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Molecular typing of *Legionella pneumophila* serogroup 1 clinical strains isolated in Italy

Q1 Stefano Fontana[a], Maria Scaturro[a], Maria Cristina Rota[b], Maria Grazia Caporali[b], Maria Luisa Ricci[a,*]

[a] Istituto Superiore di Sanità (ISS, National Institute of Health), Department of Infectious Parasitic and Immune-mediate diseases, Rome, Italy
[b] Istituto Superiore di Sanità (ISS, National Institute of Health), National Centre for Epidemiology, Surveillance and Health Promotion, Rome, Italy

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ABSTRACT

Molecular typing methods for discriminating different bacterial isolates are essential epidemiological tools in prevention and control of *Legionella* infections and outbreaks. A selection of 56 out of 184 *Legionella pneumophila* serogroup 1 (Lp1) clinical isolates, collected from different Italian regions between 1987 and 2012, and stored at the National Reference Laboratory for *Legionella*, were typed by monoclonal antibody (MAb) subgrouping, amplified fragment length polymorphism (AFLP) and sequence based typing (SBT). These strains were isolated from 39 community (69.6%), 14 nosocomial (25%) and 3 travel associated (5.4%) Legionnaires'disease cases. MAb typing results showed a prevalence of MAb 3/1 positive isolates (75%) with the Philadelphia subgroup representing 35.7%, followed by Knoxville (23.2%), Benidorm (12.5%), Allentown/France (1.8%), Allentown/France-Philadelphia (1.8%). The remaining 25% were MAb 3/1 negative, namely 11 Olda (19.6%), 2 Oxford (3.6%) and 1 Bellingham (1.8%) subgroups.

AFLP analysis detected 20 different genomic profiles. SBT analysis revealed 32 different sequence types (STs) with high diversity of STs (IOD_{STs} = 0.952) 12 of which were never described before. ST1 and ST23 were most frequently isolated as observed worldwide. A helpful analysis of data from SBT, MAb subgrouping and AFLP is provided, as well as a comparison to the Lp1 types investigated from other countries. This study describes the first Italian Lp1 strains database, providing molecular epidemiology data useful for future epidemiological investigations, especially of travel associated Legionnaires' diseases (TALD) cases, Italy being the country associated with the highest number of clusters.

Introduction

Legionella pneumophila is a Gram-negative waterborne pathogen causing a severe form of pneumonia known as Legionnaires' disease (LD), which is commonly acquired by inhalation or micro-aspiration of aerosol particles originating from contaminated man-made water systems (Carratalà and Garcia-Vidal, 2010). Sources of infection were indeed demonstrated to be hot water systems, cooling towers, spa pools, dental unit waterlines, etc. (Rota et al., 2005; Ricci et al., 2012; Lee and Lee, 2013). Lp includes 16 serogroups are able to cause disease, but serogroup 1 is the most frequently isolated from LD patients. Lp1 can be further divided into several subgroups according to the expression of different epitopes on the lipopolysaccharide. MAbs directed against these antigens have differentiated Lp1 strains in MAb 3/1 positive, associated to higher virulence, and MAb 3/1 negative (Helbig et al., 2002). For epidemiological investigations, AFLP genomic typing has been demonstrated to have a greater discriminatory power and to be efficient in establishing the clonal relatedness between clinical and environmental strains of Lp. However several cons were observed when using this method, including the difficulty in determining the fragment size and the consequent trouble in comparing genomic patterns among different laboratories and platforms (Fry et al., 2002).

Afterwards, a new typing scheme based on the partial sequence of 7 genes, named SBT, was developed by the EWGLI and a dedicated database was then created (Gaia et al., 2005; Scaturro et al., 2005; Ratzow et al., 2007). The SBT method is now considered the gold standard for Lp molecular typing and represents a useful tool for easy, rapid and efficient exchange of molecular epidemiology data (Scaturro et al., 2005; Rota et al., 2011).

* Corresponding author at:. Istituto Superiore di Sanità (ISS, National Institute of Health), Department of Infectious Parasitic and Immune-mediate Diseases, Rome, Italy. Tel.: +39 06 49902856; fax: +39 06 49387112.
E-mail address: marialuisa.ricci@iss.i (M.L. Ricci).

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Typing methods are applied not only for LD epidemiological investigations to determine the sources of infection, but also to investigate the distribution of Lp strains in specific areas (Borchardt et al., 2008; Chasqueira et al., 2009; Harrison et al., 2009; Vekens et al., 2010; Kozak et al., 2013).

In Italy, notification of legionellosis to health authority has become mandatory since 1983. The Legionellosis National Register was established and managed by Istituto Superiore di Sanità (Italian Institute of Health, ISS). In addition to epidemiological data collection, the National Reference Laboratory for Legionella performs diagnosis confirmation and typing of the isolated strains. Since 2008 more than 1000 cases of legionellosis per year have been notified either as sporadic or clusters/outbreaks associated. Most cases have been diagnosed by detection of Lp urinary antigen, while the isolation of the pathogen occurred only in 2.3% of the cases (Rota et al., 2013a, 2013b).

From 1987 to 2012, 206 *Legionella* clinical strains were isolated and stored in our laboratory, among them 184 were Lp1. In this study 56 strains were analyzed by SBT after a preliminary selection that took into account molecular typing results, obtained from MAb and AFLP, as well as geographical distribution and year of occurrence. The aim of this investigation was to describe the molecular characteristics and distribution of Lp1 strains isolated in Italy. Results were then compared to those obtained in other countries (Borchardt et al., 2008; Den Boer et al., 2008; Chasqueira et al., 2009; Harrison et al., 2009; Kozak et al., 2009; Amemura-Maekawa et al., 2010; Vekens et al., 2010; Kozak et al., 2013) and to the ones present in the EWGLI SBT database.

Material and methods

Legionella strains

The 56 clinical strains, isolated during the period 1987–2012, were from 10 out of 21 Italian regions and accompanied by epidemiological data obtained from patients' medical history. These strains were selected from a collection of 184 Lp1 clinical strains. The selection criteria were the molecular patterns (MAb and AFLP), the year and the region of isolation, epidemiological data, so that the strains chosen were representative of the whole collection.

A LD case was considered as confirmed nosocomial acquired (NA) if the patient was continuously hospitalized during the incubation period (2–10 days) or travel associated (TA) if the patient spent at least one night away from home in Italy. Cases where both NA and TA origins could be excluded were considered as community acquired (CA).

Strains stored at −80 °C were thawed and cultured for two days in Buffered Charcoal Yeast Extract (BCYE, Oxoid, UK) agar plates at 36 ± 1 °C with 2.5% CO_2 before being analyzed with molecular typing assays.

MAb, AFLP and SBT typing

The 56 Lp1 strains were typed serologically by monoclonal antibodies, according to the Dresden Panel of MAb (Helbig et al., 2002).

Genotyping was performed by AFLP and SBT (Fry et al., 2002; Gaia et al., 2005) on genomic DNA prepared from a single Lp1 colony with 20% Chelex 100 (Sigma-Aldrich, Germany). Briefly, the Lp1 colony was suspended in 1 mL sterile distilled H_2O in a microfuge tube and centrifuged for 1 min at 12,000 × *g*. Then, supernatant was removed and DNA extracted by adding 200 μL 20% Chelex 100 to pellet followed by boiling for 10 min. AFLP was carried out as described elsewhere (Fry et al., 2002) and the obtained genomic patterns were compared by visual analysis using the CANVAS software (ACD system 2005). SBT was carried out according to the EWGLI "Sequence-based typing (SBT) protocol for epidemiological typing of *Legionella pneumophila*" (Version 4.2) (www.hpa bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php). Raw sequence data were analyzed using the Legionella Sequence Quality Tool available at www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi. New allelic profiles were submitted to the EWGLI SBT database (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php) in order to designate new STs.

Statistical analysis

The index of diversity of STs (IOD_{STs}) was calculated as previously reported (Simpson et al., 1949; Hunter and Gaston, 1988), and implemented through the V-Dice application (www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl). In addition the IOD of allelic profiles (IOD_{AP}) was calculated as average of IOD values found for each of the 7 SBT loci, and was determined both for the allelic profiles of all the 56 strains and separately for the allelic profiles of the strains that showed the two most common AFLP types, found throughout this study.

UPGMA analysis

The examination of relationships among the STs was conducted using the Unweighted Pair Group Method With Arithmetic mean algorithm (UPGMA) constructing a dendrogram based on the distance matrix of allelic profiles among the 56 strains selected (START 2© software, University of Oxford).

Results

Legionella strains

The 56 Lp1 strains were from 39 CA (69.6%), 14 NA (25%) and 3 (5.4%) TA cases. These strains, like the others present in the collection, were isolated in 10 Italian regions, with Lazio ($n = 19$, 34%) and Lombardia ($n = 12$, 21.5%) being the most represented. The remaining strains (44.5%) were isolated in regions prevalently of the North of Italy, except for 3 Lp1 isolates originated from the Campania region in Southern Italy (Fig. 1).

On the basis of epidemiological information, present in the Italian Legionellosis National Register, the 56 Lp1 strains were classified as related (R) and unrelated (UR) (Fig. 1). Among the strains associated to CA infections, the R1, R2 and R3 groups included strains isolated either during an outbreak or they were part of a cluster of LD. All the other CA strains were epidemiologically UR (Fig. 1).

The strains cultured from NA cases were linked to six different hospitals and isolated over several years. Among them, strains responsible of cases occurred in the same hospital were considered potentially related each other. Twelve out of fourteen NA strains were clustered in R4, R5, R6 and R7 correlation groups corresponding to four different hospitals. Two NA strains from other two hospitals were UR (Fig. 1). The three strains isolated from TA cases were 1 isolated from cluster (Rota et al., 2011) and two from single cases. They were all UR each other.

MAb typing

MAb typing demonstrated a prevalence ($n = 42$, 75%) of MAb 3/1 positive isolates. Indeed, 20 *Legionella* isolates were Philadelphia (35.7%), 13 Knoxville (23.2%), 7 Benidorm (12.5%), 1 Allentown/France (1.8%) and 1 Allentown/France -Philadelphia (1.8%)

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