Whole-genome sequencing of *Klebsiella pneumoniae* MDR strain isolated in a Syrian refugee

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Introduction

In the spring 2011 civil war becoming in Syria providing condition for diseases outbreaks [1]. During the war, the precariousness living condition dropped down the health system, resulting in a lack of shelter and sanitation services. Many Syrians remained injured with scarce possibility to have adequate care consequently to deterioration in the functionality of medical equipment, to the shortages of drugs and medical supplies due to sanctions application. Since 2013, Syrian patients, especially those wounded in the civil war, have been admitted for care in hospitals of the Northern Israel. Lerner et al. [2], reported the frequent isolation of carbapenem-resistant Enterobacteriaceae (CRE) in Syrian patients, prevalently *Klebsiella pneumoniae* and *Escherichia coli*. Recently, CRE emerged as one of the most important group of antibiotic resistant pathogens causing infections with high rates of morbidity and mortality and the potential for rapid dissemination in healthcare settings [3,4]. These isolates are characterized by multidrug resistance phenotypes leaving few therapeutic choices so that the spread of these strains is a great clinical challenge and a public health problem, in particular because these strains are associated to nosocomial infections [5–8].

During the last decades, a rapid increase of CRE have been documented worldwide [9] and within CRE, *K. pneumoniae* strains represent a major health problem [9]. Infectious disease as well as the isolation of resistant microorganisms to antimicrobial agents in migrants moving from a country to another can represents a potential source of spreading of fearsome infective agents. Lerner et al. isolated *K. pneumoniae* ST101 and

ST1633 strains from hospitalized Israeli patients as well as from Syrian patients admitted in the intensive care unit, suggesting the potential nosocomial spread of these pathogens and the importance to adopt infection control measures.

Carbapenemase-producing *Klebsiella pneumoniae* (KPC-KP) can cause serious infections with a mortality rates, ranging from 24 to 70% [3,10]. In Italy, KPC-KP strains have been documented for the first time in 2008 [11], undergoing rapid dissemination and causing nosocomial outbreaks [12,13]. Polymyxins, tigecycline and gentamicin represent the few therapeutic options that are recommended for treatment of infections caused by these pathogens [7,14]. Recently, colistin-resistant KPC-KP isolates have been described. In Italy, colistin resistant strains account for 43% suggesting the importance of monitoring this resistance [15].

Patients from foreign countries in which CRE are endemic or with unknown epidemiology should be subjected to microbiological surveillance at hospital admission looking for multidrug-resistant (MDR) bacteria to prevent further spread in nosocomial settings. The surveillance could aid the public health regulatory authority in the containment of the spread of Multi Drug Resistant (MDR) pathogens adopting adequate preventive measures, such as contact isolation upon MDR bacteria detection [2].

In this study, the complete genome of a *Klebsiella pneumoniae* MDR strain isolated in a Syrian patient has been analysed. Towards this aim the phylogenetic tolls have been applied to improve the microbiological surveillance of MDR strains.

Materials and methods

On October 2015, a 30-year-old man, Syrian refugee, arrived in Italy and was admitted at the Asylum Seekers Centre (ASC) of Castelnuovo di Porto, Rome, Italy, where he received social, legal and health assistance.

Upon his arrival, the Syrian received physical examination by the medical staff of the ASC. The headquarter of the forensic medicine at the University Hospital of Aleppo certified that the patient had the amputation of the right lower limb, of the left upper limb and an injury of the left foot where at the fifth toe an amputation was made through the metatarsophalangeal joint, as consequence of a bomb explosion in 2014. A functional disability by 60% of the total body functions and a loss of worktime of nine months consequently to the bomb injuries, were estimated. At his arrival in Italy, the patient reported pain at the right leg and left foot especially at nighttime and for this he took Pregabalin (GABA analog), 75 mg per day. At the physical exam, the presence of purulent material in correspondence of the metatarsophalangeal area of the left foot was observed. The patient previously received an unspecified antimicrobial therapy in Syria to treat this infection.

Purulent material was collected through a wound swab from the leg and inoculated on blood agar, McConkey agar and CNA agar selective media (BD BBL™ Prepared Plated Media, Beckton Dickinson) and incubated at 35 °C in 5% CO₂. Mucoviscous colonies lactose-fermenting growing on Mc Conkey Agar media were isolated and identified as *Klebsiella pneumoniae* by MALDI-TOF using the MALDI Biotyper 3.0 software version (Bruker Daltonics, GmbH, Bremen, Germany) [16]. Antimicrobial susceptibility test of the strain of *Klebsiella pneumoniae* was performed by Vitek2 Compact (bioMérieux, Marcy l'Etoile, France) and the resistant phenotype was further confirmed with the Kirby-Bauer method according to Clinical Laboratory Standard Institute (CLSI) and European Committee for Antimicrobial Susceptibility Test (EUCAST) [17].

Bacterial DNA was extracted by the EZ1 DNA tissue kit (Qiagen, Dusseldorf, Germany) following manufacturer's instructions starting from 200 µL of bacterial pellet suspension. After the quantification, using Invitrogen Quant-iT Picogreen dsDNA assay, purified DNA was diluted to obtain a final concentration of 0.7 ng/ml. Library preparation for Illumina sequencing was done using a Nextera® XT DNA Sample Preparation and Index kit (Illumina, San Diego, CA, USA) according to the manufacturer's manual. Resulting libraries were not normalized, quantified to reach a final concentration of 4nM, and pooled for subsequent sequencing on an Illumina MiSeq platform using the 2 × 150 cycle paired-end sequencing protocol. The *Klebsiella pneumoniae* genome from Syria was assembled using Velvet the algorithms for *de novo* short read assembly [18]. From the genome sequence it was possible to extrapolate the Multilocus Sequence

Typing (MLST) sequence type (ST) of the Syrian *Klebsiella pneumoniae* strain by submission of the whole genome at the Institute Pasteur MLST website (<http://bigsdb.pasteur.fr/klebsiella/klebsiella.html>). At the MLST analysis, the Syrian *Klebsiella pneumoniae* strain resulted ST 101.

For the phylogenetic analysis, a data-set of 43 sequences was built. This data-set included 21 *Klebsiella pneumoniae* genome from the Hospital Campus Bio-Medico of Rome (Italy), 21 *Klebsiella pneumoniae* genomes downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/>) (USA *n* = 3; Canada *n* = 2; China *n* = 3; United Arab Emirates *n* = 1; Nepal = 1; India *n* = 1; Australia *n* = 1; Hong Kong *n* = 1; Greece *n* = 2; Italy *n* = 5 and Lebanon *n* = 1) plus the Syrian *Klebsiella pneumoniae* genome (the whole genome sequence has been submitted in GenBank accession number MLQU000000000). All the sequences were aligned with progressive Mauve [19]. The resulting multiple alignment column positions containing a non-ambiguous, non-deletion and non-insertion base change from the consensus (93923 SNPs) were concatenated in a final alignment. Phylogenetic signal was assessed by likelihood mapping using TreePuzzle [20]. Maximul Likelihood (ML) phylogenetic analysis was performed using PhyML [21] (<http://www.atgc-montpellier.fr/phyml/>). The statistical robustness and reliability of the branching order within the phylogenetic tree, were confirmed with the bootstrap analysis by PhyML. The General Time Reversible (GTR) 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 version 3.7 [22].

Results and discussion

The *Klebsiella pneumoniae* strain isolated from the Syrian refugee showed a multidrug resistance (MDR) phenotype. The minimal Inhibitory Concentration (MIC) for the currently used antibiotics were as follows: Amoxicillin/clavulanic acid MIC > 32 mg/L (resistant); Piperacillin/tazobactam MIC > 128 mg/L (resistant); Cefotaxime MIC > 64 mg/L (resistant); Ceftazidime MIC > 64 mg/L (resistant); Cefepime MIC > 64 mg/L (resistant); Imipenem MIC > 16 mg/L (resistant); Meropenem MIC > 16 mg/L (resistant); Amikacin MIC > 64 mg/L (resistant); Gentamicin MIC > 16 mg/L (resistant); Ciprofloxacin MIC > 4 mg/L (resistant); Trimetoprim/sulfamethoxazol MIC = 40 mg/L (susceptible) and colistin MIC < 0.5 mg/L (susceptible).

The phylogenetic analysis showed that the Syrian genome of the *Klebsiella pneumoniae* did not cluster with any Italian strains. ML tree showed two main clades (I and II) statistically supported, as reported in Figure 1. In the clade I, Italian and American strains were represented. The clade II included genomes from strains isolated in Canada, United Arab Emirates, China, Australia, Greece,

Figure 1. Maximum Likelihood (ML) of *Klebsiella pneumoniae* complete genome SNPs alignment.

Notes: The asterisk (*) along the branches indicates a statistical value from bootstrap. The different locations are indicated in the strain labels. The Syrian strain is in bold. A focus of the clade including the Syrian genome has been highlighted.

India, Nepal, Italy, Lebanon and Hong Kong. Interestingly, these last strains clustered in two sub clades (IIa and IIb) statistically supported. The Syrian genome belongs to the cluster IIa closely related with the Greek sequences in a cluster statistically supported.

Recently, CRE strains have been isolated in Syrian wounded patients admitted to northern Israel hospitals. Even if the source for bacterial infection was unknown, the molecular characterization of these strains showed that some patients admitted in different hospital wards carried the same sequence type [2]. It has been recently reported that refugees migration towards Europe may represents a challenge to public health and clinical services [23–25].

In the present case report, phylogenetic analysis was used to analyze the evolution of the *K. pneumoniae* MDR strain isolated in a Syrian refugee. The phylogenetic analysis suggested that the Syrian refugee probably, did not acquire the MDR *K. pneumoniae* infection at his arrival in Italy, in fact the Syrian genome clustered in a statistically supported separate clades from Italian strains (Figure 1) suggesting that he probably acquired infection during his travel. Moreover, we can hypothesize a relative and temporary permanence in Greece where thousands of people have been stranded by the EU-Turkey agreement.

Lastly, MLST analysis revealed that the *K. pneumoniae* Syrian strain was ST 101, as reported also in Syrian patients admitted in Israeli hospitals [2]. This ST is completely different from Italian strains reported in this study. Italian *K. pneumoniae* were all ST512 belonging to the clonal complex 258 the most prevalent in Italy [16,26]. Recently, *K. pneumoniae* ST 101 strains were isolated in Italy [27,28] and other European Countries [29]. Based on

MLST analysis the possibility that the Syrian migrant was infected upon its arrival in Italy, should not have been excluded, because *K. pneumoniae* ST101 strains circulation has been reported also in Italy. Conversely, the phylogenetic analysis of the whole genome was more informative, suggesting that the Syrian migrant could have been infected during his journey through different countries, including Greece.

In conclusion, the molecular microbiological surveillance performed on migrants as well as routine surveillance in nosocomial settings could be a valid tool to evidence MDR strains and limit their spread [25]. A molecular sentinel surveillance system could aid to determine the healthy status of migrants even in cases where there is carriage of microorganisms, and support an effective strategy for disease prevention.

Abbreviation

CRE	Carbapenem resistant Enterobacteriaceae
KPC-KP	Carbapenemase-producing <i>Klebsiella pneumoniae</i>
ASC	Asylum Seekers Centre
CLSI	Clinical Laboratory Standard Institute
EUCAST	European Committee for Antimicrobial Susceptibility Test
MDR	Multidrug resistance
MLST	Multilocus Sequence Typing
ST	Sequence Type
ML	Maximul Likelihood
GTR	General Time Reversible
HLRT	Hierarchical Likelihood Ratio Test

Disclosure statement

No potential conflict of interest was reported by the authors.

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