime	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
Levofloxacin	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 Sulfamethoxazole	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.

Figure 1. Circular genome representation of IMP 1078b (A) and IMP 1078s (B) (IMP1078b accession number is SAMN14404320 and IMP1078s accession number is SAMN14404321).

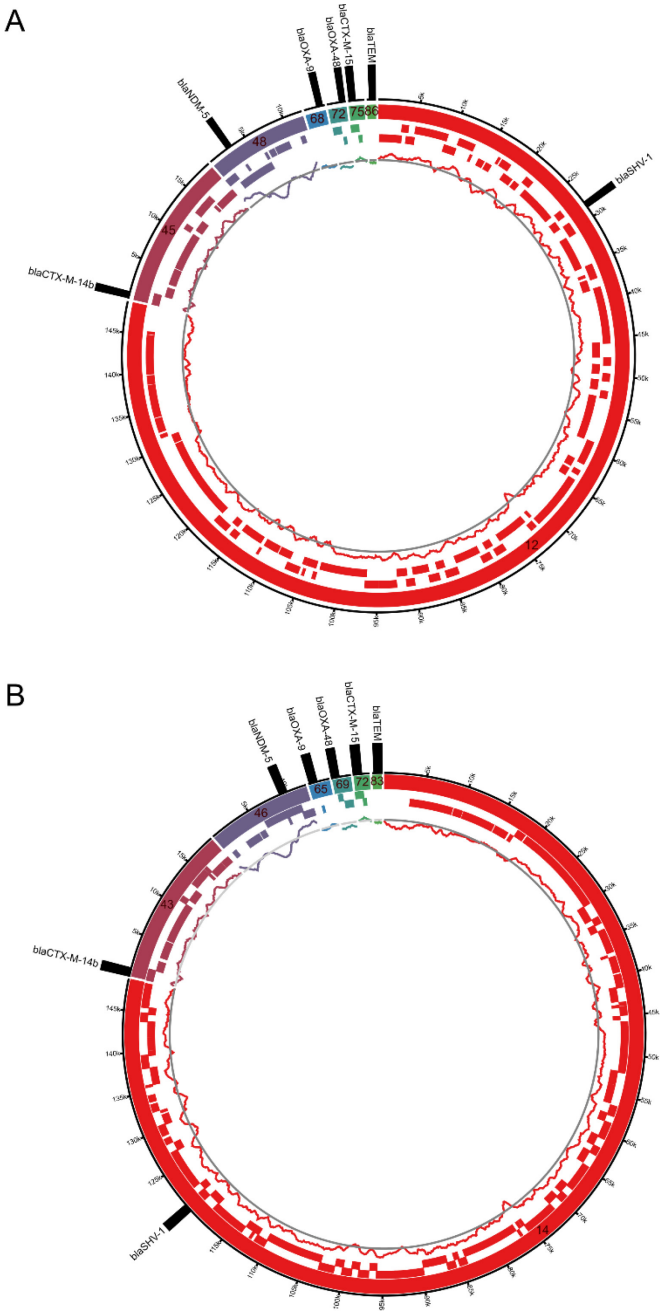
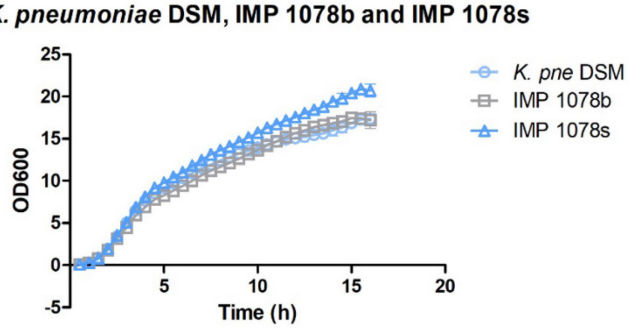


Figure 2. Fitness cost of IMP 1078b and IMP 1078s compared to *K. pneumoniae* DSM 30104.



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	Fluoroquinolone	+	+	
parC	Fluoroquinolone	+	+	*Escherichia coli* parC conferring resistance to fluoroquinolone
gyrA	Fluoroquinolone	+	+	*Salmonella enterica* gyrA conferring resistance to fluoroquinolones
aac(6')-Ib	Fluoroquinolone and aminoglycoside	+	+	
aadA1	Aminoglycoside	+	+	
aph(3'')-Ib	Aminoglycoside	+	+	
aph(3')-Ia	Aminoglycoside	+	+	
aph(3')-VI	Aminoglycoside	+	+	
aph(3')-VIb	Aminoglycoside	+	+	
aph(6)-Id	Aminoglycoside	+	+	
armA	Aminoglycoside	+	+	
fosA	Fosfomycin	+	+	
tet(A)	Tetracycline	+	+	
dfrA5	Trimethoprim	+	+	
sul1	Sulfonamide	+	+	
sul2	Sulfonamide	+	+	
catA1	Phenicol	+	+	
mph(A)	Macrolide	+	+	
mph(E)	Macrolide	+	+	
msr(E)	Macrolide, Lincosamide and Streptogramin B	+	+	
$bla_{\text{CTX-M-14b}}$	Beta-lactam	+	+	
$bla_{\text{CTX-M-15}}$	Beta-lactam	+	+	
$bla_{\text{NDM-5}}$	Beta-lactam	+	+	
$bla_{\text{OXA-48}}$	Beta-lactam	+	+	
$bla_{\text{OXA-9}}$	Beta-lactam	+	+	
$bla_{\text{SHV-1}}$	Beta-lactam	+	+	
bla_{TEM}	Beta-lactam	+	+	
ampH	Beta-lactam	+	+	*Escherichia coli ampH* beta-lactamase
PBP3	Cephalosporin, cephamycin, carbapenem	+	+	*Haemophilus influenzae PBP3* conferring resistance to beta-lactam antibiotics
Ble	Bleomycin resistant protein against glycopeptide antibiotic	+	+	*BRP*(MBL)
UhpT	Fosfomycin	+	+	*Escherichia coli UhpT* with mutation conferring resistance to fosfomycin
EF-Tu	Elfamycin antibiotic	+	+	*Escherichia coli EF-Tu* mutants conferring resistance to Pulvomycin
KpnE	Macrolide, aminoglycoside, cephalosporin, tetracycline, peptide, and rifamycin	+	+	*Klebsiella pneumoniae KpnE* (MFS antibiotic efflux pump)
KpnF	Macrolide, aminoglycoside, cephalosporin, tetracycline, peptide, and rifamycin	+	+	*Klebsiella pneumoniae KpnF* (MFS antibiotic efflux pump)
KpnG	Macrolide, fluoroquinolone, aminoglycoside, carbapenem, cephalosporin, penam, peptide, and penem	+	+	*Klebsiella pneumoniae KpnG* (MFS antibiotic efflux pump)
KpnH	Macrolide, fluoroquinolone, aminoglycoside, carbapenem, cephalosporin, penam, peptide, and penem	+	+	*Klebsiella pneumoniae KpnH* (MFS antibiotic efflux pump)
emrD	Fluoroquinolone	+	+	MFS antibiotic efflux pump
OmpK37	Monobactam, carbapenem, cephalosporin, cephamycin, penam, and penem	+	+	*Klebsiella pneumoniae OmpK37* (General Bacterial Porin with reduced permeability to beta-lactams)
baeR	Aminoglycoside and aminocoumarin	+	+	RND antibiotic efflux pump
CRP	Macrolide, fluoroquinolone antibiotic, and penam	+	+	RND antibiotic efflux pump
adeF	Fluoroquinolone and tetracycline	+	+	RND antibiotic efflux pump
msbA	Nitroimidazole	+	+	ABC antibiotic efflux pump
marR	Fluoroquinolone, cephalosporin, glycylcycline, penam, tetracycline, rifamycin, phenicol, and triclosan	+	+	*Escherichia coli marR* mutant conferring antibiotic resistance (RND antibiotic efflux pump)
H-NS	Macrolide, fluoroquinolone, cephalosporin, cephamycin, penam, and tetracycline	+	+	MFS antibiotic efflux pump and RND antibiotic efflux pump
marA	Fluoroquinolone, monobactam, carbapenem, cephalosporin, glycylcycline, cephamycin, penam, tetracycline, and rifamycin	+	+	RND antibiotic efflux pump, and General Bacterial Porin with reduced
oqxA	Fluoroquinolone, glycylcycline, tetracycline, diaminopyrimidine, and nitrofuran	+	+	RND antibiotic efflux pump

Table 4. Virulence genes harbored by both IMP 1078b and IMP 1078s.

VFclass	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; orf04629	orf01752; orf04626
		fimE	orf01618	orf01748
		fimF	orf01613	orf01753
		fimG	orf01612	orf01754
		fimH	orf01611	orf01755
		fimI	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; orf01214; orf01215; orf01216; orf01217; orf01218; orf04070	orf00893; orf00894; orf00895; orf00896; orf00897; orf00899; orf00900; orf00901; orf00902; orf00903; orf00904; orf00905; orf00906; orf00907; orf00908; orf00909; orf00910; orf04187
Efflux pump	AcrAB	acrA	orf00340	orf00095
		acrB	orf00341; orf02038	orf00094; orf02035
Iron acquision	Aerobactin	iucA	orf04473	orf04505
		iucB	orf04474	orf04504
		iucC	orf04475	orf04503
		iucD	orf04476	orf04502
		iutA	orf04445; orf04477	orf02907; orf04501
	Ent siderophore	entA	orf00156	orf00278
		entB	orf00157	orf00277
		entC	orf00159	orf00275
		entD	orf00169	orf00265
		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
		fes	orf00167	orf00267
	Salmochelin	iroE	orf02963	orf03244
		iroN	orf03392	orf03626
	Yersiniabactin	ybtU	orf02612	orf02806
Regulation	RcsAB	rcsA	orf03201	orf02189
		rcsB	orf01085	orf01026
Secretion system	T6SS-I	clpV/tssH	orf04519	orf04568
		dotU/tssL	orf04522	orf04565
		hcp/tssD	orf04520	orf04567
		icmF/tssM	orf02208	orf02402
		impA/tssA	orf02207	orf02401
		ompA	orf04521	orf04566
		sciN/tssJ	orf02203	orf02397
		tssF	orf02205	orf02399
		tssG	orf02204	orf02398
		vasE/tssK	orf04523	orf04564
		vgrG/tssI	orf04518	orf04569
		vipA/tssB	orf04525	orf04562
		vipB/tssC	orf04524	orf04563
	T6SS-II	clpV	orf04765	orf04763

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	orf00886; orf00887; orf00888; orf00889; orf00890
Fimbrial adherence determinants	Stb(Salmonella)	stbA	orf04623	orf04620
		stbB	orf04622	orf04619
		stbC	orf04621	orf04618
		stbD	orf04620	orf04617

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 30104 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_{NDM-5}) and class D β-lactamases (bla_{OXA-48} and bla_{OXA-9}). 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

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

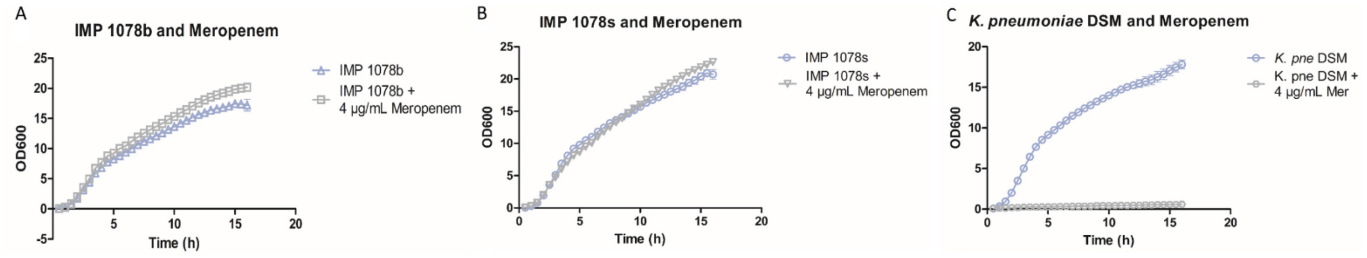
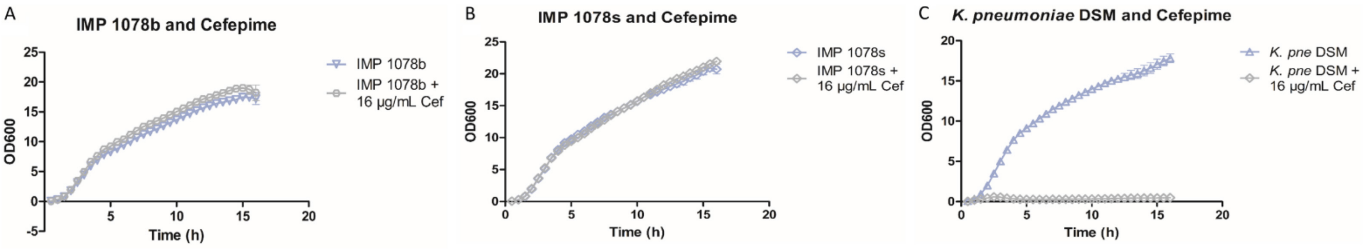


Figure 4. Fitness cost of *K. pneumonia* DSM, IMP 1078b and IMP 1078s against cefepime.



avibactam alone, the MIC of meropenem remained constant (256 μg/mL) for both isolates. On the other hand, the MIC of meropenem declined to 4 μg/mL and 16 μg/mL for IMP 1078 b and IMP 1078s respectively when combining both Ca-EDTA and avibactam. Taken together, these data indicate that $bla_{\text{NDM-5}}$ represents the main enzyme that these isolates use to hydrolyze carbapenems.

E. coli 1176 and *E. coli* 57 are clinical isolates that express solely one carbapenemase: $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ respectively. These bacteria were used as control strains to check the efficiency of EDTA and Avibactam in combination with Meropenem.

Discussion

We report the highest number of AMR genes ever detected in a *K. pneumoniae* isolate. These 47 AMR genes encode for resistance to all the antimicrobial agents used in clinical setting, except for colistin. Colistin resistance in IMP 1078b may be the reason behind the 1% difference between the genomes of both isolates. Colistin resistance is occasionally caused by the acquisition of the *mcr gene* [13], which is not the case in our isolate. Other ways that *K. pneumoniae* can acquire resistance to colistin are: (i) the *lpxM* gene that leads to the formation of hexa-acylated lipid A by encoding an enzyme that is involved in the addition of the myristoyl group to lipid A [14], and (ii) LPS modification due to the inactivation of *mgrB*, upregulation of the PhoP/PhoQ signalling system, activation of the PmrA-regulated *pmrHFIJKLM* operon, and the presence of ArnB [15]. Moreover, several studies were done at the country level that led to the detection of several β-lactamase genes in *K. pneumoniae*, such as: $bla_{\text{CTX-M-15}}$ [16-18], $bla_{\text{TEM-1}}$ [17,18], $bla_{\text{SHV-28}}$ [17], $bla_{\text{OXA-1}}$ [16-18], $bla_{\text{OXA-48}}$ [16,18,19], $bla_{\text{NDM-1}}$ [16-18], and $bla_{\text{NDM-7}}$ [20]. However, we hereby report the first *K. pneumoniae* isolate harboring $bla_{\text{NDM-5}}$, $bla_{\text{OXA-48}}$, and $bla_{\text{OXA-9}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14b}}$, 7 different bla_{SHV} genes, and 2 bla_{TEM} genes at the same time. Seventy-two virulence genes were detected in each isolate encoding for 15 different virulence factors. We are the first to report type IV pilli (*Yersinia)* and the fimbrial adherence determinant Stb (*Salmonella*) in *K. pneumoniae*. StbA, stbB, stbC, and stbD are genes encoding for the fimbrial adherence determinant Stb (*Salmonella*) present in our 2 isolates. Stb are a type 1 fimbriae and are, in addition to 7 other clusters, one of the most abundant fimbrial clusters in the genome of *Salmonella* spp. [21]. They function by binding to the intestinal epithelial cells or by participating in the colonization of avian or mammalian intestines [22]. Furthermore, type IV pilli (*Yersinia)* are multifunctional surface structures that function in: biofilm formation, adhesion to host cells and other surfaces, cellular invasion, formation of bacterial aggregates or microcolonies, DNA and phage uptake, electron transfer, and twitching or gliding motility [23]. The cost of harboring AMR genes is believed to reduce bacterial fitness, especially in the absence of antibiotic pressure [24,25]. However, multiple studies have already refuted this hypothesis for Enterobacterales that acquired either carbapenemases [26] or extended-spectrum β-lactamases [27]. Our hypervirulent isolates which garnered an incredible sum of resistance genes further support this evidence. Even in the absence of antibiotics, both IMP 1078b and IMP 1078s did not show a significant decrease in growth rates. However, both isolates demonstrated a slightly enhanced fitness in the presence of antibiotics used. A reason behind the lack of a burden on the growth in these isolates might be from the resistance-conferring plasmids themselves. IncHI family of plasmids such as IncHI1B which is present in both our isolates, can carry a gene called histone-like nucleoid-structuring protein or H-NS [28]. This pleotropic regulator has been shown to regulate a multitude of pathogenicity factors in *K. pneumoniae*, as well as reducing the fitness cost of plasmid acquisition [29]. The latter effect might be a consequence of the abilities of H-NS: it allows the entry of plasmids to the host with a minimal change on global gene expression patterns, then integrates other plasmidic genes into the established gene expression regulation networks [28]. Thus, H-NS might be allowing plasmid-encoded resistance genes to be expressed constitutively without being affected by antibiotic exposure. This assumption is based on our RT-PCR data (Supplementary Figures 2-4), where the

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 $bla_{\text{NDM-5}}$. 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 $bla_{\text{OXA-9}}$. Although the action of avibactam on $bla_{\text{OXA-48}}$ has been repeatedly proven [30,31], no study has directly linked avibactam to an inhibitory activity on $bla_{\text{OXA-9}}$. 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 conjugable 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 Haraoui.

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Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

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

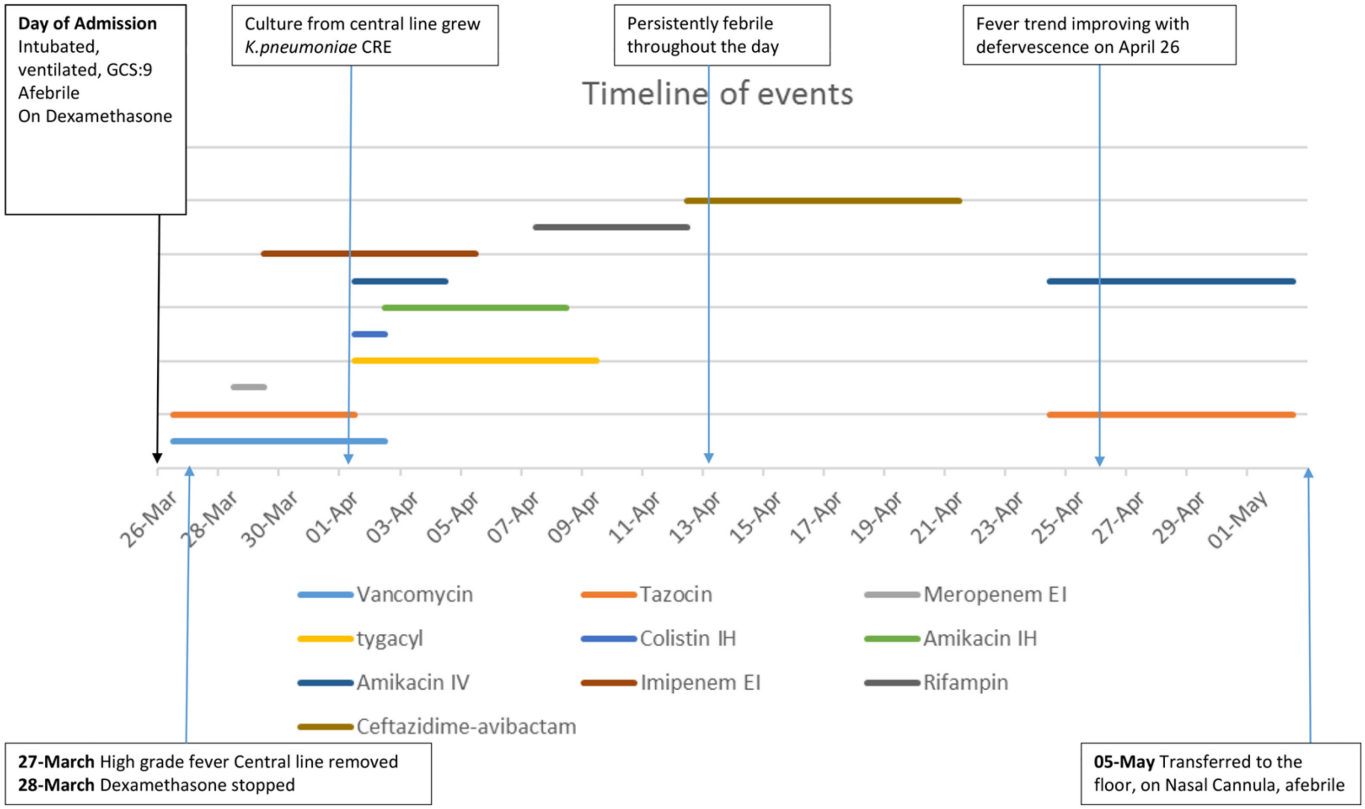
		IMP 1078b	IMP 1078s
Sequences	TotalSeqs	140	135
	AlignedSeqs	140 (100.00%)	135 (100.00%)
	UnalignedSeqs	0 (0.00%)	0 (0.00%)
Bases	TotalBases	5722168	5721916
	AlignedBases	5721714 (99.99%)	5721908 (100.00%)
	UnalignedBases	454 (0.01%)	8 (0.00%)
Alignments	1-to-1	143	143
	TotalLength	5739542	5739509
	AvgLength	40136.66	40136.43
	AvgIdentity	100	100
	M-to-M	147	147
	TotalLength	5740303	5740268
	AvgLength	39049.68	39049.44
	AvgIdentity	100	100
Feature Estimates	Breakpoints	15	31
	Relocations	1	1
	Translocations	1	6
	Inversions	0	0
	Insertions	7	10
	InsertionSum	767	471
	InsertionAvg	109.57	47.1
	TandemIns	0	1
	TandemInsSum	0	16
	TandemInsAvg	0	16
SNPs	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%)
	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.

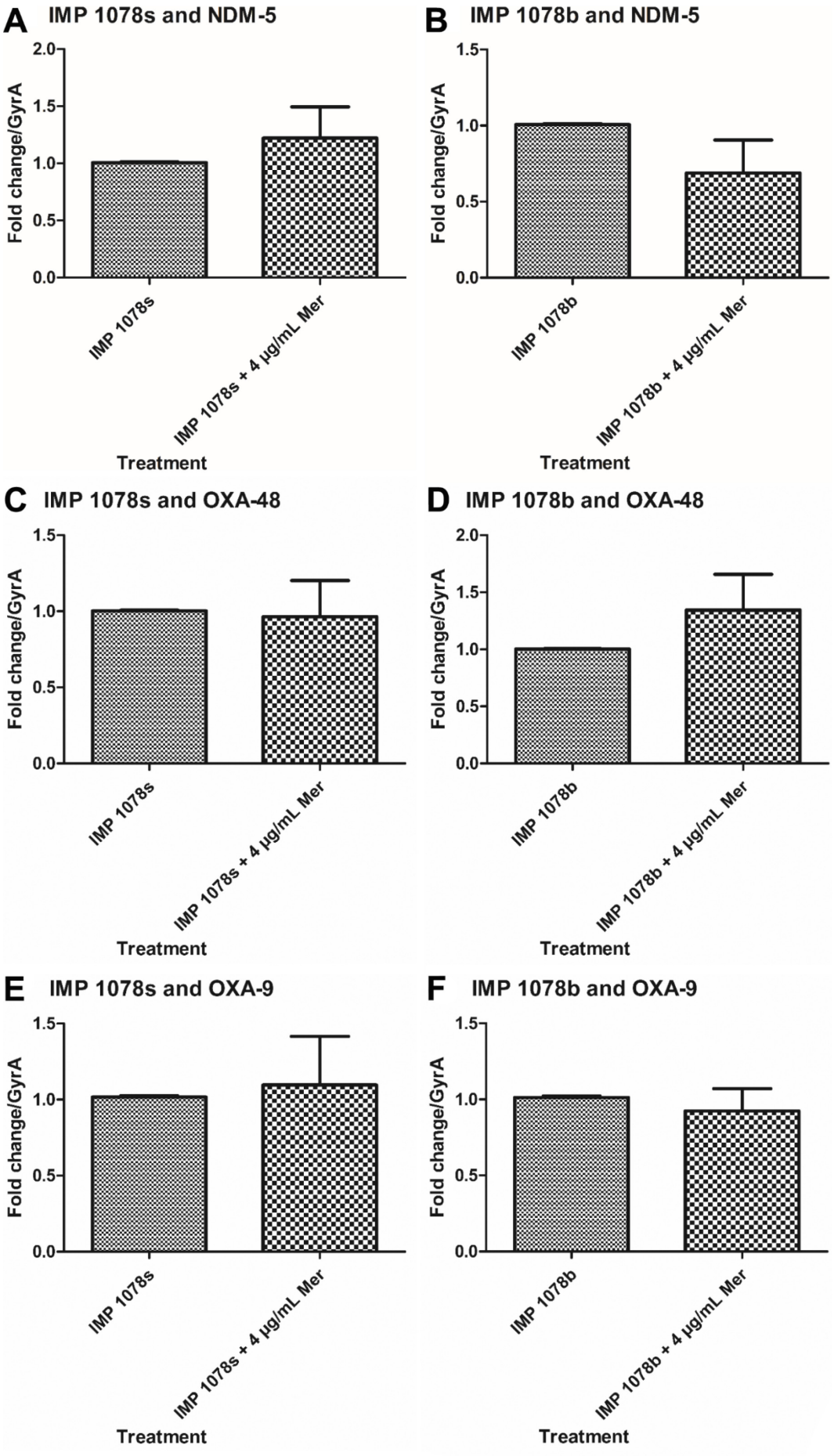
	DTA	Skin screening	Urine culture	Blood culture	Miscellaneous	MIC
26/3	Klebsiella pneumoniae Pure mod. growth *CRE Tigecycline: S; Colistin: I; Fosfomycin: I	Klebsiella pneumoniae CRE Tigecycline: S; Fosfomycin: I	negative			MIC vs: Ertapenem: 8 ug/ml Imipenem : 0.5 ug/ml Meropenem: 4 ug/ml
27/3				Negative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

5/5	Klebsiella pneumoniae - Heavy growth *CRE 2-Tigecycline: S; Colistin: I; Fosfomycin: S Klebsiella pneumoniae - Few growth *CRE 1-Tigecycline: S; Colistin: R; Fosfomycin: R Proteus mirabilis -Few growth	Klebsiella pneumoniae *CRE Tigecycline: R; Fosfomycin: R	Negative

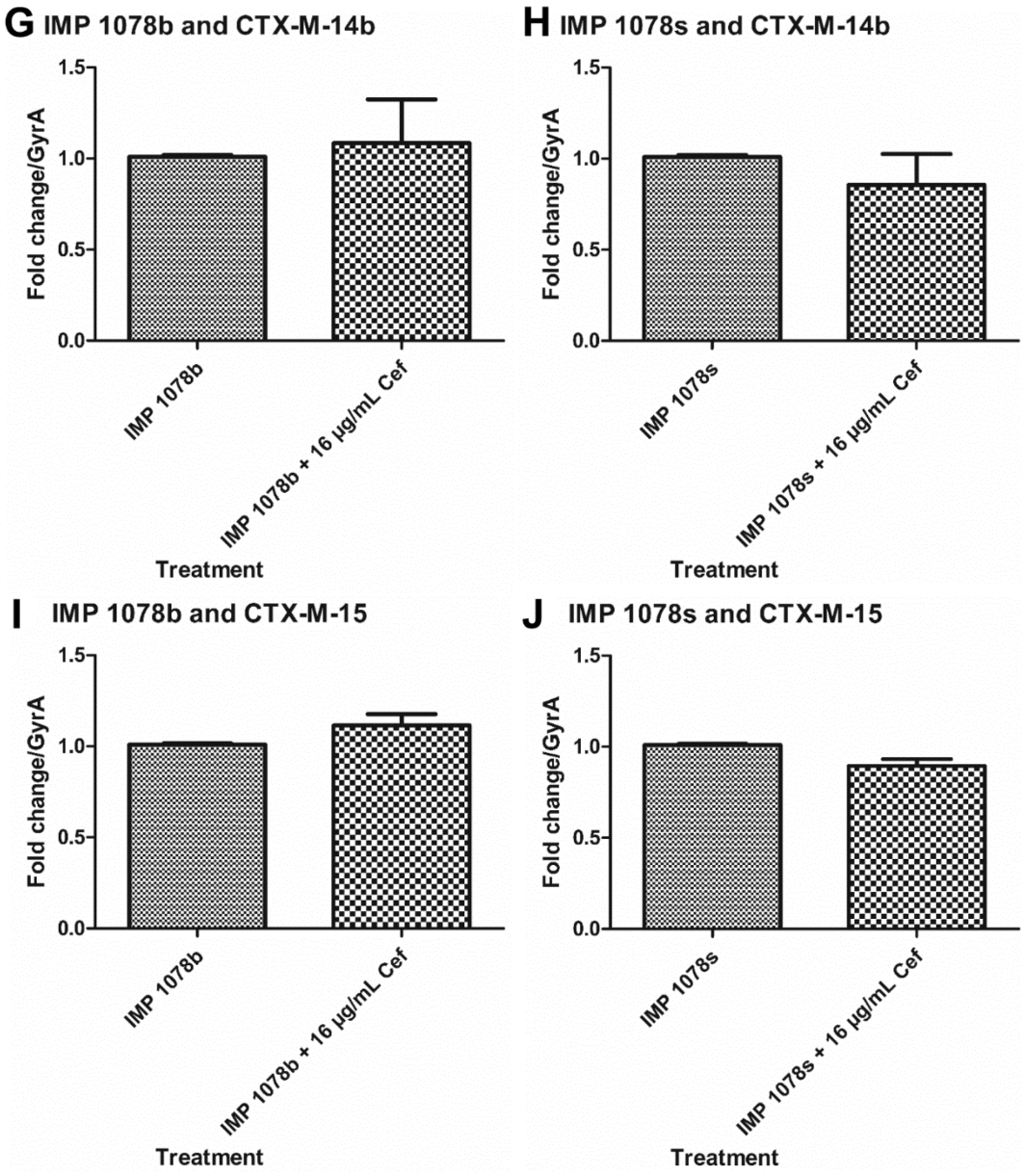
Supplementary Figure 1. Timeline of infection.



Supplementary Figure 2. qRT-PCR results of IMP 1078b and IMP 1078s against *bla*$_{NDM-5}$, *bla*$_{OXA-48}$, and *bla*$_{OXA-9}$.



Supplementary Figure 3. qRT-PCR results of IMP 1078b and IMP 1078s against $bla_{\text{CTX-M-14b}}$ and $bla_{\text{CTX-M-15}}$.



Supplementary Figure 4. qRT-PCR results of IMP 1078b and IMP 1078s against *bla*$_{SHV}$ and *bla*$_{TEM}$.