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Rapid Resistome Fingerprinting and Clonal Lineage Profiling of Carbapenem-Resistant *Klebsiella pneumoniae* Isolates by Targeted Next-Generation Sequencing

Fabio Arena,[a] P. Alexander Rolfe,[b] Graeme Doran,[b] Viola Conte,[a] Sarah Gruszka,[b] Thomas Clarke,[b] Yemi Adesokan,[b] Tommaso Giani,[a] Gian Maria Rossolini[a,c,d]

Department of Medical Biotechnologies, University of Siena, Siena, Italy[a]; Pathogenica Inc., Boston, Massachusetts, USA[b]; Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy[c]; Clinical Microbiology and Virology Unit, Florence Careggi University Hospital, Florence, Italy[d]

Thirty-two carbapenem-resistant *Klebsiella pneumoniae* isolates, representative of different resistance mechanisms and clonal lineages, were analyzed with the Pathogenica HAI BioDetection system, based on targeted next-generation sequencing (NGS) technology. With most strains, the system simultaneously yielded comprehensive information on relevant β-lactam resistance determinants and accurate discrimination of clonal lineages, in a shorter time frame and in a less labor-intensive manner than currently available methods for molecular epidemiology analysis. Results supported the usefulness of targeted NGS-based technologies for similar applications.

The dissemination of carbapenem-resistant *Enterobacteriaceae* (CRE) has become a major public health problem of global dimensions, although with notable geographical variability (1, 2). Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains constitute the majority of CRE in clinical settings (3, 4), and their epidemic diffusion is often sustained by the expansion of successful "high-risk" carbapenemase-producing clones, such as *K. pneumoniae* strains of sequence type (ST) 258 producing KPC-type enzymes (5, 6).

Surveillance and efficient infection control practices are of paramount importance to limit the spread of CRE within health care structures (7, 8), their crucial role being emphasized by the dearth of available therapeutic options (9) and by the high mortality rates observed in infected patients (2, 10–12). To this purpose, rapid identification of resistance mechanisms and of high-risk clonal lineages of CRE are essential steps. However, the two reference methods for CRE typing, i.e., multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE), are time-consuming, expensive, and technically demanding procedures (13), and the same is partially true for the conventional methods for molecular detection and identification of resistance determinants (14).

Next-generation sequencing (NGS) technologies can simultaneously provide comprehensive information on the presence of resistance genes and on the clonal lineage of different isolates (15–18) and are promising for similar applications.

The Pathogenica HAI BioDetection system (Pathogenica Inc., Boston, MA, USA) is a commercial in vitro diagnostic (IVD) CE marked system, which yields simultaneous information on species identification, relevant resistance determinants, and clonal profiling of several bacterial pathogens. The system includes a set of probes targeted to bind specific genes. Each probe enriches by PCR the abundance of these loci and appends adaptor sequences to enable sequencing of this library of amplicons using an NGS platform. The product software performs multiple alignments across these sequenced amplicons and matches the contigs obtained with an internal database. Results are automatically incorporated in an electronic format, enabling comparison with new tested isolates, and also includes analytical software able to automatically and quickly process the sequence output into simple reports of species and resistance genes present.

In this work, we investigated the performance of this system for rapid characterization of the β-lactam resistome and clonal profiling of a collection of CRKP representative of different β-lactam resistance mechanisms and clonal lineages.

Thirty-two CRKP strains collected between 2009 and 2012, from various health care centers distributed across Italy, were investigated. Four strains of other Gram-negative species (*Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*), previously identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Vitek MS, bioMérieux, Marcy l'Etoile, France) and/or amplification and sequencing of 16S rRNA, were also included as a control for identification of common Gram-negative species other than *K. pneumoniae* by the system.

All CRKP strains had been previously characterized in the Microbiology Laboratory of the Department of Medical Biotechnologies, University of Siena, by MLST (19) and PFGE (20) and for carbapenem resistance mechanisms and had been selected as being representative of different resistance mechanisms and clonal lineages (Fig. 1). Interpretation of PFGE results had been made according to the work of van Belkum et al. (21). The presence of carbapenemases and other relevant ß-lactamase genes (including bla_{IMP}, bla_{VIM}, bla_{OXA-48}, bla_{NDM}, bla_{KPC}, and bla_{FOX}) had been investigated using primers and methods previously described (5, 22). Alleles encoding the outer membrane proteins OmpK35 and OmpK36 had been characterized as previously described (23).

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Address correspondence to Gian Maria Rossolini, gianmaria.rossolini@unifi.it.

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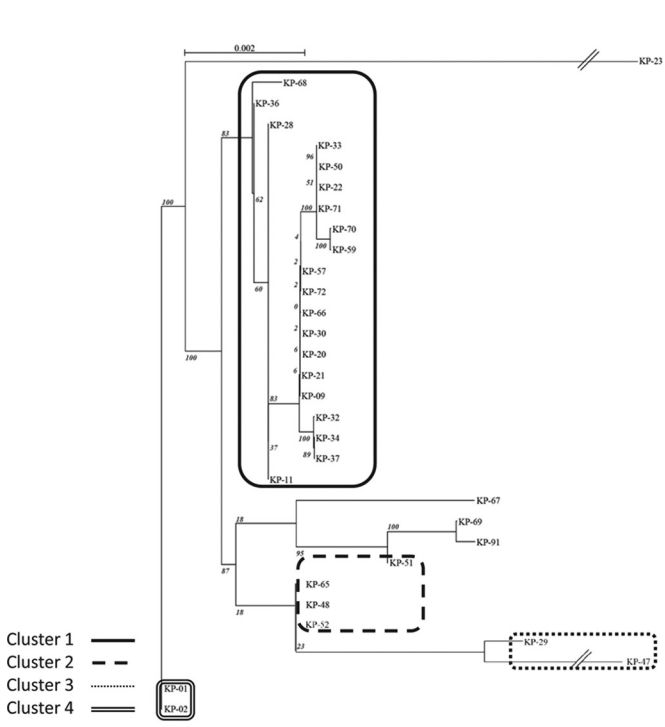

Strain ID	Year	Sequence Type	PFGE profile
23	2011	299	V0
68	2012	258	E3
36	2011	258	A3
28	2011	258	A6
33	2012	512	A4
50	2012	512	A4
22	2012	512	A4
71	2012	512	A4
70	2012	554	A7
59	2012	258	A1
57	2012	258	A2
72	2012	512	A4
66	2012	258	A3
30	2012	512	A0
20	2012	512	A4
21	2012	512	A4
9	2009	258	A11
32	2011	512	A4
34	2011	258	A2
37	2011	512	A0
11	2011	258	A10
67	2012	147	C0
69	2012	15	D0
91	2010	14	F0
51	2012	101	B2
65	2012	101	B1
48	2012	101	B2
52	2012	101	B2
29	2012	16	X0
47	2012	16	X0
1	2008	37	N0
2	2008	37	N0

FIG 1 Cluster groups of isolates obtained from different outbreak episodes. Isolates in cluster 1 are representative of the recent nationwide oligoclonal diffusion of KPC-producing isolates in Italy (5). Isolates in clusters 2, 3, and 4 were obtained from different earlier outbreaks of CRKP infection in Italian hospitals (22; also this work). The phylogenetic tree was generated using RAxML software. After tree construction, each tree's leaves were ordered using the optimal leaf-ordering algorithm of Bar-Joseph et al. (28). Numbers at nodes represent the probability of each node, based on the bootstrap method used for resampling.

To verify the performance of Pathogenica HAI BioDetection system in the detection of other β-lactamases, when revealed, the presence of $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} was confirmed by PCR and sequencing (24, 25). Amplicons were sequenced on both strands at an external facility (Macrogen Inc., Seoul, South Korea), and sequences were compared with those available in GenBank using BLAST.

For analysis with the Pathogenica HAI BioDetection system, genomic DNA of each strain was extracted, processing 400 μl of a 0.5 McFarland suspension in normal saline using the Complex400_V3_DSP protocol with the DSP virus/pathogen midikit on a QIAsymphony SP (Qiagen, Valencia, CA). Each sample was diluted to a working DNA concentration of 0.5 ng/μl and prepared with the Pathogenica HAI BioDetection kit according to the manufacturer's instructions. Template preparation was carried out with the Ion PGM template OT2 200 kit (Life Technologies, Gaithersburg, MD, USA), and sequencing was performed using the Ion PGM sequencing 200 kit v2 with a PGM sequencer (Life Technologies) according to the manufacturer's instructions. Data were analyzed using an internal version of the Pathogenica HAI BioDetection software v1.2. The RAxML software was used to generate a phylogenetic tree for the samples with a GTR+Γ model (26).

The analysis was run under blinded conditions. The time to perform the analysis of each strain, from purified DNA sample to results provided by the system, was 12.5 h.

All strains tested were correctly identified to the species level by the Pathogenica system. Concerning analysis of the β-lactam resistance mechanisms, the system correctly detected the presence of all NDM-1, KPC-type, and VIM-type ß-lactamase genes. All TEM-type, SHV-type, and CTX-M-type variants detected by the system were confirmed (Table 1). On the other hand, the genes encoding the OXA-48 and FOX-7 ß-lactamases were not detected, since the current version of the system does not include probes targeting those genes. The system was also unable to detect genetic alterations of porin genes leading to permeability defects that can cause decreased carbapenem susceptibility in strains producing extended-spectrum β-lactamases (ESBLs).

Results on ß-lactamase genotypes are provided in terms of ß-lactamase types (e.g., TEM, SHV, CTX-M, KPC, VIM, and NDM). With TEM and SHV types, the possibility of an ESBL genotype is also reported in the presence of mutations known to be associated with ESBL activity (Table 1). However, since the sequences targeted for amplification and sequencing do not cover the entire coding sequences of the bla_{TEM} and bla_{SHV} genes, some mutations associated with ESBL activity (e.g., that leading to G238S substitution in TEM-type enzymes) can remain undetected with the current version of the kit.

Additional sequence analysis of the contigs, automatically generated by the system with ß-lactamase genes, could provide additional information about the nature of their groups/allelic variants. For instance, with $bla_{\text{CTX-M-type}}$ genes, the sequence analysis allowed distinction between group 1 and group 9 CTX-M-type lineages, although they cannot discriminate the entire repertoire of known allelic variants of each group. Within $bla_{\text{KPC-type}}$ genes, it was possible to identify some point mutations associated with

TABLE 1 Results of analysis of β-lactamase content of the 32 CRKP strains by the Pathogenica HAI BioDetection system

Strain	Beta-lactam resistance determinants detected with:	
	PCR and sequencing	Pathogenica
KP-01	SHV-12, CTX-M-15, TEM-1 plus OmpK35 porin loss	SHV, CTX-M, TEM
KP-02	SHV-12, CTX-M-15, TEM-1 plus OmpK35 porin loss	SHV, CTX-M, TEM
KP-09	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-11	KPC-2, SHV-12, TEM-1	KPC, SHV, TEM
KP-20	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-21	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-22	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-23	VIM-1, SHV-12	VIM, SHV
KP-28	KPC-2, SHV-12, TEM-1	KPC, SHV, TEM
KP-29	OXA-48, CTX-M-15, SHV-1, TEM-1	CTX-M, SHV, TEM
KP-30	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-32	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-33	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-34	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-36	KPC-3, SHV-12	KPC, SHV
KP-37	KPC-2, SHV-12, TEM-1	KPC, SHV, TEM
KP-47	OXA-48, CTX-M-15, SHV-1, TEM-1	CTX-M, SHV, TEM
KP-48	KPC-2, SHV-1, TEM-1	KPC, SHV, TEM
KP-50	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-51	KPC-2, SHV-1, TEM-1	KPC, SHV, TEM
KP-52	KPC-2, SHV-1, TEM-1	KPC, SHV, TEM
KP-57	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-59	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-65	KPC-2, SHV-1, TEM-1	KPC, SHV, TEM
KP-66	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-67	KPC-3, SHV-1, TEM-1	KPC, SHV, TEM
KP-68	KPC-2, SHV-1	KPC, SHV
KP-69	KPC-2, SHV-1, TEM-1	KPC, SHV, TEM
KP-70	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-71	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-72	KPC-3, SHV-11, TEM-1	KPC, SHV, TEM
KP-91	FOX-7, SHV-1, TEM-1 plus OmpK35 porin loss	SHV, TEM

KPC allelic variability (for instance enabling distinction between bla_{KPC-2} and bla_{KPC-3}). With $bla_{VIM-type}$ it was possible to distinguish between members of the VIM-1 and VIM-2 lineages.

Concerning the analysis of clonal relatedness, 17 distinct lineages were recognized by the Pathogenica system, with an overall high concordance with the reference typing methods (Fig. 1). In terms of discriminatory power, the Pathogenica system showed superiority over the other typing techniques, with a Simpson's index of discrimination (27) of 94.8%, compared to 82.1% and 92.7% for MLST and PFGE, respectively.

The largest barriers to the implementation of NGS technologies in clinical microbiology are represented by the relatively high cost of DNA sequencing platforms and sequencing reactions and by the difficulties encountered in translating the magnitude of data obtained with these technologies into information that will be useful to microbiologists in a timely manner. Altogether, the Pathogenica system was able to provide meaningful information on resistance determinants and clonal lineages with most of the tested CRKP strains, significantly reducing the bioinformatic burden and the costs (approximately $100 [U.S. dollars] per isolates) compared to a conventional whole-genome sequencing approach.

The major advantage of the Pathogenica system is the ability to simultaneously provide identification to the species level, a repertoire of relevant resistance genes, and fine-tuned clonal profiling of bacterial isolates, in a less technically demanding and labor-intensive procedure and within a shorter time frame (approximately 25 min of technician's hands-on time for library preparation and 30 min of sample and data processing time) than the standard methods currently being used in clinical microbiology laboratories for characterization of resistance genes (e.g., conventional PCR and sequencing) and clonal profiling (e.g., PFGE and MLST).

In conclusion, this work describes the first application of an innovative commercial system based on a targeted sequencing approach using an NGS platform in the characterization of a collection of CRKP strains representative of many emerging resistance determinants and clones of clinical relevance.

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