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MALDI-TOF mass spectrometry and *bla*$_{kpc}$ gene phylogenetic analysis of an outbreak of carbapenem-resistant *K. pneumoniae* strains

Silvia Angeletti[1], Giordano Dicuonzo[1], Alessandra Lo Presti[2], Eleonora Cella[2, 3], Francesca Crea[1], Alessandra Avola[1], Massimiliano Andrea Vitali[4], Marco Fagioni[5], Lucia De Florio[1]

[1]Clinical Pathology and Microbiology Laboratory, University Hospital Campus Bio-Medico of Rome, Italy;
[2]Department of Infectious, Parasitic, and Immune-Mediated Diseases, Epidemiology Unit, Reference Centre on Phylogeny, Molecular Epidemiology, and Microbial Evolution (FEMEM), National Institute of Health, Rome, Italy;
[3]Department of Public Health and Infectious Diseases, Sapienza University, Rome, Italy;
[4]Hospital Management, University Hospital "Campus Bio-Medico" of Rome, Italy;
[5]Mass Spectrometry Support, Bruker Daltonics Srl , Macerata, Italy

SUMMARY

Carbapenem-resistant *Klebsiella pneumoniae* isolates are an important cause of nosocomial infections. This study evaluated a rapid cost-saving method based on MALDI-TOF technology, was and compared it with phenotypic, genotypic and epidemiological data for characterization of KPC-Kp strains consecutively isolated during a supposed outbreak. Twenty-five consecutive KPC *Klebsiella pneumoniae* isolates were identified and clustered by the MALDI Biotyper (Bruker, Daltonics). To display and rank the variance within a data set, principal component analysis (PCA) was performed. ClinProTools models were generated to investigate the highest sum of recognition capability and cross-validation. A Class dendrogram of isolates was constructed using ClinproTool. MLST was performed sequencing *gapA*, *infB*, *mdh*, *pgi*, *rpoB*, *phoE* and *tonB* genes. *bla*$_{kpc}$ and *cps* genes were typed. Phylogenetic analysis and genetic distance of the KPC gene were performed using the MEGA6 software.
PCA analysis defined two clusters, I and II, which were identified in a dendrogram by both temporal split and different antimicrobial susceptibility profiles. These clusters were composed mostly of strains of the same sequence type (ST512), the most prevalent ST in Italy, and the same *cps* (type 2). In cluster II, *bla*$_{kpc}$ genotype resulted more variable than in cluster I. Phylogenetic analysis confirmed the genetic diversity in both clusters supported by the epidemiological data. Our study confirms that MALDI-TOF can be a rapid and cost-saving method for epidemiological clustering of KPC *K. pneumoniae* isolates and its association with *bla*$_{kpc}$ genotyping represents a reliable method to recognize possible clonal strains in nosocomial settings.

KEY WORDS: MALDI-TOF clustering, Carbapenem resistance, MLST, *bla*$_{kpc}$ gene, Phylogenetic analysis.

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INTRODUCTION

Carbapenem-resistant *Klebsiella pneumoniae* isolates of the multilocus sequence type (ST) 258 have been described as an important cause of hospital infections, associated with longer of hospital stay, increased use of antibiotic therapy and high mortality rate. Resistance to carbapenems may involve several combined mechanisms among which production of specific carbapenem-hydrolysing β lactamases (carbapenemases) is the most common in clinical settings. First discovered in USA in 1996, *Klebsiella pneumoniae* carbapenemases have spread worldwide and represent a clinical and public health problem.

Corresponding author
Silvia Angeletti
Clinical Pathology and Microbiology Laboratory
University Hospital Campus Bio-Medico of Rome
Via Alvaro del Portillo, 200 - 00128, Rome, Italy
E-mail: s.angeletti@unicampus.it

Clonal dissemination of KPC isolates within countries and between countries has been described as the principal mechanism of the diffusion of these strains: the predominant clone worldwide is the ST258 lineage, a single locus variant of ST11 described in Hungary [Samuelsen *et al*., 2009; Munoz-Price *et al*., 2013; Arena *et al*., 2014; Mathers *et al*., 2011; Adler *et al*., 2014; Damjanova *et al*., 2008).

In Italy, the first KPC strain was isolated in 2008 from an inpatient with a complicated intra-abdominal infection in Florence (Giani *et al*., 2009). The isolate harbored the KPC-3 gene located in the transposon Tn*4401*, like the previously described ST258 isolated in Israel (Samra *et al*., 2007).

A first paper and subsequent countrywide survey have shown that the KPC ST258/512 isolates are the prevalent strains circulating in Italy (Arena *et al*., 2014; Giani *et al*., 2013). A recent study reported that ST258 strains can be divided into two clades, with different capsule polysaccharide gene (*cps*) regions (Deleo *et al*., 2014; Chen *et al*., 2014a).

The polysaccharide capsule is one of the primary determinants of antigenicity and antigenic diversity among *K. pneumoniae* and contributes to innate host defense elusion important for ST258 *K. pneumoniae* survival and spread in the host (Domenico *et al*., 1994).

A variation in the *cps* gene has also been described in the Italian epidemic ST 258/512 KPC strains which harbored at least two different types of *cps* gene clusters (D'Andrea *et al*., 2014).

Rapid identification of resistance mechanisms and of high-risk clonal lineages of carbapenem-resistant *Enterobacteriaceae* (CRE) and particularly of KPC are essential to take appropriate measures of prevention as well as transmission-based precautions (D'Andrea *et al*., 2014).

The two reference methods for KPC *Klebsiella pneumoniae* typing are multilocus sequence typing (MLST) and the pulsed-field gel electrophoresis (PFGE), time-consuming, expensive, and technically demanding procedures.

The aim of the present study was to evaluate the ability of MALDI-TOF technology to characterize KPC isolates according to their different pattern of TOF peaks. This method was applied to an outbreak of 25 KPC *Klebsiella pneumoniae* isolated at the University Hospital Campus Bio-Medico of Rome and compared to phenotypic, genotypic and epidemiological data. Furthermore, a phylogenetic analysis of the KPC-gene, conferring carbapenem resistance to each strain, was performed to evaluate the genetic relationship between the gene and the strains.

MATERIALS AND METHODS

Bacterial isolates. Between January 2012 and February 2013 a total of 25 consecutive non-replicate clinical KPC *Klebsiella pneumoniae* strains were isolated from inpatients at the University Hospital Campus Bio-Medico in Rome. Such isolates were recovered as follows: 10 from urinary tract infections, 7 from surgical site infections, 5 from bloodstream infections and 3 from respiratory tract infections. Antimicrobial susceptibility of the strains, selectively isolated in McConkey agar, was performed by the Vitek-2 Compact instrument (bio-Merieux, France).

MALDI-TOF MS (Bruker Biotyper; Bruker Daltonics, Bremen, Germany). Bacterial colonies were subjected to ethanol-formic acid protein extraction according to the MALDI Biotyper protocol (Bruker Daltonics GmbH, Bremen, Germany) (Matsumura *et al*., 2014). One microliter of the samples was prepared on a 96-spot polished steel target with 1µl of matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid (Bruker, Daltonics, Bremen, Germany) in 50% acetonitrile and 2.5% trifluoroacetic acid (Sigma-Aldrich, Milan, Italy). MALDI-TOF MS was performed on a Microflex LT controlled by FlexControl version 3.4 software (Bruker, Daltonics, Bremen, Germany). Spectra were acquired by the standard recommended proprietary method utilizing the Biotyper preprocessing standard method and the Biotyper MSP identification standard method (2,000 to 20,000 Da; linear positive method). Species were identified using the MALDI Biotyper 3.1 (Bruker) and its standard database (Bruker Taxonomy database version 3.3.1). Identification was provided with accompanying scores as follows: <1.7 = no reliable identity; ≥1.7 and <2.0 = identity at genus level; ≥2.0 = identity at species level (Matsumura *et al*., 2014).

ClinProTools model. The ClinProTools models

were generated using the three available algorithms (Genetic Algorithm, Supervised Neural Network, and Quick Classifier) in ClinProTools 3.0 software (Bruker, Daltonics, Bremen, Germany). The best models with the highest sum of recognition capability and cross-validation, corresponding to reliability and accuracy respectively, were used. ClinProTools model was used for MALDI-TOF clustering and dendrogram construction.

MALDI-TOF Bruker MS (Bruker Daltonics, Bremen, Germany) clustering and dendrogram construction. Each isolate was loaded on ClinproTools by spectra grouping function in order to allow the software to group all the technical replicates in one biological replicate, named Class by software. A Class dendrogram of all the study isolates was constructed using the ClinproTool dendrogram creation standard method (with the correlation distance measured by the average linkage algorithm) of the Biotyper 3.1 software (Bruker Daltonics, Germany). Clusters were then detailed and analyzed according to an arbitrary distance levels cutoff of 10 (logarithmic transformation of the value 500) in a maximum of 20 (logarithmic transformation of 1000).

Single-peak analysis. The ten peaks of the spectra with the highest intensity were collected for the peak statistics calculation and for each peak the area under the curve (AUC) was obtained. The detection performances of the peaks with the highest AUC in each comparison were studied using FlexAnalysis 3.4 (Bruker, Daltonics, Bremen, Germany).

After smoothing and baseline subtraction, the mass lists for each isolate were obtained using the centroid algorithm with a signal-to-noise threshold of 0.5 and a maximum of 500 peaks and exported to Microsoft Excel. ROC curves were constructed, and their optimal cutoff values were determined with the maximum Youden index.

PCA analysis. PCA is a widely used mathematical technique designed to extract, display and rank the variance within a data set (Shao *et al*., 2012). The overall goal of PCA is to reduce the dimensionality of a data set, simultaneously retaining the information present in the data. Statistical testing of the datasets was performed on the basis of principle component analysis (PCA). The loading values provided by PCA (established between -1 and 1) made it easy to select the contributing peaks for further analysis. The reduced datasets, the so-called PCs (principle components) were displayed in a score plot illustration, which was generated automatically by the software.

MLST analysis. MLST was performed according to the protocol described by Diancourt and colleagues (Diancourt *et al*., 2005) based on seven housekeeping genes: *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *infB* (translation initiation factor 2), *mdh* (malate dehydrogenase), *pgi* (phosphoglucose isomerase), *phoE* (phosphorine E), *rpoB* (betasubunit of RNA polymerase) and *tonB* (periplasmic energy transducer). The MLST database used for *K. pneumoniae* was found at http://www.pasteur.fr.

Cps gene analysis. *cps* was amplified following the protocol described by Chen L. *et al.* using two different primer pairs, one specific for *cps* type I (wzy 258-I-f TACGGGGATTCCGGGAACAGCA and wzy258-I-r ACAAAACCTCAATTGCTCTTCGGCT) and the other for cps type II (wzy258-II- f GCACAAGAGAAATTGGATCTGACAACG; wzy258-II-r ACTTCCAAATCCCATTGCAA CTG CT) (Chen *et al*., 2014b). *cps* type I or II results were confirmed by direct PCR product sequencing.

bla$_{kpc}$ gene analysis. bla$_{kpc}$ resistance gene was amplified using the primers described by Chen L. *et al.* (KPC-F107 TCGAACAGGACTTTGGCGGCT and KPC-R860 CC CTC GAGC GCGA G TCTA) changing the protocol by coupling the forward primer KPC-F107 with the reverse primer KPC-R860 to detect the 10 currently described *bla*KPC variants in a single amplification and sequencing reaction (Chen *et al*., 2011).

Phylogenetic analyses. The dataset included bla$_{kpc}$ gene sequences of 25 KPC *Klebsiella pneumoniae* isolates. All the sequences were aligned using CLUSTAL X software (Ciccozzi *et al.*, 2012) then manually edited with the Bioedit software (version 7.0.9) (Ciccozzi *et al.*, 2012). Phylogenetic analysis and genetic distance was carried out using the MEGA6 software (Tamura *et al*., 2013) with the Kimura 2-parameters model of substitution and the Neighbor-Joining (NJ) tree building method. The mean distance in Clade II among and within the groups was calculated (Gp1 and Gp2).

RESULTS

All of the 25 clinical isolates included in the study were identified as *Klebsiella pneumoniae* by MALDI-TOF MS with scores >2.0 (categorized as highly probable species identification) at the Biotyper software analysis.

ClinProTools model. Table 1 lists the performances of the best models for the detection or discrimination of the isolates grouped into different clusters, I and II. More than 90% of both the cross-validation and recognition capability was found in the models used for the discrimination of the Clusters.

MSP dendrogramm and clustering (ClinproTools). At the arbitrary distance level cutoff of 10 (logarithmic transformation of the value 500) in a maximum of 20 (logarithmic transformation of 1000), two clusters (Cluster I, n=14; Cluster II, n=11) were identified in a dendrogram (Figure 1). Isolates in Cluster I were from clinical specimens collected from January to

TABLE 1 - *Performances of the best models for the detection or discrimination of the isolates in the Cluster I and in the Cluster II.*

Algorithm	*Recognition Capability %*	*Cross Validation%*	*n Peak used*
Supervised Neural Network	100	95,83	2*
Quick Classifier	100	93,75	4*
Genetic Algorithm	100	100	8
Genetic Algorithm	100	100	9
Genetic Algorithm	100	100	10

*Automatic detected by ClinProt Tools.

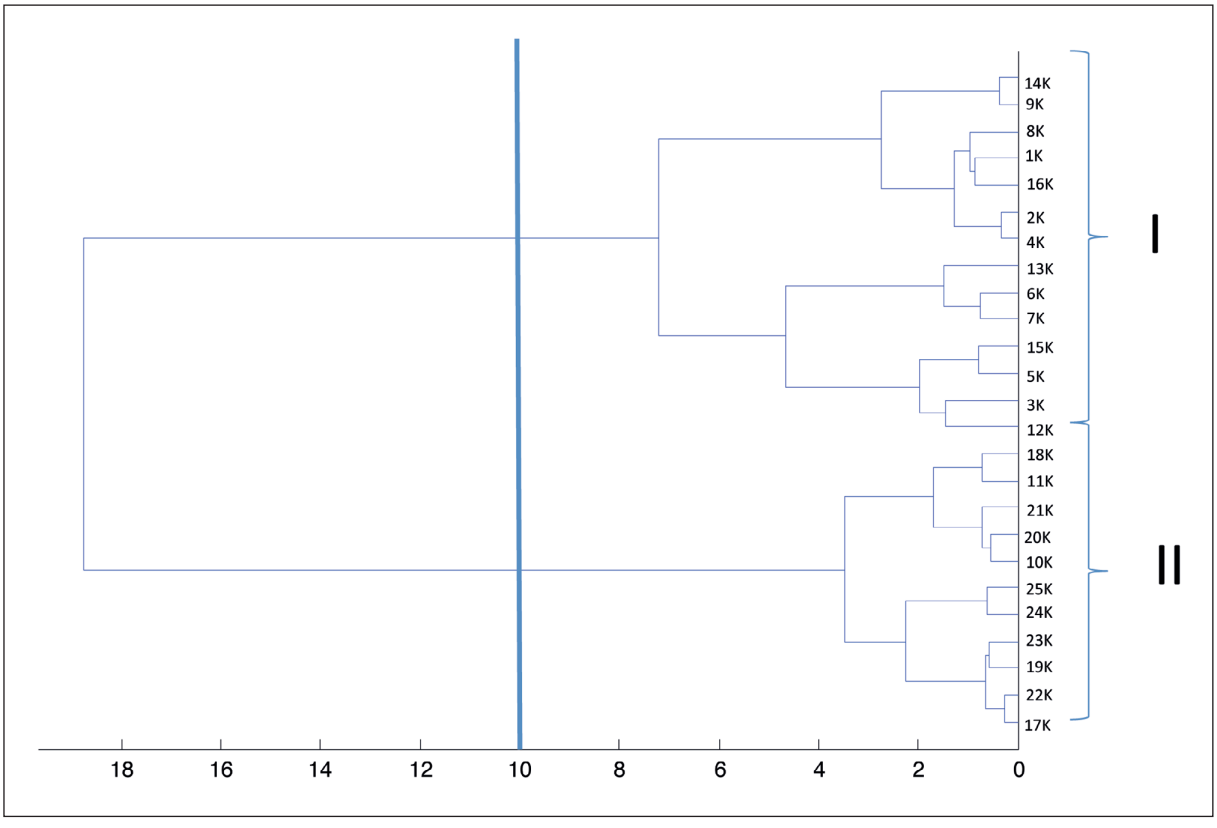

FIGURE 1 - *MSP classification dendrogram. Line position indicates the arbitrary distance levels at 10 used for strain clustering analysis.*

December 2012, while cluster II consisted of 11 strains isolated from October-November 2012 to February 2013.

Single peak analysis. The first ten peaks showing the highest AUC in the detection or discrimination of the strains used for the clustering and

TABLE 2 - *ClinProTools analyses: first 10 peaks with the highest Area Under the Curve (AUC) for the detection or discrimination of the isolates clustered in the Cluster I and Cluster II.*

Peak Mass	*AUC*	*Dave*	*PTTA*	*PWKW*	*PAD*	*Ave1*	*Ave2*	*Ave3*	*StdDev1*	*StdDev2*	*StdDev3*	*CV1*	*CV2*	*CV3*
4770	1	17.17	<0.000001	0.0000514	0.00214	7.49	4.09	21.26	2.28	1.06	3.29	30.43	25.95	15.49
6152	1	95.91	<0.000001	0.0000514	0.00666	53.19	101.44	5.53	8.39	22.46	4.23	15.78	22.14	76.49
7244	1	14.23	<0.000001	0.0000562	0.0605	8.41	4.39	18.62	2.3	1.06	4.05	27.32	24.14	21.74
6096	1	76.38	<0.000001	0.0000514	0.00105	34.76	78.93	2.55	12.21	11.35	1.07	35.13	14.38	42.12
8308	1	45.67	0.0000012	0.000067	0.0173	51.73	38.2	6.06	15.05	7.41	3.59	29.09	19.4	59.25
4738	0.94	13.81	0.00000208	0.000291	0.0267	16.09	8.25	22.06	5.31	2.75	3.68	32.97	33.32	16.69
9476	0.85	11.42	0.00000255	0.000596	0.159	16.62	8.27	19.69	4.9	2.36	3.14	29.45	28.51	15.97
6289	1	15.08	0.0000083	0.0000749	0.00368	7.28	5.57	20.65	2.97	0.84	6.06	40.73	15.04	29.33
4154	1	33.35	0.000016	0.000067	0.066	39.3	28.62	5.95	12.36	9.17	3.85	31.46	32.04	64.68
5381	1	21.84	0.000016	0.0000769	0.00105	6.08	5.12	26.96	2.17	0.77	9.74	35.67	15.07	36.12

Dave Difference between the maximal and the minimal average peak area/ intensity of all classes. PTTA P-value of ANOVA test (range 0-1; 0= good). PWkW P-value of Wilcoxon test, (range 0-1; 0= good). PAD P-value of Anderson-Darling test; gives information about normal distribution (range 0-1; 0= not normal distributed). AveN Peak area/intensity average of class N. StdDevN Standard deviation of the peak area/ intensity average of class N. CVN Coefficient of variation in % of class N.

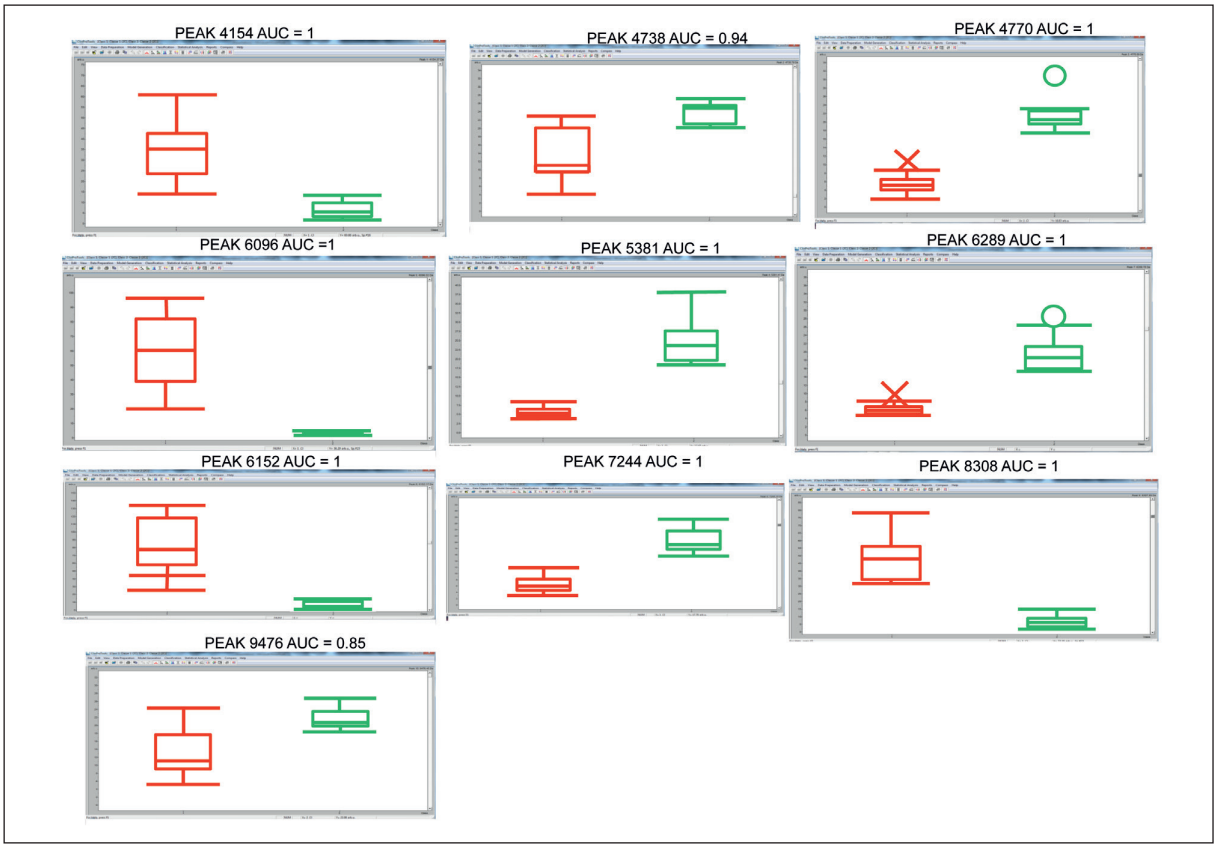

FIGURE 2 - *AUC values of the first 10 peaks with the highest performance in strain detection and clustering.*

MSP dendrogram construction (Cluster I or II) are reported in Table 2 and Figure 2.

PCA analysis. The PCA analysis, applied to the spectra of the 25 clinical isolates of *Klebsiella pneumoniae* identified two different clusters on the basis of the first three principal components (PC1, PC2 and PC 3) as displayed in the score plot illustration, reported in Figure 3.

Clusters I and II characterization. Isolates belonging to cluster I were from clinical samples collected during the year 2012 while those belonging to cluster II were isolated between the end of the year 2012 and the beginning of 2013 (October 2012/February 2013. The temporal split is well represented in the dendrogram (Figure 1) and in Table 3.

Besides being KPC, the 25 *K. pneumoniae* KPC strains distributed in the two clusters were also multidrug-resistant (MDR). The susceptibility to gentamicin, fosfomicin, colistin and cotrimoxazol (SXT) the only drugs showing variability, are reported in Table 3. Most of the isolates belonging to cluster I resulted fosfomicin- and

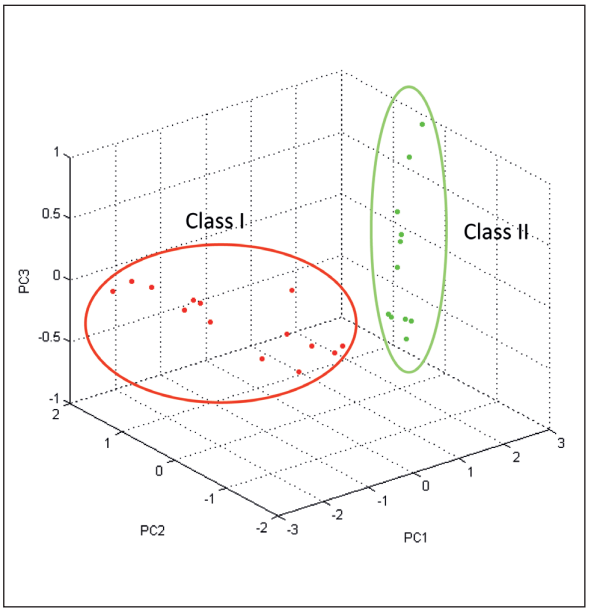

FIGURE 3 - *Tridimensional representation of the results of PCA analysis (3 principle components PC1, PC2, PC3) by ClinproTools software: clusters I and II spatial distribution.*

TABLE 3 - *Phenotypic and genotypic characteristics of isolates belonging to Cluster I and II.*

Isolates	*Collection date*	*Antibiotics MIC (μg/ml) and interpretation*	*ST*	*bla$_{kpc}$*	*cps*
Cluster I					
6K	20/01/2012	Gentamycin 4 I; SXT >320 R; Fosfomycin >64 R; Colistin <1S	512	3	II
7K	01/03/2012	Gentamycin 4 I; SXT >320 R; Fosfomycin <16 S; Colistin <1S	512	3	II
5K	22/05/2012	Gentamycin >4 R; SXT >320 R; Fosfomycin 32 S; Colistin <1S	**650**	3	II
15K	20/06/2012	Gentamycin 4 I; SXT <20 S; Fosfomycin 32 S; Colistin <1S	512	3	II
3K	27/06/2012	Gentamycin 4 I; SXT <20 S; Fosfomycin 32 S; Colistin <1S	512	3	II
12K	14/07/2012	Gentamycin >4 R; SXT >320 R; Fosfomycin <16 S; Colistin >16R	512	3	II
13K	01/08/2012	Gentamycin 4 I; SXT >320 R; Fosfomycin <16 S; Colistin >4 R	512	3	II
8K	08/04/2012	Gentamycin 4 I; SXT >320 R; Fosfomycin <16 S; Colistin <1S	512	3	II
1K	11/04/2012	Gentamycin 4 I; SXT <20 S; Fosfomycin 32 S; Colistin <1S	512	3	II
2K	08/05/2012	Gentamycin 4 I; SXT >320 R; Fosfomycin <16 S; Colistin >4 R	**650**	3	II
4K	01/08/2012	Gentamycin 4 I; SXT >320 R; Fosfomycin <16 S; Colistin <1S	512	3	II
9K	02/08/2012	Gentamycin 4 I; SXT >320 R; Fosfomycin <16 S; Colistin <1S	512	3	II
14K	15/11/2012	Gentamycin 4 I; SXT <20 S; Fosfomycin >64 R; Colistin >16R	512	3	II
16K	04/12/2012	Gentamycin <1 S; SXT >320 R; Fosfomycin >64 R; Colistin <1S	512	2	II
Cluster II					
10K	11/10/2012	Gentamycin 4 I; SXT >320 R; Fosfomycin >64 R; Colistin <1S	512	2	II
11K	07/11/2012	Gentamycin 4 I; SXT >320 R; Fosfomycin >64 R; Colistin <1S	512	2	II
20K	23/11/2012	Gentamycin 4 I; SXT <20 S; Fosfomycin >64 R; Colistin <1S	**37**	3	II
18K	26/11/2012	Gentamycin 4 I; SXT >320 R; Fosfomycin >64 R; Colistin <1S	512	3	II
21K	26/11/2012	Gentamycin <1 S; SXT >320 R; Fosfomycin >64 R; Colistin <1S	512	3	II
19K	09/01/2013	Gentamycin 4 I; SXT >320 R; Fosfomycin >64 R; Colistin <1S	512	3	II
17K	02/01/2013	Gentamycin 2 S; SXT >320 R; Fosfomycin >64 R; Colistin <1S	512	3	II
22K	24/01/2013	Gentamycin 4 I; SXT >320 R; Fosfomycin >64 R; Colistin <1S	512	3	II
23K	25/01/2013	Gentamycin 4 I; SXT >320 R; Fosfomycin >64 R; Colistin <1S	512	2	II
24K	15/02/2013	Gentamycin 4 I; SXT >320 R; Fosfomycin >64 R; Colistin <1S	512	3	II
25K	26/02/2013	Gentamycin 4 I; SXT >320 R; Fosfomycin >64 R; Colistin <1S	512	3	II

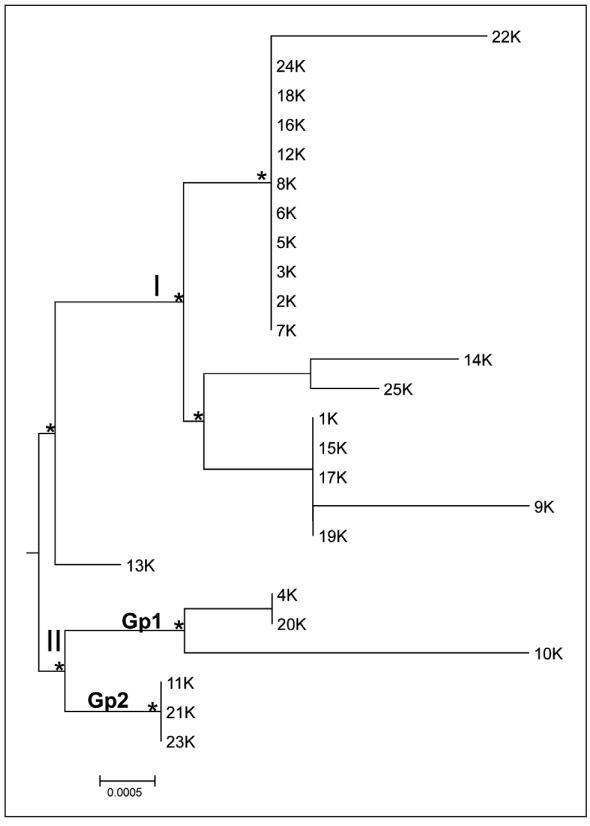

FIGURE 4 - *Neighbor-joining phylogenetic (NJ) tree of Klebsiella pneumoniae blakpc gene sequences. The scale bar at the bottom indicates 0.0005 nucleotide substitutions per site. One * along a branch represents a significant statistical support for the clade subtending that branch (p<0.001 in the zero-branch-length test and bootstrap support >75%).*

colistin-susceptible, while all isolates of cluster II were fosfomicin-resistant and colistin-susceptible. All strains belonging to the cluster II were SXT-resistant except one effectively resulting a ST different from the others (ST 37) (Table 3).

For gentamicin susceptibility, no significant differences between the two clusters were found except for one strain belonging to Cluster I (gentamicin-susceptible with bla_{kpc} gene type 2) (Table 3).

The molecular analysis by MLST, *cps* and bla_{kpc} genes was performed to reveal any difference between the strains clustered in the MSP dendrogram. MLST analysis showed that all 25 *K. pneumoniae* KPC strains were ST512 except three (two ST650 belonging to Cluster I and one ST 37 belonging to Cluster II) (Table 3). All isolates in Cluster I carried bla_{kpc} type 3 except one that was type 2, while 8/11 isolates of Cluster II had bla_{kpc} type 3 and 3/11 bla_{kpc} type 2 (Table 3). All 25 *K. pneumoniae* KPC strains bore c*ps* gene type II (Table 3).

Phylogenetic analyses. The NJ tree (Figure 4) shows two statistically different clades (clade I and clade II). Inside these clades statistically significant clusters were considered. Isolates belonging to these clusters are closely related each other. In clade II, two statistically significant clusters are displayed (Gp1 and Gp2). Within Gp2 two bla_{kpc} sequences (KPC-2 type within cluster II of the dendrogram) were strongly related.

The mean genetic distance calculated between clusters Gp1 and Gp2 was 0.04, the mean distance within cluster Gp1 was 0.03 while the mean distance within cluster Gp2 was 0.00.

DISCUSSION

Clonal dissemination of *K. pneumoniae* KPC isolates is an important cause of hospital infections with a high mortality rate. Rapid identification of these isolates to prevent clonal spread in a hospital setting is essential.

The results of this study demonstrated that the MALDI-TOF technique could represent a more rapid and inexpensive method to identify these isolates and characterize their short-term epidemiology than conventional methods. An evaluation of the time and cost required for each MALDI-TOF test was performed. Each identification is completed in 30 minutes at a cost of 50 Euro cents compared with conventional methods requiring at least 4-6 hours at a cost of 6 Euros.

By MALDI-TOF clustering, the 25 KPC isolates were divided in two clusters characterized by a defined temporal split: strains of Cluster I were isolated during the year 2012 and strains of Cluster II from the end of 2012 to the beginning of 2013. This temporal split confirms the ability of MALDI-TOF clustering to differentiate those isolates presumably not related.

A further analysis of the KPC isolates revealed that strains grouped by MALDI-TOF in Cluster I were phenotypically characterized by more

heterogeneous AST profiles than those in Cluster II (all fosfomicin-resistant and colistin-susceptible), thus suggesting the ability of MALDI-TOF to separate isolates into different clusters according to their phenotypic properties.
These results confirm data reported by Berrazeg *et al*., affirming that MALDI-TOF represents a successful tool for *K. pneumoniae* strains biotyping able to identify specific clusters associated with particular phenotypes from different clinical and geographical sources as well as from different seasonal periods (Berrazeg *et al*., 2013).
Genotyping of the 25 *K. pneumoniae* KPC isolates by MLST, *cps* and bla_{kpc} genes showed that MLST was not able to disclose substantial differences between the isolates. In fact, all isolates resulted ST 512 except two in Cluster I (ST 650, included with ST512 in the same clonal complex ST258) and one in Cluster II (ST 37). The two strains ST650 were clustered by MALDI-TOF in the same cluster (Cluster I) together with strain ST512. This could be justified because ST512 and ST650 represent different locus variants belonging to the same clonal group (ST258) (Chen *et al*., 2014c). On the other hand, strain ST37 was clustered by MALDI-TOF separately from ST650 in Cluster II: this strain is not part of the clonal group ST258 and its belonging to Cluster II should be due to other reasons of similarity with strains of Cluster II classified as ST512. Therefore, our data confirm that MLST does not seem able to disclose variations occurring in a narrow time frame probably because it is based on conserved genes analyses and is not sufficient for contact tracing in epidemics or single-clone pathogen characterization, as reported by other authors (Maiden *et al*., 2013; Snitkin *et al*., 2012; Berrazeg *et al*., 2013; Sachse *et al*., 2014). *cps* gene resulted type II in all isolates screened and it does not seem useful to follow isolates spread in a reduced temporal frame. Likewise, MLST *cps* gene analysis is based on a conserved gene. MLST, as other authors reported (Berrazeg *et al*., 2013), classifies bacteria according to their core genome representing less than 10% of the genome, while 90% of the genome is composed of accessory genes, as mobile genetic elements, largely influencing the bacterial phenotype. The conserved genes do not represent the majority of expressed proteins in contrast to the MALDI-TOF dendrogram analysis which is based on the functional and expressed proteins of the whole cell that is more representative of the whole phenotype. bla_{kpc} gene, carried by mobile genetic elements, resulted type 3 except in four cases (type 2) one isolate in Cluster I and three isolates in Cluster II. Phylogenetic analysis was performed to confirm if bla_{kpc} genotyping could really differentiate isolates on the basis of their KPC type and to compare this clustering with that obtained by MALDI-TOF. The phylogenetic analysis effectively confirmed the relationship between the three KPC-2 isolates belonging to Cluster II of the dendrogram and the lack of a relationship with the KPC-2 isolate in Cluster I; in fact the latter is located in clade I of the phylogenetic tree. To confirm these data, the genetic distance was computed and the three KPC-2 isolates belonging to Cluster II of the dendrogram resulted related. Interestingly, a very strong relationship between two KPC-2 isolates of clade II (Gp-2) was demonstrated by the absence of genetic distance.
These data were cross-checked with the epidemiological information available through the integrated informatics platform (MedArchiver) that manages patients records. The epidemiological analysis showed that the two KPC-2 isolates of clade II, closely related, were from patients that had shared a portable X-ray instrument on the same day. It is possible that this instrument was the carrier that transmitted the same strain *K. pneumoniae* KPC type-2.
Recently, other authors reported evidence of a horizontal transfer of carbapenemase encoding plasmids between *K. pneumoniae*, *E. cloacae*, and *C. freundii* within hospitalized patients and that this horizontal transfer could represent a mechanism of possible connections between patients and the hospital environment (Conlan *et al*., 2014).
In conclusion, MALDI-TOF clustering was comparable to that obtained by bla_{kpc} phylogenetic analysis, even if based on different principles. Probably, as other authors reported, genotyping based on a gene carried by genetic mobile elements (bla_{kpc} gene) could be more representative of the bacterial phenotypes than genotyping based on conserved genes (MLST) Berrazeg *et al*., 2013). This study confirmed that MAL-

DI-TOF, a rapid and cost saving method, could be appropriate to study local epidemiology in nosocomial settings (Berrazeg *et al*., 2013; Bernaschi *et. al*., 2013), and that its association with bla_{kpc} genotyping offers an advantage. These complementary approaches represent a reliable method to identify the clonal spreading of KPC isolates and to prevent the further diffusion of these strains in a nosocomial setting.

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