Ministério da Saúde
FIOCRUZ
Instituto Oswaldo Cruz
LAPIH

Poster 2010

IDWeek 2016
October 26-30 • New Orleans, LA
Advancing Science, Improving Care

Evolution of Single Susceptible Profile in CC258-ST437 KPC-2-Producing *Klebsiella pneumoniae* Strains Causing Untreatable Infections Supported by *in vitro* Synergy Testing

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Abstract

Background:
Pan (PDR) or extensively drug-resistant (XDR) *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* (*Kp*) has become a growing threat.

Methods:

We retrospectively investigated phenotypic profile of 353 *Kp* isolates from 258 patients hospitalized from December 2014 to August 2015. Chart review was performed on cases with PDR- or XDR-*Kp* possible untreatable infection (UI). Proved UI was defined as any systemic infection caused by PDR/XDR strain in which the susceptible drugs are not recommended for the site of infection (including biofilm) or available in local market, the infection cannot be surgically removed and antagonism or non-synergistic action was evidenced by any combination therapy synergy testing.

Results:

Among 196 *Kp* detected from clinical samples, 16% (32/196) was PDR (n=4) /XDR (n=28)-*Kp* strains, corresponding to 38% (32/84) of MDR *Kp* isolates. High MICs ≥16µg/ml were found in 97% (29/30) of meropenem-resistant *Kp*. 15% (17/113) was polymyxin B (E-test)/colistin (Vitek-2) resistant. The incidence densities of clinical MDR, XDR and PDR *Kp* cases per 1,000 patient days were 1.0, 0.4 and 0.05, respectively (Fig. 1). We diagnosed two cases with proved UI caused by PDR KPC-2 *Kp* strains with none enough synergistic activity achieved at 4, 6, 8 and 24 h at target concentrations of meropenem 0.5 × MIC plus colistin 1 × MIC. Three strains recovered from proved untreatable cases (CCBH 17440 and CCBH 17428) and a patient with XDR *Kp* infection (CCBH 19496), who had close contact during hospitalization, pertained to same PFGE clone (Fig. 1) and MLST 437. Through whole genome sequencing (CCBH 17440) we detected resistance genes corroborating the resistance phenotype. Virulence genes and other features demonstrated several potential abilities to invade tissues and be maintained within hospital environment.

Conclusions:

ST437 is one of the most disseminated in Brazil among KPC-2 *Kp* and belongs to the worldwide clonal complex 11. Our findings emphasize the urgent need for global actions in the fight against PDR/XDR KPC-2 *Kp* and new treatment options. The association of important resistance and virulence factors makes this pathogen successful at infections and to expansion and evolution, leading to PDR phenotype and UI in Brazilian hospitals.

Acknowledgements

No financial support was received for the work performed that contributed to this poster.

Introduction

- Evolution of *Klebsiella pneumoniae* (*Kp*) to a pandrug resistant (PDR) phenotype poses an urgent serious warning to the world[1].
- Mortality of bloodstream infections (BSI) caused by a more susceptible phenotype as carbapenem-resistant (CR), polymyxin-susceptible *Kp* has been reported as high as 50%[2].
- We describe the emergence of clonal KPC-2-*Kp* strains with single susceptibility profile (SSP) in Brazil, pertained to the most prevalent worldwide clonal complex 258[3-5], causing untreatable infections (UI), investigated initially by time kill curve analysis with old and novel antimicrobial combination therapies. We also report the results of the hospital-wide survey for multidrug resistance phenotypes in *Kp* after the first detection of SSP-KPC-2-*Kp* strains in a 500-bed federal tertiary hospital in Rio de Janeiro, Brazil.

Methods

Bacterial Identification, Susceptibility Testing, and Molecular Typing

- *Kp* strains (CCBH17440 and CCBH17428) recovered from the patients' blood (Case-1) and tracheal aspirate (Case-2) were identified by automated Vitek-2 system and classical recommended biochemical tests.
- Broth microdilution, Etest (Biomérieux) and disk diffusion (Oxoid; Hampshire, UK) methods according to CLSI criteria[6] and EUCAST 2015 breakpoints for tigecycline[7].
- Multiplex PCR assay to detect carbapenemase genes bla_{KPC-2}, bla_{NDM-1} and bla_{OXA-48} [9].
- Pulsed field gel electrophoresis (PFGE) with XbaI digestion was performed (http://www.cdc.gov/pulsenet/index.html) (Fig. 1).

Whole-genome Sequencing and Phylogenetic Analysis

- Genomic DNA of CCBH17440 was sequenced using an Illumina MiSeq platform (Illumina Inc, USA).
- Resistance, virulence genes, and sequence type were analyzed using ResFinder, VirulenceFinder, and MLST platforms respectively (http://www.genomicepidemiology.org) and manual curation resorting Geneious (http://www.geneious.com), Blastn (NCBI) and Bigsdb database (http://bigsdb.pasteur.fr/klebsiella).
- PlasmidFinder to identify plasmids and Provean platform (http://provean.jcvi.org/index.php) to predict alterations on biological function of proteins, using *Kp* MGH 78578 (CP000647.1) as reference.

Antimicrobial Synergy Testing

- Time–kill studies of CCBH17440 and CCBH17428 strains were performed in duplicate to target initial inoculums of ~1x 10^6 cfu/mL, and combination of colistin at 16 mg/L (0.5xMIC of both organisms) and meropenem at 49mg/L (*f*Cmax of meropenem 1 g) against each strain.
- Daptomycin at 9.39 mg/L (*f*Cmax of daptomycin 6 mg/kg) was added to investigate the potential additional benefit compared to meropenem plus colistin alone.
- Synergy was defined as a > 2 $\log_{10}$ cfu/mL reduction compared to the most active single agent of the combination while also achieving ≥ 1 $\log_{10}$ cfu/mL reduction from the initial inoculum at 24 hours.

Hospital-wide Surveillance for PDR *Kp* Phenotype

- All routine antimicrobial susceptibility testing of *Kp* strains recovered from clinical and surveillance cultures were followed to investigate all *Kp* phenotypes, from December 2014 to August 2015.
- Clinical strains with initial possible-PDR or -XDR profiles with non-susceptibility to polymyxins were preserved for additional microbiological testing.
- Incidence rates per 1,000 patient-days were monthly calculated by dividing the number of *Kp* isolates with the specific phenotypes, according to Magiorakos et al (2012)[10], by the total number of inpatient-days.

Untreatable *Kp* Infections

- Chart review was performed in all hospitalized patients harboring *Kp* with possible-PDR or -XDR profile and polymyxins non susceptibility.
- UI was arbitrarily defined for surveillance purpose only as any systemic monomicrobial infection caused by possible-PDR-*Kp* or -XDR-*Kp* strain in which the susceptible drugs are not recommended for the site of infection, including infections possibly forming biofilm, or available in the local market, and the infection cannot be removed surgically or by device withdrawal.

Results

Table 1. Patient data and susceptibility profile of CC258-ST437-KPC-2-producing *Klebsiella pneumoniae* isolates.

Patient Data				Strain	Antimicrobial Susceptibility Testing[a] MIC (mg/L)																							
Gender Age	Co-morbidities	Admission	Sample	Outcome	CCBH	AKN	GEN	NET	TOB	SAM	PTZ	CAZ	CTT	CZ	CZA	FEP	FOX	XM	ERT	IMP	MEM	CIP	LEV	SUT	TCG	FOS	COL	PB	DOXY
M/72	Tetanus, chronic renal failure, MDR *Acinetobacter baumannii* VAP, suspected CDAD	ID ICU	Blood	Death	**17440**	>64	>64	>64	>64	≥32	≥128	16	>64	>64	0.5	≥64	≥64	>256	64	≥32	>64	≥4	>64	>64	2	>64	32	24	8
F/25	Rhombencephalitis, *Stenothrophomonas maltophilia* VAP, suspected CDAD, acute renal failure, hemodialysis	ID ICU, MS ICU Tracheal aspirate		Death	**17428**	≥64	≥64	≥64	>64	≥32	≥128	16	≥64	≥64	0.5	≥64	≥64	>256	64	≥32	>64	≥4	>64	>64	2	>64	64	32	8

A Dendrogram generated with Small Digestion PFGE, Phenotype Profiles and MLST of KPC-2 producing Polymyxin-Resistant *Klebsiella pneumoniae* Strains

B Incidence Density of *Klebsiella pneumoniae* Phenotypes. Temporal Occurrences of Clonally PFGE-Related KPC-2 producing Polymyxin-Resistant *Klebsiella pneumoniae* Strains (red circles)

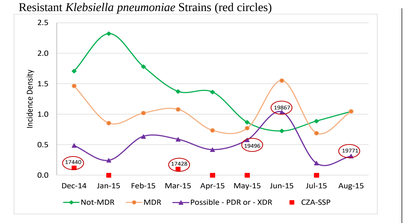
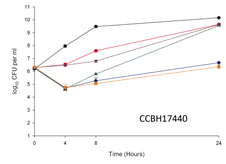


Figure 1. *Klebsiella pneumoniae* detected in clinical samples in a tertiary hospital of Rio de Janeiro, Brazil



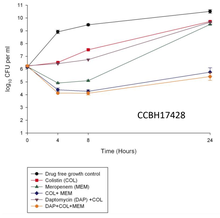


Figure 2. Time Kill curves.

Table 2. Genetic and other features detected in CC258-ST437-KPC-2 producing *Klebsiella pneumoniae* strain CCBH17440.

Features	Potential Abilities
armA, aac(6')Ib-cr-2	aminoglycosides resistance[a]
oqxB, oqxA, qnrA1, Ser83Ile mutation in *gyrA, aac(6')Ib-cr-2*	fluoroquinolones resistance
Val130 to Ala mutation in *oqxR*	tygecycline resistance
bla_{OXA-1}, bla_{TEM-1A}, $bla_{CTX-M-9}$, bla_{SHV-11}, bla_{KPC-2}	beta-lactams resistance, including extended-spectrum beta-lactamases (ESBL) and a carbapenemase
catB3, catA1, oqxB, oqxA	cloramphenicol resistance
dfrA8	trimethoprim resistance
arr-3	rifampim resistance
mph(E), msr(E)	macrolide resistance
fosA	fosfomycin resistance
mgrb gene disrupted by a IS5-like	colistin and polymyxin B resistance
terD, terW, terX, terZ	heavy metals resistance, protection from other forms of oxidative stress or agents causing membrane damage
iutA, entB	siderophore, iron-sequestering compounds - more efficiently acquire iron
sitA	iron transport system, required for virulence of *Salmonella typhimurium*
mrkA, mrkC, mrkD, mrkF, mrkH, mrkI, mrkJ. fimH,	adhesin types 1 and 3 fimbriae, bind host derived matrix components, the ability to colonize, invasion, and biofilm formation

Conclusions

- Our findings emphasize the urgent need for coordinated actions in the fight against PDR/XDR KPC-2 *Kp* possibly linked to untreatable infections.
- Confluence of virulence and resistance in one of the most disseminated multilocus sequence types in Brazil, pertained to an international high-risk clonal complex, may represent a new and serious problem in the global management of *Kp* infection.
- These findings must be useful as alert to speed access and development of therapeutic options and other unmet needs against this exceptionally prepared *Kp*.