

One Health Perspective on Infections in Nephrology: A Parallel Survey on Carbapenem- and Multidrug-resistant Klebsiella Pneumoniae and Pseudomonas Aeruginosa in High-risk Renal Patients and Farm Animals in Italy

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Abstract

One Health is a multisectorial approach to achieve optimal health and well-being outcomes, recognizing the interconnections between people, animals, plants, and their environment. A relevant application is represented by antimicrobial resistance. Noteworthy, intensive farming has been frequently associated with abuse of antimicrobials and antimicrobial resistance is common among microorganisms from food-producing animals, whose transmission to humans cannot be excluded. In our survey, prevalence of carbapenem- and multidrug resistant *K. pneumoniae* and *P. aeruginosa* in renal patients and pigs in northern Italy was evaluated. During 2017–2018, 300 urine samples were collected from 187 high risk patients with kidney disease (mainly Acute Kidney Injury, 59/187, 31.5%; or kidney transplant, 90/187, 48.1%) at the Parma University Hospital. Additionally, 300 faecal samples were collected from slaughtered pigs. Carbapenemase-producing *K. pneumoniae* was detected in 4.8% of patients (9/187). Specifically, ST554 *bla*_{KPC-3} (61.5%), ST37 *bla*_{VIM-1} (15.4%), ST 258 *bla*_{KPC-2} (15.4%) and ST4525 *bla*_{VIM-1} (7.7%) were found. To our knowledge, ST4525 is a novel ST-type and ST37 *bla*_{VIM-1} was never reported before. ESBL genes (*bla*_{SHV}, *bla*_{TEM}, *bla*_{OXA} and *bla*_{CTX}) were differently distributed among isolates, as well as several AMR genes. Carbapenem-resistant *K. pneumoniae* was not detected in pigs. Carbapenem-resistant *P. aeruginosa* was detected in 1.1% patients (2/187) and in 1.3% pigs (4/300). Interestingly, *P. aeruginosa* ST938 *bla*_{PAO} *bla*_{OXA396} was isolated from one patient (83 years old male with Acute Kidney Injury) and one pig. Although no direct epidemiological link was demonstrable, SNP calling suggested a close genetic relationship of the isolates (only 41 SNPs difference).

1 Introduction

One Health is a collaborative, multisectorial, and trans-disciplinary approach - working at local, regional, national, and global levels - to achieve optimal health and well-being outcomes, recognizing the interconnections between people, animals, plants and their shared environment; thus, the One Health concept is based on the definition of communication spaces among different environments and hosts, and antimicrobial resistance (AMR) represents an important field of this approach [1]. Resistance to carbapenems is of particular concern since they are the “last-line defence” to threat human infections by multiresistant Gram-negative bacilli. Carbapenem-resistance can be mediated either by porin deficiency combined with expression of AmpC-β-lactamases or ESBLs or by biosynthesis of carbapenemases [2]. Among carbapenemases, class A KPC enzymes can hydrolyse all β-lactams in use. Class B enzymes belonging to the IMP, VIM and NDM families show the highest carbapenemase activity [3]. Class D carbapenem-hydrolyzing oxacillinas (OXA) have emerged in several bacterial species as responsible for carbapenem resistance [4]. The ESBLs (Extended-Spectrum β-Lactamases) hydrolyse most β-lactams but show a weak affinity for carbapenems. Families of ESBL enzymes include SHV, TEM-type-ESBLs and CTX-M [5].

Klebsiella pneumoniae and *Pseudomonas aeruginosa* infections are commonly healthcare associated. In Italy, 26.8% of *K. pneumoniae* and 15.8% of *P. aeruginosa* human invasive isolates were found to be carbapenem resistant (CR) in 2018 [6]. Hospital outbreaks have been predominantly attributed to *K. pneumoniae* ST258 *bla*_{KPC-3} [7] but recently ST307 *bla*_{KPC-3} and ST512 *bla*_{KPC-3} have been emerging [8, 9] and a severe outbreak of NDM-1 producing *K. pneumoniae* has been reported [10].

Patients with kidney diseases are a population at high risk for multi drug resistance (MDR)-associated complications, as well a potential reservoir of antibiotic-resistant pathogens, owing to the coexistence of many factors increasing the risk of colonization and infection [11]. High prevalence of colonization/infection by MDR organisms has been documented in recent years in patients with kidney disease (both acute and chronic), who are disproportionately affected by antimicrobial resistance (AMR) as compared to the general population [11]. Along with cardiovascular disease, infection represents a leading cause of death among patients with chronic kidney disease, end-stage renal disease on dialysis and in those who have undergone kidney transplantation [12], as well as during and after an episode of Acute Kidney Injury (AKI) [13].

Carbapenems are not licenced for use in food-producing animals in the EU [14], but other β-lactams are commonly used, such as extended-spectrum cephalosporins, which could provide a selection pressure favouring the expression of carbapenem-resistance [15]. In the last decades, intensive farming has been frequently associated with use/abuse of antimicrobials and AMR is common among microorganisms from food-producing animals, whose transmission to humans cannot be excluded [16].

On these grounds, the present study was aimed at testing for CR *K. pneumoniae* and *P. aeruginosa* samples from both high-risk renal patients hospitalized in the Nephrology Unit at the Parma University Hospital and pigs at slaughter in the same area of the country (Parma, northern Italy).

The main objective of the study was focused on the One Health concept of AMR bacteria sharing between human and animal compartments.

The specific aims were focused on: *i*) the detection of CR bacteria in hospitalized patients and pigs; *ii*) the distribution of carbapenemases and other β-lactamases co-responsible for resistance to carbapenems in human and porcine strains; *iii*) the characterization of genetic clones of carbapenemase-producing (CP) *K. pneumoniae* and *P. aeruginosa*; *iv*) the investigation on additional AMR genes in CR isolates.

2 Materials And Methods

2.1 Sample collection

2.1.1 Patient sampling

A total of 300 urine samples were collected from patients once every week in the Renal Intensive Care Unit (ICU) and the Renal Transplant Unit of the Parma University-Hospital. The samples were collected from indwelling bladder catheters in 187 patients (mean age 60 years, standard deviation 15) since 113 of them (60.4%) were tested more than once because of prolonged hospitalization. The patients had been consecutively admitted between February 1st, 2017 and May 31st, 2018 with a diagnosis mainly of AKI (59/187, 31.5%) or kidney transplant (90/187, 48.1%).

Written informed consent was obtained from the patients when possible or from their closest relatives when available. The Ethics Committee of the Area Vasta Emilia Nord (AVEN) approved the study (protocol n. 24497).

2.1. 2 Pig sampling

From October 2016 to December 2017, 300 finishing pigs were randomly selected during 23 visits in a slaughterhouse of Parma province. The animals belonged to 300 batches and were reared in 95 farms. The caecal content of pigs was aseptically collected immediately after evisceration.

2.2 Sample testing

2.2.1 Patient sample testing

From each urine sample, 1 ml was transferred into 5 ml of Trypticase Soy Broth (TSB; Oxoid, Basingstoke, UK) added with a 10-µg Meropenem disk (Oxoid). After overnight incubation at $35 \pm 2^\circ\text{C}$, 100 µl were streaked onto Mac Conkey agar (Oxoid) plates added with a 10-µg Meropenem disk and 10 µl were streaked onto Brilliance CRE Agar (Oxoid), which provides presumptive identification of CR microorganisms. Plates were incubated aerobically at $35 \pm 2^\circ\text{C}$ for 18–24 h. Suspect colonies were subcultured onto Trypticase Soy Agar plates (TSA; Oxoid) at $37 \pm 1^\circ\text{C}$ for 18–22 h to obtain pure cultures for Gram-staining and further testing.

2.2.2 Pig sample testing

A 10 g aliquot of faecal material was suspended in 90 ml of Buffered Peptone Water (BPW; Biolife Italiana, Italy) and incubated at $37 \pm 1^\circ\text{C}$ for 18–22 h. Thereafter, cultures plating and colonies selection were performed as described in 2.2.1.

2.3 Kirby-Bauer Disk Diffusion Susceptibility test

Human and porcine Gram-negative isolates were screened by the Kirby-Bauer Disk Diffusion Susceptibility test following EUCAST (2018) [17] and considering both clinical and screening breakpoints.

2.4 Species identification

Oxidase-negative isolates were tested by API® 20 E (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions. Non-fermenting, oxidase-positive isolates were tested by the API® 20 NE (bioMérieux) system.

2.5 MIC test

The CR isolates were analysed by the Minimal Inhibitory Concentration (MIC) test. Following EUCAST (2018) [17], *K. pneumoniae* and *P. aeruginosa* strains with meropenem MIC > 8 µg/ml (clinical breakpoint) and *K. pneumoniae* strains with meropenem MIC > 0.125 µg/ml (screening breakpoint) were selected for whole genome sequencing (WGS). *E. coli* ATCC 25922 was tested periodically as quality control microorganism.

2.6 Whole genome sequencing

Based on MIC, selected *K. pneumoniae* and *P. aeruginosa* isolates were tested by WGS. Whole-genomic DNA was extracted from *K. pneumoniae* and *P. aeruginosa* isolates using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany). The purified DNA concentration and the quality parameter ratio 260/280 were measured by BioSpectrometer fluorescence (Eppendorf). Whole genomes were sequenced on Illumina MiSeq platform (Nextera library, paired-end reads). The INNUca v3.2 pipeline was used to quality check and *de novo* assemble reads into contigs. The pipeline includes SPAdes 3.11 as *de novo* assembler and provides Multi Locus Sequence Typing (MLST) profile.

SNP calling was performed on reads using snippy 4.0 pipeline with default setting (<https://github.com/tseemann/snippy>). An alignment of core SNPs was produced to infer a high-resolution phylogeny. A maximum likelihood (ML) tree was constructed using the iQTree program and was viewed using iTOL v4.2.3 software [18]. *Klebsiella pneumoniae* str. HS11286 (Ref Seq GCA_000240185.2) and *Pseudomonas aeruginosa* PAO1 (Ref Seq NC_002516.2) were used as references for SNP calling on *K. pneumoniae* and *P. aeruginosa* newly sequenced reads, respectively.

Analysis of resistome of genomes was performed using ABRicate [19]. With this tool a BLAST search of genes included in the Resfinder database was performed on *de novo* assemblies [20, 21].

De novo assemblies were annotated by RAST version 2.0 [22]. Gbk files were used as input files for visualisation of the genetic environment of carbapenemase encoding genes by SnapGene [23]. Assembled sequences are available at NCBI [24] under BioProject PRJNA587603.

3 Results

3.1 *Klebsiella pneumoniae* of human origin

Seventeen *K. pneumoniae* strains were isolated from 12 patients (Table 1). Long-lasting infections in four patients were revealed by repeated detection of *K. pneumoniae* over time.

Table 1
Carbapenem-resistant strains isolated from humans and pigs in the study period

source	ID code	MIC values ($\mu\text{g/ml}$)	MLST	<i>bla</i> genes
K. pneumoniae				
human	NEF 48	16 ± 0	37	VIM-1, OXA-1, CTX-M-15, SHV-12
human	NEF 87	32 ± 0	258	KPC-2, OXA-9, SHV-187
human	NEF 88	13.33 ± 4.62	258	KPC-2, OXA-9, SHV-12
human	NEF 112	10.67 ± 4.62	37	VIM-1, OXA-1, CTX-M-15
human	NEF 116	85.33 ± 36.95	554	KPC-3, OXA-9, SHV-182
human	NEF 119	256 ± 0	554	KPC-3, OXA-9, SHV-182
human	NEF 123	16 ± 0	4525	VIM-1, OXA-1, CTX-M-15, SHV-12
human	NEF 130	>256	554	KPC-3, OXA-9, SHV-182
human	NEF 131	>256	554	KPC-3, OXA-9, SHV-182
human	NEF 145	>256	554	KPC-3, OXA-9, SHV-182
human	NEF 196/2	64 ± 0	554	KPC-3, OXA-9, SHV-182
human	NEF 197	64 ± 0	554	KPC-3, OXA-9, SHV-182
human	NEF 198	2 ± 0	277	-
human	NEF 199	1.67 ± 0.58	277	SHV-27
human	NEF 261	0.027 ± 0.009	307	SHV-28, CTX-M-15
human	NEF 278	0.15 ± 0.1	307	OXA-1, SHV-106, CTX-M-15
human	NEF 297	53.33 ± 18.48	554	KPC-3, OXA-9, SHV-182
P. aeruginosa				
human	NEF 23	21.33 ± 9.24	938	OXA-396, PAO
human	NEF 156	8 ± 0	395	OXA-488, PAO
pig	CRE 98	2 ± 0	274	OXA-486, PAO
pig	CRE 102	4.67 ± 3.06	938	OXA-396, PAO
pig	CRE 153	16 ± 0	782	OXA-50, PAO
pig	CRE 295	1.67 ± 0.58	885	OXA-50, PAO

WGS identified carbapenemase genes in 13 (76.5%) strains from 9 patients. Apparent prevalence of CP *K. pneumoniae* infected patients was 4.8% (9/187) CI₉₅[2.6–8.9]. Sequence identity ($\geq 98\%$) and coverage minimum sequence length ($> 60\%$) were used as selection criteria for gene detections. The 13 CP *K. pneumoniae* isolates harboured *bla*_{KPC-3} (61.5%), *bla*_{VIM-1} (23.1%) and *bla*_{KPC-2} (15.4%), as well as other β -lactamase-producing (*bla*) genes (Tables 1 and 2). CP *K. pneumoniae* belonged to ST554 (61.5%), ST37 (15.4%), ST258 (15.4%) and ST4525 (7.7%). The *bla*_{KPC-2} and *bla*_{KPC-3} determinants were linked to specific ST-types, namely ST258 and ST554, respectively. The *bla*_{VIM-1} gene was found in ST37 and ST4525 (Table 2).

Table 2
Resistomes of CP *K. pneumoniae* strains of human origin

Antimicrobial class	Mechanism (*)	Carbapenemase-producing <i>K. pneumoniae</i>							
		ST37 (NEF 48)	ST37 (NEF 112)	ST258 (NEF 87)	ST258 (NEF 88)	ST554 (NEF 116)	ST554 (NEF 119)	ST554 (NEF 130)	ST554 (NEF 131)
Aminoglycosides	acetyltransferase		<i>aac(6')-lb</i>	<i>aac(6')-lb</i>	<i>aac(6')-lb</i>	<i>aac(6')-lb</i>	<i>aac(6')-lb</i>	<i>aac(6')-lb</i>	<i>aac(6')-lb</i>
	adenyltransferase			<i>aadA2</i>	<i>aadA2</i>	<i>aadA2</i>		<i>aadA2</i>	<i>aadA2</i>
	nucleotidyltransferase	<i>ant(3")-la</i>	<i>ant(3")-la</i>						
	phosphotranferase	<i>aph(3')-XV</i>	<i>aph(3')-XV</i>				<i>aph(3')-la</i>	<i>aph(3')-la</i>	<i>aph(3')-la</i>
				<i>aph(3')-lb</i>	<i>aph(3')-lb</i>				
				<i>aph(6')-ld</i>	<i>aph(6')-ld</i>				
	16 rRNA methylase	<i>armA</i>	<i>armA</i>						
β-lactams	Class A	<i>bla_{CTX-M-15}</i>	<i>bla_{CTX-M-15}</i>						
		<i>bla_{SHV-12}</i>			<i>bla_{SHV-12}</i>				
						<i>bla_{SHV-182}</i>	<i>bla_{SHV-182}</i>	<i>bla_{SHV-182}</i>	<i>bla_{SHV-182}</i>
					<i>bla_{SHV-187}</i>				
	Class D	<i>bla_{OXA-1}</i>	<i>bla_{OXA-1}</i>						
				<i>bla_{OXA-9}</i>	<i>bla_{OXA-9}</i>	<i>bla_{OXA-9}</i>	<i>bla_{OXA-9}</i>	<i>bla_{OXA-9}</i>	<i>bla_{OXA-9}</i>
Carbapenems	Class A			<i>bla_{KPC-2}</i>	<i>bla_{KPC-2}</i>				
						<i>bla_{KPC-3}</i>	<i>bla_{KPC-3}</i>	<i>bla_{KPC-3}</i>	<i>bla_{KPC-3}</i>
	Class B	<i>bla_{VIM-1}</i>	<i>bla_{VIM-1}</i>						
Fluoroquinolones	Efflux	<i>oqxA</i>	<i>oqxA</i>	<i>oqxA</i>	<i>oqxA</i>	<i>oqxA</i>	<i>oqxA</i>	<i>oqxA</i>	<i>oqxA</i>
		<i>oqxB</i>	<i>oqxB</i>	<i>oqxB</i>	<i>oqxB</i>	<i>oqxB</i>	<i>oqxB</i>	<i>oqxB</i>	<i>oqxB</i>
Macrolides	phosphotranferase	<i>mph(A)</i>	<i>mph(A)</i>	<i>mph(A)</i>	<i>mph(A)</i>	<i>mph(A)</i>	<i>mph(A)</i>	<i>mph(A)</i>	<i>mph(A)</i>
		<i>mph(E)</i>	<i>mph(E)</i>						
	ribosomal protection	<i>msr(E)</i>	<i>msr(E)</i>						
Major facilitator superfamily (MFS)								<i>mdf(A)</i>	<i>mdf(A)</i>
Phosphonic acids	thiol transferase	<i>fosA6</i>	<i>fosA6</i>	<i>fosA6</i>	<i>fosA6</i>	<i>fosA6</i>	<i>fosA6</i>	<i>fosA6</i>	<i>fosA6</i>
Phenicols	acetyltransferase	<i>catA1</i>	<i>catA1</i>	<i>catA1</i>	<i>catA1</i>			<i>catA1</i>	<i>catA1</i>
		<i>catB2</i>	<i>catB2</i>						
		<i>catB3</i>	<i>catB3</i>						
Sulphonamides	dihydropteroate synthase	<i>sul1</i>	<i>sul1</i>	<i>sul1</i>	<i>sul1</i>	<i>sul1</i>	<i>sul1</i>	<i>sul1</i>	<i>sul1</i>
		<i>sul2</i>	<i>sul2</i>						
Trimethoprim	dihydropholate reductase	<i>dfrA5</i>	<i>dfrA5</i>						
				<i>dfrA12</i>	<i>dfrA12</i>	<i>dfrA12</i>	<i>dfrA12</i>	<i>dfrA12</i>	<i>dfrA12</i>
		<i>dfrA14</i>	<i>dfrA14</i>						
No. of genes		22	22	13	13	13	11	15	15

(*) Comprehensive Antibiotic Data Base [34]

ST4525 is a novel ST-type with the following allelic profile: gapA (2); infB (9); mdh (2); pgi (1); phoE(13); rpoB (214); tonB (16). This allelic profile differs from ST37 by only one nucleotide within *rpoB* gene: T at position 100 of allele *rpoB_1* (ST37) is substituted by a C in allele *rpoB_214* (ST4525) (Fig. 1).

In three *K. pneumoniae* isolates (3/17; 17.6%), ESBL genes belonging to *bla_{SHV}*, *bla_{TEM}*, *bla_{Oxa}* and *bla_{CTX}* families were found. These isolates were characterized by MIC values lower than the clinical breakpoint (< 2 µg/ml) (Table 3).

Table 3
Resistomes of ESBL *K. pneumoniae* strains of human origin

Antimicrobial class	Mechanism *	ESBL <i>K. pneumoniae</i>		
		ST277 (NEF 199)	ST307 (NEF 261)	ST307 (NEF 278)
Aminoglycosides, non-specific	acetyltransferase	<i>aac(3')-Ila</i>	<i>aac(3')-Ila</i>	<i>aac(6')-Ib-cr_1</i>
	adenyltransferase		<i>aadA1_4</i>	
	nucleotidyltransferase		<i>ant(3')-Ia</i>	
	phosphotransferase		<i>aph(3")-Ib_5</i>	<i>aph(3")-Ib_5</i>
				<i>aph(6)-Id</i>
β-lactams	Class A			<i>bla_{CTX-M-15}</i>
		<i>bla_{SHV-27}</i>		
			<i>bla_{SHV-106}</i>	<i>bla_{SHV-106}</i>
				<i>bla_{TEM-1B}</i>
			<i>bla_{TEM-102}</i>	
	Class D			<i>bla_{OXA-1}</i>
Fluoroquinolones	efflux	<i>oqxA</i>	<i>oqxA</i>	
		<i>oqxB</i>	<i>oqxB</i>	
				<i>qnrB1</i>
Macrolides	phosphotransferase		<i>mph(E)</i>	
Major facilitator superfamily (MFS)		<i>mdf(A)</i>	<i>mdf(A)</i>	<i>mdf(A)</i>
Phosphonic acids	thiol transferase		<i>fosA6</i>	
			<i>fosA7</i>	
Sulphonamides	dihydropteroate synthase			<i>sul2</i>
Tetracyclines	efflux			<i>tet(A)_6</i>
Trimethoprim	dihydropholate reductase			<i>dfrA14_5</i>
N° of genes		4	10	15
(*) Comprehensive Antibiotic Data Base [34]				

ST258 *bla_{KPC-2}* isolates carried other *bla* genes, namely *bla_{OXA-9}*, *bla_{SHV-12}*, and *bla_{SHV-187}*. Their resistome consisted of 13 genes conferring resistance to nine antimicrobial classes. ST554 *bla_{KPC-3}* isolates carried *bla_{OXA-9}* and *bla_{SHV-182}*, with resistomes consisting of 11–15 genes. One ST37 *bla_{VIM-1}* strain carried *bla_{OXA-1}* and *bla_{CTX-M-15}*, whereas the other ST37 *bla_{VIM-1}* additionally carried *bla_{SHV-12}*. Their resistome consisted of 22 AMR genes. (Table 2)

The novel ST4525 *bla_{VIM-1}* (NEF123) was characterized by a resistome of 17 genes, which included *bla_{CTX-M-15}*, *bla_{SHV-12}*, *bla_{OXA-1}* and genes for resistance to aminoglycosides, fluoroquinolones, macrolides, *fosfomycin*, chloramphenicol, sulphonamides, and trimethoprim (Table 2).

Among the ESBL ST307 isolates, one strain harboured *bla_{SHV-106}* and *bla_{TEM-102}*, and the other carried *bla_{SHV-106}*, *bla_{TEM-1B}*, *bla_{CTX-M-15}* and *bla_{OXA-1}*. One ST277 strain carried *bla_{SHV-27}*, whereas the other ST227 did not harbour any *bla* genes. Their resistomes contained from 4 to 15 genes and showed a great variety (Table 3).

3.2 *Pseudomonas aeruginosa* of human and porcine origin

Two CR *P. aeruginosa* strains were isolated from patients. Apparent prevalence was 1.1% (2/187) (95% CI: 0.3%-3.8%) (Table 1). The NEF23 strain belonged to ST938 and its resistome consisted of five genes, including *bla_{OXA-396}* and *bla_{PAO}*, while the NEF156 strain belonged to ST395 and its resistome consisted of six genes, including *bla_{OXA-488}* and *bla_{PAO}* (Table 4).

Table 4
Resistomes of *P. aeruginosa* of human and porcine origin

Antimicrobial class	Mechanism *	<i>P. aeruginosa</i>					
		ST-938 (NEF 23)	ST-938 (CRE 102)	ST-274 (CRE 98)	ST-782 (CRE 153)	ST-885 (CRE 295)	ST-395 (NEF 156)
Aminoglycosides, non-specific	phosphotransferase	<i>aph(3')-Iib</i>	<i>aph(3')-Iib</i>	<i>aph(3')-Iib</i>	<i>aph(3')-Iib</i>	<i>aph(3')-Iib</i>	<i>aph(3')-Iib</i>
β-lactams	Class D				<i>bla</i> _{OXA-50}	<i>bla</i> _{OXA-50}	
		<i>bla</i> _{OXA-396}	<i>bla</i> _{OXA-396}				
				<i>bla</i> _{OXA-486}			<i>bla</i> _{OXA-488}
	PAO genes	<i>bla</i> _{PAO}	<i>bla</i> _{PAO}	<i>bla</i> _{PAO}	<i>bla</i> _{PAO}	<i>bla</i> _{PAO}	<i>bla</i> _{PAO}
Fluoroquinolones (Ciprofloxacin)	phosphorylase			<i>crpP</i>	<i>crpP</i>	<i>crpP</i>	<i>crpP</i>
Phosphonic acids	thiol transferase	<i>fosA4</i>	<i>fosA4</i>	<i>fosA4</i>	<i>fosA4</i>	<i>fosA4</i>	<i>fosA4</i>
Phenicols	acetyltransferase	<i>catB7</i>	<i>catB7</i>	<i>catB7</i>	<i>catB7</i>	<i>catB7</i>	<i>catB7</i>
N° of genes		5	5	6	6	6	6
(*) Comprehensive Antibiotic Data Base [34]							

In pigs, four CR *P. aeruginosa* strains were detected. Specifically, one ST938 isolate (CRE102) was identical to the NEF23 from a 83-old male patient hospitalized one month after pig slaughter for an episode of AKI due to sepsis, on previous chronic kidney disease. Moreover, based on SNP calling, the two genomes differed by only 41 SNPs suggesting the close genetic relationship among the porcine and the human isolates (Fig. 3, Table S3). No direct epidemiological link was found.

The remaining *P. aeruginosa* isolates of porcine origin belonged to ST274, ST782 and ST885 and all of them harboured the *bla*_{PAO} gene. In addition, *bla*_{OXA-50} (ST782 and ST885) and *bla*_{OXA-486} (ST274) were identified together with other AMR genes (Tables 1 and 4).

3.3 Single Nucleotide Polymorphisms (SNPs) calling

Results on *de novo* assembly statistics are reported in table S1. SNP calling confirmed results of MLST typing with a higher level of discriminatory power (Figs. 2 and 3). Regarding *K. pneumoniae*, within the same ST types pairwise SNP differences were 41 (ST37), 51 (ST277), 55 (ST258), ranging from 29 and 89 (ST554) and 1071 (ST307). Isolate NEF123 (ST4525) showed 292 and 296 SNPs differences to NEF112 and NEF48 both belonging to ST37, demonstrating the close genetic relationship of the two ST-types (Fig. 2, table S2). Not surprisingly, between genomes of different ST-types the genetic distance increased with pairwise SNPs differences ranging from 905 to 32805.

Regarding *Pseudomonas*, CRE102 and NEF23 (ST938) of porcine and human origin respectively showed pairwise SNPs distances of 41 SNPs confirming their close genetic relationship (Fig. 3, table S3). Not surprisingly, comparing genomes of different ST-types (ST274, ST782, ST885, ST395) the genetic distance increased with pairwise SNPs differences ranging from 17359 to 20981.

3.4 Localisation of carbapenemase-encoding genes

Within the meropenem-resistant *K. pneumoniae* isolates, the carbapenemase-encoding genes *bla*_{KPC-2}, *bla*_{KPC-3} or *bla*_{VIM-1} were detected. To evaluate their mobilisation potential, their genetic environments were investigated. *bla*_{KPC-2} and *bla*_{KPC-3} were included in a complete Tn4407 (Figs. 4 and 5). Tn4407 was previously described as a transposon belonging to the Tn3 family ad involved in *bla*_{KPC} mobilisation [25].

*bla*_{VIM-1} was located close to other AMR determinant genes (Fig. 6). Although the contig was too short to confirm the localisation of the gene in a class 1 integron, the sequence showed 99% identity in comparison to *K. pneumoniae* genomes (CP032178.1, MF344563.1) in which the sequence was included in a class 1 integron within a transposon (data not shown). Moreover, this gene was already detected within class 1 integrons as part of a Tn402 transposon [26].

Regarding *P. aeruginosa*, *bla*_{OXA} and *bla*_{PAO} showed genetic environments which did not show any specific sequence of mobilizable genetic elements (MGEs) confirming previous data (Figs. 7 and 8) [27].

4 Discussion

4.1 *Klebsiella pneumoniae* in humans

In our study, *K. pneumoniae* ST258 *bla*_{KPC-2}, which is one of the most common in Italian hospital settings [7], was identified in two patients only (22.2%) tested on the same day. Instead, *K. pneumoniae* ST554 *bla*_{KPC-3} has been the only clone isolated from the two renal wards for 10 months (July 2017-May 2018), suggesting a long-lasting outbreak involving six patients. MDR to amynoglycosides, β-lactams, macrolides, phenicols, sulphonamides, fluoroquinolones and fosfomycin was associated to carbapenem resistance.

To our knowledge, this is the first report of *K. pneumoniae* ST37 producing VIM-1 in Italy. The two isolates were detected from the same patient (72 years old male with AKI) tested twice during a three-month period (April-July 2017). The isolates carried the *bla*_{VIM-1} gene within a class 1 integron, along with additional ESBL-encoding genes (*bla*_{CTX-M-15}, *bla*_{SHV-12}, *bla*_{OXA-1}) and several AMR determinants. High levels of AMR were identified also in the new sequence type ST4525 *bla*_{VIM-1} (NEF123), including ESBL genes (*bla*_{CTX-M-15}, *bla*_{SHV-12}, *bla*_{OXA-1}) and genes for resistance to aminoglycosides, fluoroquinolones, macrolides, fosfomycin, chloramphenicol, sulphonamides, and trimethoprim.

4.2 *K. pneumoniae* in pigs

K. pneumoniae has been associated with sporadic cases of disease in pigs, including pneumonia, mastitis and septicaemia [28]. In our study, CR *K. pneumoniae* was never isolated from swine, not only because carbapenems are banned in veterinary treatments, but also for the high biosecurity measures commonly applied in pig farms, which exclude access of pigs to the natural environment or anthropic sources of AMR bacteria [29].

4.3 *Pseudomonas aeruginosa* in humans

Although *P. aeruginosa* β-lactams resistance is usually mediated by the chromosomally encoded AmpC type β-lactamase induced by contacts with sub-MIC levels of β-lactams, the microorganism can acquire resistance through chromosomal mutations as well as acquisition of antimicrobial resistance genes horizontally transferred [30].

The two CR ST938 and ST395 strains did not harbour carbapenemase-encoding genes, but *bla*_{OXA} and *bla*_{PAO} genes, confirming that decreased susceptibility to meropenem could be conferred by oxacillinases [31].

4.4 *Pseudomonas aeruginosa* in pigs

In most of cases, *P. aeruginosa* has not a primary role in pig infections [32]. Interestingly, two porcine strains harboured β-lactamases genes, such as *bla*_{OXA-50} which is an intrinsic oxacillinase able to confer decreased susceptibility to ampicillin, ticarcillin and meropenem [31].

4.5 Shared *P. aeruginosa* ST938 between humans and pigs and implications for safety

This is the first report of an ESBL-MDR *P. aeruginosa* strain isolated both from humans and pigs in Italy, whose resistance to carbapenems is due to β-lactamases genes other than carbapenemases. *P. aeruginosa* ST938 carrying *bla*_{OXA-396} and *bla*_{PAO} was isolated from a pig slaughtered in February 2017 and from an 83-old male patient hospitalized one month later for an episode of AKI due to sepsis, on previous chronic kidney disease. Even if no epidemiological links between these findings were found, the occurrence of the same CR strain in humans and pigs reflects the One Health concept of bacterial sharing between humans and food animals.

Since other isolates of MDR *P. aeruginosa* harbouring ESBL genes were found in pigs, the potential risk of pig meat contamination and subsequent human exposure to MDR opportunistic pathogens could not be excluded. Especially raw pork and products thereof (e.g. sausages, salami) could represent a risk for humans.

5 Conclusions

In recent years, the concept of One Health has been reformulated underlying the role of geographically close ecosystems in the occurrence of traits which have an impact on human, animal, plant, and environmental health, such as AMR [33].

In our study based on the One Health perspective of bacterial exchange between human and animal compartments, *K. pneumoniae* strains harbouring *bla*_{KPC-2}, *bla*_{KPC-3} and *bla*_{VIM-1} were isolated from patients only. This finding is consistent with the European ban of carbapenem use in food-producing animals. Nevertheless, despite treatments bans, environmental overlapping could cause microbial and MGEs exchange between humans and animals, causing wide distribution of AMR bacteria in different settings. On the contrary, *P. aeruginosa* was found both in human and porcine entities, but the isolates did not carry carbapenemase genes and their carbapenem-resistance was associated to *bla*_{OXA-50} family and *bla*_{PAO} genes. In addition, MDR microorganisms were mostly detected in *K. pneumoniae* strains from hospitalized patients, probably because of the selection pressure following antimicrobial treatments. The surprising similarity in AMR of *P. aeruginosa* strains detected from pigs and patients could be due to improper use in pig husbandry of the same antimicrobial agents used in the hospital setting. However, the circulation of bacterial strains between the animal and human compartments cannot be excluded.

Declarations

Funding:

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Conflicts of interest:

The authors declare no conflicts of interest.

Ethics approval:

The study adhered to the Helsinki Declaration for Ethical Treatment of Human Subjects, with local ethics committee approval (The Ethics Committee of the Area Vasta Emilia Nord, AVEN n. 24497). The study respects international ethics standards for anonymity and data protection.

Consent to participate:

Written informed consent was obtained from the patients when possible or from their closest relatives when available.

Availability of data:

Silvia Bonardi, Enrico Fiaccadori and Frederique Pasquali had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Code availability:

not applicable

Authors' contributions:

S.B., E.F., F.P.: study design, data analysis; paper writing A.P.: WGS analyses; C.S.C., C.S., M.L.D.E.: MIC testing; G.M., S.C.: paper supervision; G.P., G.P.: microbiological testing; C.C.: data analyses; A.S.; F.R.: samples collection.

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Figures

CLUSTAL O(1.2.4) multiple sequence alignment

rpoB1_NEF48	ACCCACTACGGTCGCGTATGTCGATCGAACGCCTGAAGGTCCGAAACATCGGCTGATT	60
rpoB1_NEF112	ACCCACTACGGTCGCGTATGTCGATCGAACGCCTGAAGGTCCGAAACATCGGCTGATT	60
rpoB214_NEF123	ACCCACTACGGTCGCGTATGTCGATCGAACGCCTGAAGGTCCGAAACATCGGCTGATT	60

rpoB1_NEF48	AAACTCCCTGTCCGTACCGCGAGACCAACGAATATGGCTTCTTGAGACGCCGTATCGT	120
rpoB1_NEF112	AAACTCCCTGTCCGTACCGCGAGACCAACGAATATGGCTTCTTGAGACGCCGTATCGT	120
rpoB214_NEF123	AAACTCCCTGTCCGTACCGCGAGACCAACGAATATGGCTTCTTGAGACGCCGTATCGT	120

rpoB1_NEF48	AAAGTGACCAACGGTTGTTACTGACGAAATTCACTACCTGTCTGCTATCGAAGAAGGC	180
rpoB1_NEF112	AAAGTGACCAACGGTTGTTACTGACGAAATTCACTACCTGTCTGCTATCGAAGAAGGC	180
rpoB214_NEF123	AAAGTGACCAACGGTTGTTACTGACGAAATTCACTACCTGTCTGCTATCGAAGAAGGC	180

rpoB1_NEF48	AAACTACGTTATCGCTCAGGGCAACTCCAACCTGGATGAAAACGCCACTTCGAGAAGAT	240
rpoB1_NEF112	AAACTACGTTATCGCTCAGGGCAACTCCAACCTGGATGAAAACGCCACTTCGAGAAGAT	240
rpoB214_NEF123	AAACTACGTTATCGCTCAGGGCAACTCCAACCTGGATGAAAACGCCACTTCGAGAAGAT	240

rpoB1_NEF48	CTGGTTACCTGCCGTAGCAAAGCGAATCCAGCTTGTTCAGCCCGGACCAAGGTTGACTAC	300
rpoB1_NEF112	CTGGTTACCTGCCGTAGCAAAGCGAATCCAGCTTGTTCAGCCCGGACCAAGGTTGACTAC	300
rpoB214_NEF123	CTGGTTACCTGCCGTAGCAAAGCGAATCCAGCTTGTTCAGCCCGGACCAAGGTTGACTAC	300

rpoB1_NEF48	ATGGACGTATCCACCCAGCAGGGTGTATCGCTGGTGTGTCCTGATCCGTTCTGGAA	360
rpoB1_NEF112	ATGGACGTATCCACCCAGCAGGGTGTATCGCTGGTGTGTCCTGATCCGTTCTGGAA	360
rpoB214_NEF123	ATGGACGTATCCACCCAGCAGGGTGTATCGCTGGTGTGTCCTGATCCGTTCTGGAA	360

rpoB1_NEF48	CACGATGACGCCAACCGTGCATTGATGGGTGCGAACATGCAACGTCAGGCCGTTCCGACT	420
rpoB1_NEF112	CACGATGACGCCAACCGTGCATTGATGGGTGCGAACATGCAACGTCAGGCCGTTCCGACT	420
rpoB214_NEF123	CACGATGACGCCAACCGTGCATTGATGGGTGCGAACATGCAACGTCAGGCCGTTCCGACT	420

rpoB1_NEF48	CTGCGCGCTGATAAGCCGCTGGTGGTGGTACCGGTATGGAAACGTGCTGTTGGCGTTGACTCC	480
rpoB1_NEF112	CTGCGCGCTGATAAGCCGCTGGTGGTGGTACCGGTATGGAAACGTGCTGTTGGCGTTGACTCC	480
rpoB214_NEF123	CTGCGCGCTGATAAGCCGCTGGTGGTGGTACCGGTATGGAAACGTGCTGTTGGCGTTGACTCC	480

rpoB1_NEF48	GGTGTACTGCCGTGGCTAAA	501
rpoB1_NEF112	GGTGTACTGCCGTGGCTAAA	501
rpoB214_NEF123	GGTGTACTGCCGTGGCTAAA	501

Figure 1

alignment of rpoB gene of NEF 48 (ST37), NEF112 (ST37) and NEF123 (ST4525)

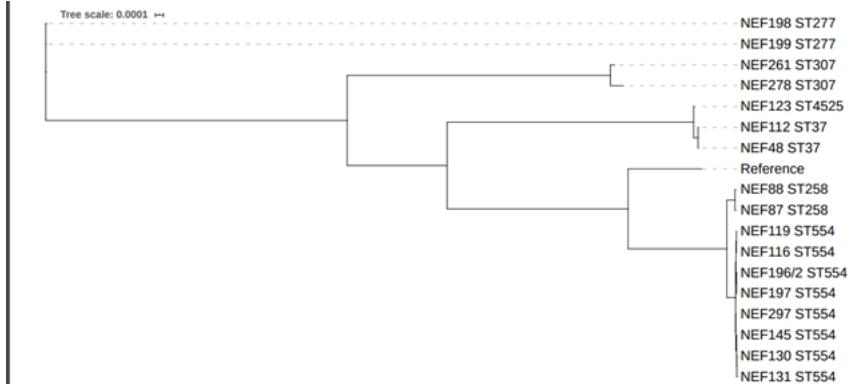


Figure 2

Core SNPs Maximum Likelihood tree showing genetic relationships among *K. pneumoniae* genomes included in this study and the reference *Klebsiella pneumoniae* str. HS11286 (Ref Seq GCA_000240185.2)

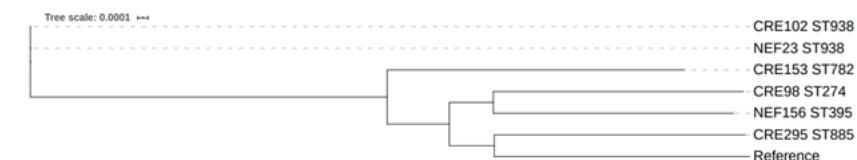


Figure 3

Core SNPs Maximum Likelihood tree showing genetic relationships among *P. aeruginosa* and *P. fluorescens* genomes included in this study and the reference *P. aeruginosa* PA01 (Ref Seq NC_002516.2)

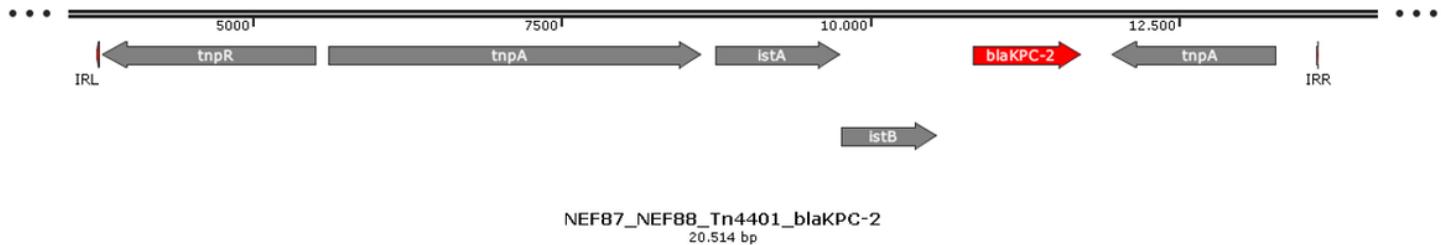


Figure 4

Genetic environment of blaKPC-2 gene in NEF 87 (ST258). The same genetic environment was observed in NEF 88 (ST258) genome

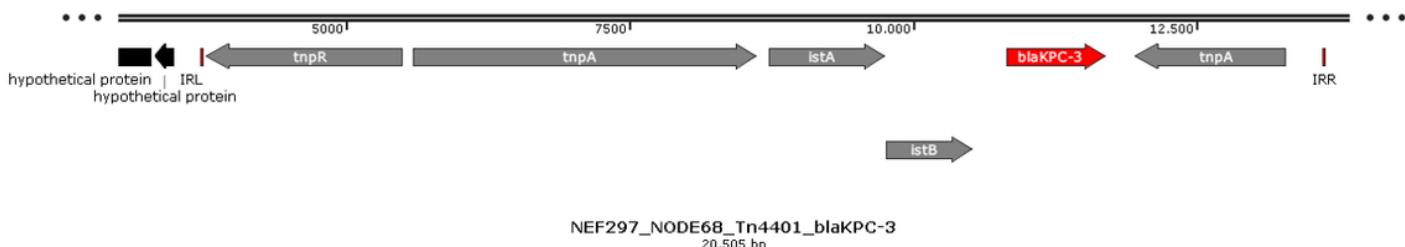


Figure 5

Genetic environment of blaKPC-3 gene in NEF197 (ST554) genome. The same genetic environment was observed in NEF 116, NEF119, NEF 130, NEF 131, NEF 145, NEF 196/2, NEF 197 (ST554) genomes

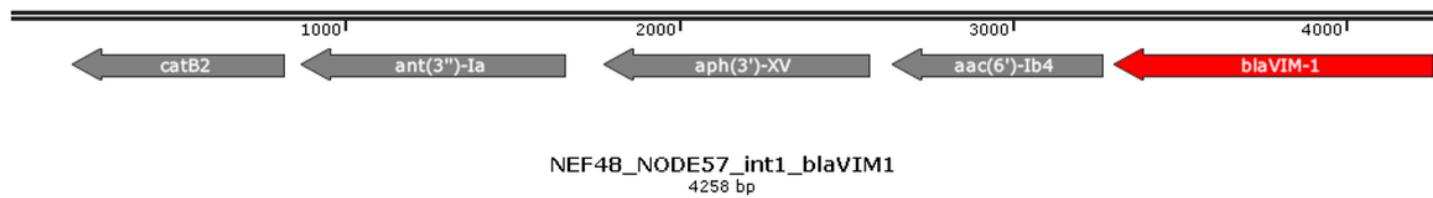


Figure 6

Genetic environment of blaVIM-1 gene in NEF 48 (ST37) genome. The same genetic environment was observed in NEF 112 (ST37) and NEF 123 (ST4525) genomes.

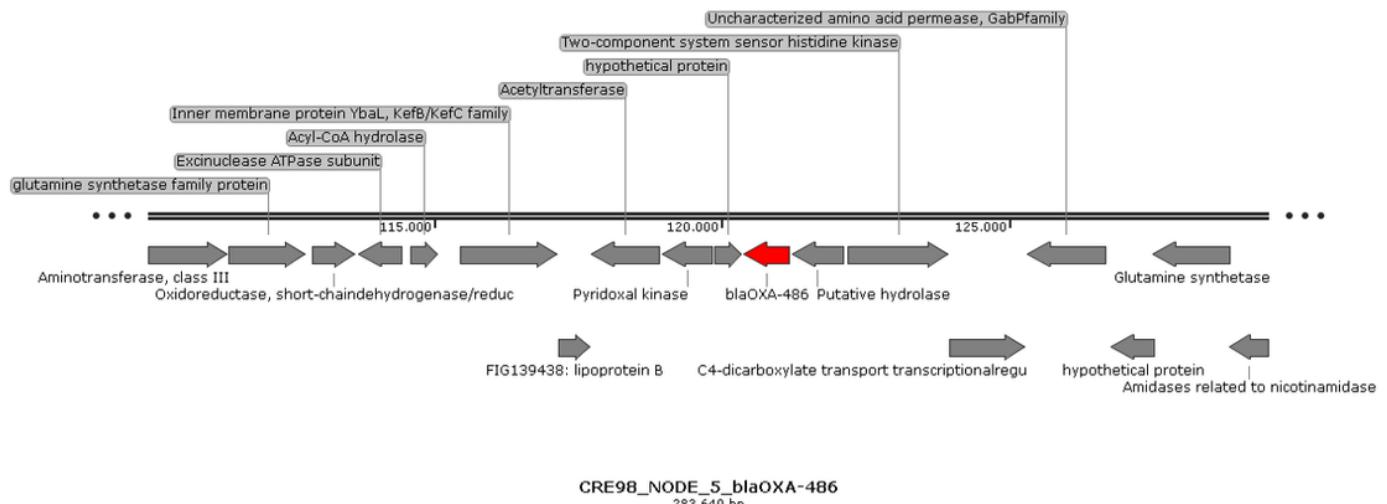


Figure 7

Genetic environment of blaOXA-486 gene in CRE 98 (ST274). The same genetic environment was observed for all detected genes belonging to the blaOXA-50 family in NEF 23 (ST938) , CRE 102 (ST938), CRE 153 (ST782), CRE 295 (ST885) and NEF 156 (ST395) genomes

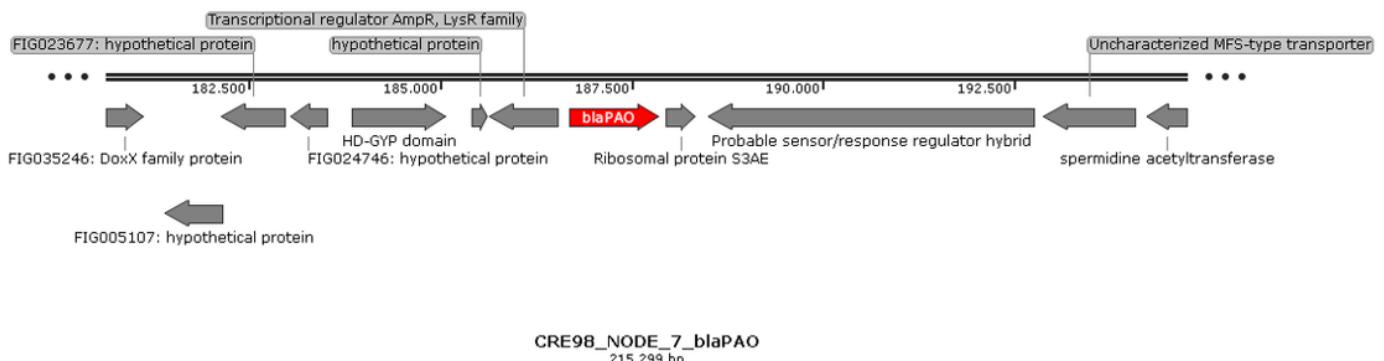


Figure 8

Genetic environment of blaPAO gene in CRE 98 (ST274) genome. The same genetic environment was observed in NEF 23 (ST938) , CRE 102 (ST938), CRE 153 (ST782), CRE 295 (ST885) and NEF 156 (ST395) genomes

Supplementary Files

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