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Research paper

Klebsiella pneumoniae blaKPC-3 nosocomial epidemic: Bayesian and evolutionary analysis

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ABSTRACT

K. pneumoniae isolates carrying *bla*KPC-3 gene were collected to perform Bayesian phylogenetic and selective pressure analysis and to apply homology modeling to the KPC-3 protein. A dataset of 44 bla_{kpc}-3 gene sequences from clinical isolates of *K. pneumoniae* was used for Bayesian phylogenetic, selective pressure analysis and homology modeling. The mean evolutionary rate for bla_{kpc}-3 gene was 2.67×10^{-3} substitution/site/year (95% HPD: 3.4×10^{-4}–5.59×10^{-3}). The root of the Bayesian tree dated back to the year 2011 (95% HPD: 2007–2012). Two main clades (I and II) were identified. The population dynamics analysis showed an exponential growth from 2011 to 2013 and the reaching of a plateau. The phylogeographic reconstruction showed that the root of the tree had a probable common ancestor in the general surgery ward. Selective pressure analysis revealed twelve positively selected sites. Structural analysis of KPC-3 protein predicted that the amino acid mutations are destabilizing for the protein and could alter the substrate specificity. Phylogenetic analysis and homology modeling of *bla*KPC-3 gene could represent a useful tool to follow KPC spread in nosocomial setting and to evidence amino acid substitutions altering the substrate specificity.

1. Introduction

Enterobacteriaceae are a family of Gram-negative bacteria causing many human infections, particularly healthcare associated (ECDC, 2008–2009; Siegel et al., 2007). *Enterobacteriaceae* resistance to antibiotics is of great concern in the clinical management of patients, has become a public health problem, all over the world. (Mathers et al., 2015).

Beta-lactam antibiotics have represented the first-line therapy for bacterial infections. The introduction of this antimicrobial compounds in the clinical practice determined the progressive bacterial acquisition of different resistance mechanism inducing the scientific community to look for wide spectrum antibiotics such as ampicillin, first, second and third generation cephalosporins, till carbapenems, the last generation of beta-lactam antibiotics. (Rodríguez-Rojas et al., 2013).

Carbapenems resistance among *Enterobacteriaceae* (CRE), mostly caused by the presence of carbapenemase, a specific class A serine beta-lactamase (KPC), is increasing worldwide and represents an important public health problem (Grundmann et al., 2010). CRE pathogens are associated with higher morbidity and mortality as a consequence of the complex multidrug resistance phenotypes giving very few therapeutic options to the clinicians (Hirsch and Tam, 2010; Falagas et al., 2011; Borer et al., 2009; Nordmann et al., 2012). KPC mostly codified in a transposon, Tn4401, is located in different plasmids with a great potential for horizontal antibiotic resistant gene transfer, as reported in both clinical and laboratory settings (Sheppard et al., 2016; Mathers et al., 2015; Hardiman et al., 2016). KPC enzyme is Ambler class A beta-lactamase able of hydrolyzing carbapenems compounds. A large number of studies on class A β-lactamases, showed that mutations can alter the protein function leading to enzyme decreased stability (Meiering et al., 1992; Brown et al., 2010).

In Europe, several outbreaks of CRE have been reported in different countries (Canton et al., 2012). The highest prevalence of the CRE can be found in Greece, Italy, Turkey, and Israel, whereas the lowest is

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reported in Nordic countries, Switzerland, Germany and the Czech Republic. The CRE type prevalence changes between different countries because it has been associated with the population exchange occurring between them (Canton et al., 2012).

Evidence-based guidelines to prevent transmission of CRE exist and are currently applied in nosocomial settings (available at http://www.cdc.gov/hai/pdfs/cre/CRE-guidance-508.pdf).

Nevertheless, within the guidelines the mobility and the potential spread of the gene encoding KPC, *bla*KPC gene, that can complicate the nosocomial infection tracking, has not been reported yet.

In Italy, sporadic cases or outbreaks caused by CRE have been reported since the early 2000s but only in the year 2010 a notable increase in carbapenem resistant *K. pneumoniae* strains has been reported by the EARS-NET surveillance system (Giani et al., 2013; European Centre for Disease Prevention and Control (ECDC), 2012).

The bacterial pathogen *K. pneumoniae* is responsible for roughly 15% of Gram negative infections in hospital intensive care units (ICUs), primarily affecting immunocompromised patients; furthermore carbapenem resistance is primarily caused by the plasmid *K. pneumoniae* carbapenemase (KPC) gene (Snitkin et al., 2012).

Recently, the geographic and phylogenetic distribution of *K. pneumoniae* isolates collected in six different Italian hospitals were analyzed by whole-genome sequencing and compared to that available worldwide obtaining a data set of 319 genomes. The study was performed with the aim to give new insights on *K. pneumoniae* evolution and to draw a dated epidemiological scenario for this pathogen in Italy (Gaiarsa et al., 2015). The analyses allowed to perform a whole-species phylogeny and to detect a ~1.3-Mb recombinant event in all isolates of clonal complex 258, the most common carbapenem-resistant group of *K. pneumoniae* (Gaiarsa et al., 2015).

Molecular methods such as comparative genomics have been used to track antibiotic-resistant bacteria dissemination and transmission. Single Nucleotide Variants (SNV) analysis has been applied to describe the global spread of these pathogens as well as to analyze localized outbreaks (Mutreja et al., 2011; Harris et al., 2013; Snitkin et al., 2012).

Recently, some authors used SNV analysis to perform active surveillance for CRE in nosocomial setting. Authors concluded that co-inheritance of SNVs, distributed along the whole genome, has enough resolution to infer transmission, because the evolutionary rate of these molecular markers is on the same timescale as nosocomial spread (Conlan et al., 2014; Snitkin et al., 2012).

In a previous study, we analyzed a group of *K. pneumoniae* KPC circulating in the University Hospital Campus Bio-Medico by MALDI-TOF and phylogenetic analysis and showed that *bla*KPC gene sequencing could represent a useful tool to follow strains circulation in a nosocomial setting. Indeed, the phylogenetic analysis effectively confirmed the relationship between the KPC isolates clustering in the same manner as showed by MALDI-TOF dendrogram analysis (Angeletti et al., 2015) based on the 16S bacterial proteomic.

In the present study, a larger group of *K. pneumoniae* KPC circulating within different wards of the University Hospital Campus Bio-Medico was collected during the years 2012–2015 to evaluate the movement of *bla*KPC gene within the hospital setting to improve the epidemiological surveillance of antimicrobial resistance genes. At this aim, the evolutionary rate, the time-scaled phylogeny, the population dynamics analysis and the phylogeography of *bla*KPC gene have been estimated to evidence potential hidden outbreaks. Furthermore, in the same strains selective pressure analysis and homology modelling were performed to check both the presence of positive selected sites and their influence on KPC protein structure.

2. Materials and methods

2.1. Bacterial isolates

Between January 2012 and January 2015 a total of 44 consecutive non-replicate clinical KPC *Klebsiella pneumoniae* strains isolated in monomicrobial infections were isolated from inpatients admitted in different wards at the University Hospital Campus Bio-Medico in Rome, Italy. Strains, selectively isolated in McConkey agar, were identified by MALDI-TOF (Bruker Daltonics GmbH, Bremen, Germany) and antimicrobial susceptibility tested by the Vitek-2 Compact instrument (bioMerieux, France), as previously described (Angeletti et al., 2015).

2.2. blaKPC gene sequencing

*bla*KPC resistance gene was amplified using the protocol by coupling the forward primer KPC-F107 with the reverse primer KPC-R860, producing a product of 753 bp, to detect the 10 currently described *bla*KPC variants in a single amplification and sequencing reaction (Chen et al., 2014)., as previously reported (Angeletti et al., 2015).

2.3. Multilocus sequence typing (MLST)

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

2.4. Phylogenetic analysis

The dataset included 44 *K. pneumoniae bla*KPC-3. The dataset was used to estimate the evolutionary rate, to perform the time-scaled phylogeny, the population dynamics analysis, phylogeography and the selective pressure analysis. This dataset was also used for residue conservation analysis and homology modelling.

2.5. Estimate of Klebsiella pneumonia blaKPC-3 evolutionary rate, time-scaled phylogeny and population dynamics

The sequences of the dataset were aligned with the Clustal X algorithm and then were manually edited using Bioedit v7.0 (Thompson et al., 1994; Hall, 1999). The evolutionary model was chosen as the best-fitting nucleotide substitution model in accordance with the results of the Hierarchical Likelihood Ratio Test (HLRT) implemented in MODELTEST software v. 3.7 (Posada and Buckley, 2002).

The evolutionary rate was estimated using a Bayesian Markov Chain Monte Carlo (MCMC) method implemented in BEAST package 1.8.0 and both a strict and relaxed clock with an uncorrelated log normal rate distribution. As coalescent priors, three parametric demographic models of population growth (constant size, exponential, expansion) and two non-parametric models (the Bayesian skyline plot, BSP and the Gaussian Markov randomfield (GMRF) Bayesian Skyride) were compared (Drummond and Rambaut, 2007; Drummond et al., 2012).

Eight independent MCMC runs were carried out: four with the strict clock and one of each demographic model and four with the relaxed clock and one of each demographic model. The demographic models tested were constant population size, exponential growth, non-parametric smooth skyride plot Gaussian Markov Random Field (GMRF), and non-parametric Bayesian skyline plot (BSP).Marginal likelihoods estimates for each demographic model were obtained using path sampling and stepping stone analyses (Baele et al., 2013a, 2013b; Baele and Lemey, 2013). After that, it was compared for each demographic model the clock (strict or relaxed) and each demographic model against each other (with the clock chosen).

The MCMC chains were run for at least 150 million generations, and sampled every 15,000 steps. Convergence was assessed on the basis of the effective sampling size (ESS) using tracer version 1.5 (http://www.Tree.bio.ed.ac.uk/software/tracer/) (Drummond and Rambaut, 2007).