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ORIGINAL ARTICLE

Comparative molecular and antibody typing during the investigation of an outbreak of Legionnaires' disease

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Abstract An outbreak of Legionnaires' disease with 113 confirmed cases was reported in the town of Mataró, Spain, in August 2002. In this study, we compared three different typing methods and characterized the clinical isolates by comparing them with other clinical isolates with the same ST from our own database to further characterize the outbreak. In the outbreak, a total of 16 clinical (nine patients) and 32 environmental (from four environmental sources) *Legionella pneumophila* isolates were analyzed by pulsed-field electrophoresis (PFGE), sequence-based typing (SBT), and monoclonal antibody typing (MAb). We compared the MAb and SBT profiles of the outbreak clinical isolates and other unrelated clinical isolates showing the same ST profile. We obtained seven different PFGE and SBT profiles and six MAb patterns from the outbreak isolates. PFGE and SBT showed 100 % concordance during the outbreak. SBT proved to be highly discriminatory, particularly with the addition of the new *neuA* gene. One PFGE, SBT (ST-37), and Philadelphia profile was observed among the clinical isolates. Using PFGE, this ST37 Philadelphia profile was closely related to other unrelated clinical isolates. These findings suggest that the ST37 Philadelphia profile could be a virulence marker in our area. The combination of the three methodologies was useful to further characterize and obtain additional information on a very explosive outbreak. Despite the minor discrimination of PFGE versus SBT, the two genetic methods are recommended in outbreak investigations. Further studies are currently underway in this area to obtain more definitive conclusions.

Keywords *Legionella* · PFGE · SBT · MAb · Typing · Outbreak · Virulence

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Introduction

Since *Legionella* was first identified in 1976, the diagnosis of Legionnaires' disease has increased considerably. Different studies have demonstrated how *Legionella pneumophila* has become one of the leading causes of community-acquired pneumonia in adults, accounting for 6–14 % of cases requiring hospitalization [1, 2]. Transmission of *Legionella* occurs most frequently with the inhalation of aerosols containing the microorganism, although micro-aspiration of contaminated potable water has also been implicated, especially in hospitalized patients [3].

Several environmental sources have been associated with *Legionella* outbreaks, including cooling towers, water distribution systems of homes, hotels, and ships, ornamental fountains, and whirlpool spas. Colonization of cooling towers with production of aerosols has also been identified as one of the major sources of community outbreaks of *Legionella* infection [4–8].

During an outbreak, appropriate typing methods are needed to establish the link between environmental and clinical isolates in epidemiological investigations. Pulsed-field gel electrophoresis (PFGE) is considered to be one of the most efficient methods for subtyping *L. pneumophila* strains and previously was the gold standard methodology in the epidemiological investigation of *Legionella pneumophila* outbreaks because of its high discriminatory power. Recently, however, the European Working Group for Legionella Infections (EWGLI) proposed sequence-based typing (SBT) as the new gold standard methodology [9]. SBT is a powerful epidemiological method, being both rapid and easy to perform, and provides unambiguous results [10]. SBT has shown to be useful in the investigation of prevalence and distribution of DNA sequence types among clinical and environmental *L. pneumophila* isolates [11–14]. However, only a few studies have demonstrated the usefulness of this technique in the investigation of nosocomial and community outbreaks using the combination of seven genes [15–19].

A community outbreak of *L. pneumonia* involving more than 154 people, 113 of whom had confirmed Legionnaires' disease, was detected in the district of Cerdanyola, Mataró (Catalonia, Spain). This explosive outbreak was investigated in an epidemiological, environmental, and molecular study during August 2002.

Epidemiological and molecular data identified a cooling tower as the direct cause of the community outbreak [20]. The aim of the present study was to further characterize this explosive outbreak comparing three different methods for the subtyping of *L. pneumophila* [monoclonal antibody (MAb) typing, PFGE, and SBT] and to characterize the clinical isolates, comparing them with other clinical isolates with the same subtype (ST) from our own database.

Materials and methods

Bacterial isolates

Bacterial isolates from the community outbreak We included a total of 16 clinical *Legionella pneumophila* serogroup (sg.) 1 isolates from nine patients admitted to the hospital for pneumonia-like symptoms and 32 isolates from four environmental sources collected during the epidemiological investigation in the 2002 community outbreak in Mataró [20]. All the clinical isolates and 28 environmental isolates were previously typed by PFGE. Furthermore, we analyzed the total of 48 isolates using a combination of three of the most commonly used typing methods for *Legionella* investigation: MAb typing, PFGE, and SBT.

Other clinical isolates We selected eight unrelated clinical isolates related to other community outbreaks showing the same ST as the outbreak clinical isolates [21]. Four of these isolates were epidemiologically and PFGE linked to cooling towers and three to a hot-water supply; one had an unknown origin.

MAb typing of *Legionella pneumophila*

MAb typing, performed by indirect immunofluorescence assay with the "Dresden MAb panel" [22], was used to determine the phenotypic subgroup.

Pulsed-field gel electrophoresis of *Legionella pneumophila*

Genomic DNA was prepared using a previously described protocol with some modifications [23]. Restriction digestion of genomic DNA with 50 U Sfi I (New England Biolabs, England, UK) was performed according to the manufacturer's recommendations. Fragments of DNA were separated in a 1 % agarose gel prepared and run in 0.5× Tris–borate-EDTA buffer (pH 8.3) in a contour-clamped homogeneous field apparatus (CHEF DR II system; Bio-Rad, Ivry sur Seine, France) with a constant voltage of 5 V cm^{-1} and increasing pulse times (5.6–50.6 s) at 14 °C for 25 h. The lambda ladder PFGE marker (New England Biolabs) was included as a molecular weight marker.

Pattern analysis was performed using the Gel Compar II software (Applied Maths, Kortrijk, Belgium) using the Dice band-based similarity coefficient and the UPGMA as the clustering method with a tolerance of 1 %. Isolates with a PFGE pattern that differed by one band or more were considered to belong to different genotypes and were designated with capital letters.

Sequence-based typing of *Legionella pneumophila*

Genomic DNA was extracted from the study isolates using the Chelex extraction technique (Bio-Rad Laboratories, Hercules, CA, USA). The seven target genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) were amplified using the primers and amplification protocol provided by the EWGLI (v. 4.2). The primers and the polymerase chain reaction (PCR) conditions were the same as those previously described [10, 23]. Amplified products were sequenced in both directions using the ABI PRISM BigDye Terminator v. 3.1 Cycle Sequencing kit in the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The sequences were analyzed using BioEdit v. 7.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). SBT allele numbers were assigned to each strain based on the EWGLI database [9].

Results

MAb typing

Using the Dresden panel of MAbs, 44 of 48 *Legionella pneumophila* isolates belonging to sg. 1 were divided into five different subgroups. The Philadelphia subgroup accounted for the majority of the isolates (48 %), followed by the Olda (11 %) and the Oxford subgroups (5 %). Twenty-seven percent of the isolates belonged to the designated "Oxford/Olda" subgroup. The subgroups Benidorm and Allentown/France accounted for two isolates each (4.5 %).

Table 1 summarizes the results of MAb typing for both the clinical and environmental isolates. MAb typing showed the common Philadelphia subgroup for the 16 clinical isolates and 5 of the environmental isolates obtained from the suspected cooling tower (S1). A total of 5 isolates belonged to the Olda subgroup and only 2 isolates belonged to the Oxford subgroup. One Olda isolate was also found in the suspected cooling tower (S1). The remaining Oxford and Olda subgroups were isolated from a different source (S2) along with 2 Benidorm isolates. The designated "Oxford/Olda" subgroup was retrieved from the incriminated cooling tower (S1) and accounted for 12 isolates. The last subgroup (Allentown/France) was isolated from source S3 and accounted for 2 isolates.

About 30 % of the isolates belonged to the designated subgroup "Oxford/Olda." It was difficult to establish a 100 % match with either the Oxford or Olda subgroup because of recognition problems with the MAb. Particularly, it was difficult to determine positivity to MAb 26/1. Some bacteria appeared in isolated clusters in our preparations, showing weak positivity to the MAb 26/1.

All the clinical isolates and the environmental strains responsible for the outbreak possessed the virulence-associated epitope MAb 3/1. The Benidorm and Allentown/France subgroups also showed positivity to the MAb 3/1 but were not outbreak related.

Four isolates from source S4 were *L. pneumophila* non-sg. 1 and were negative for all MAb from the Dresden panel.

Pulsed-field gel electrophoresis

The 48 isolates were discriminated into seven different PFGE profiles (Table 1; Fig. 1).

As confirmed by the other study, 5 of the environmental isolates from the incriminated cooling tower were indistinguishable from the clinical isolates because they both showed the same PFGE pattern (PFGE A), and 13 isolates showed a different pattern (PFGE B).

PFGE C, D, and E were collected from the same source, S2, whereas PFGE F was from source S3, and the *L. pneumophila* non-sg. 1 isolates from source S4 showed the PFGE G pattern.

The dendrogram in Fig. 1 shows the cluster analysis for the seven different pulsotypes. Our patterns analysis showed 65 % similarity between profiles A and E; profiles B and A, albeit isolated from the same cooling tower, only shared 52 % similarity. Profile B shared greater similarity with profile C (62 %) and profiles G and D shared 65 % similarity. Profile F was similar to profiles A and E (60 %).

Sequence-based typing

We applied SBT to the same *L. pneumophila* isolates as those used in the PFGE analysis. The sequences of *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA* were determined.

Table 1 Sequence-based typing (SBT), pulsed-field gel electrophoresis (PFGE), and monoclonal antibody typing (MAb) patterns for clinical and environmental *Legionella pneumophila* isolates

Origin	Source	Number of strains	PFGE profile	Allelic profile (SBT)	Sg	Dresden panel MAb subgroup
C	–	16	A	37 (3,4,1,1,14,9,11)	1	Philadelphia
E	S1	5	A	37 (3,4,1,1,14,9,11)	1	Philadelphia
E	S1	12	B	284 (1,6,3,10,1,1,11)	1	Oxford/Olda
E	S1	1	B	284 (1,6,3,10,1,1,11)	1	Olda
E	S2	3	C	1 (1,4,3,1,1,1,1)	1	Olda
E	S2	2	C	1 (1,4,3,1,1,1,1)	1	Oxford
E	S2	2	D	181 (3,10,1,12,14,9,9)	1	Benidorm
E	S2	1	E	3,4,1,1,14,9,43	1	Olda
E	S3	2	F	20 (2,3,18,15,2,1,6)	1	Allentown/France
E	S4	4	G	12,8,18,5,5,12,15	ns1	–

C clinical, *E* environmental, *Sg* serogroup

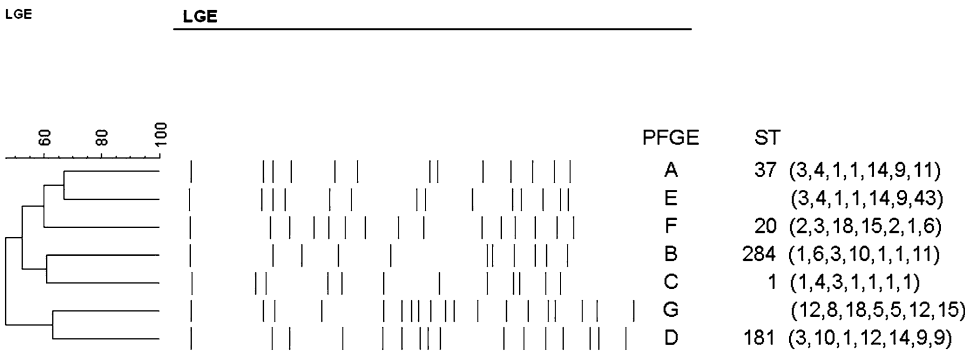

Fig. 1 Dendrogram of pulsed-field gel electrophoresis and sequence-based typing of *Legionella pneumophila* isolates

The 48 isolates were divided into seven ST types based on the sequences of the seven typed genes.

The SBT results confirmed those obtained by PFGE, and the environmental isolates from the responsible cooling tower matched the clinical isolates obtained from the nine patients (ST 37).

The remaining epidemiologically unrelated isolates showed different ST types in accordance with the PFGE results (Table 1; Fig. 1). Five isolates, represented by two different ST profiles, were different from those already in the EWGLI-SBT database, and their sequences were submitted according to the curators' instructions (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php).

The sequence of one epidemiologically unrelated environmental isolate (3,4,1,1,14,9,43) was shown to be very similar to the sequence of the outbreak-related isolates (3,4,1,1,14,9,11) because it differed in only the *neuA* gene. The *neuA* gene found in this study was recognized as a new allele type and was designed as *neuA* gene 43.

The combination of MAb typing, PFGE, and SBT

On combining the three methods, we were able to divide the clinical and environmental isolates into nine different types.

Comparison of clinical isolates versus other clinical isolates

On comparing the outbreak clinical isolates with eight other unrelated clinical isolates that showed the same ST 37, we observed that all were mAb3/1 positive and Philadelphia subtype. Furthermore, in the PFGE we observed five indistinguishable PFGE profiles that could be considered as closely related according to the Tenover criteria because they showed less than three bands of difference (Fig. 2).

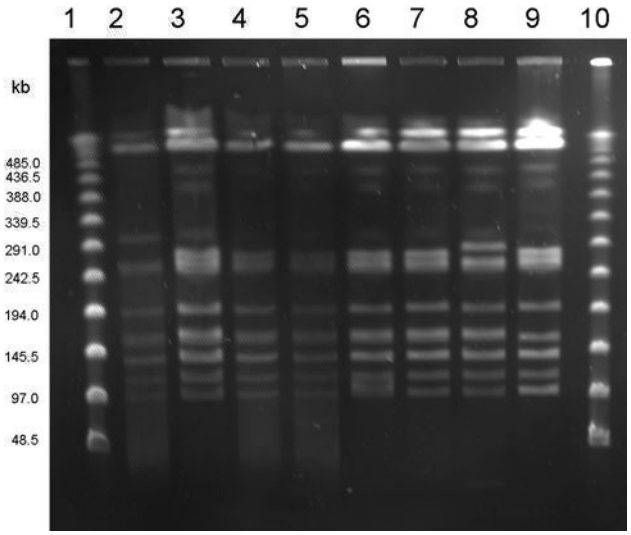

Fig. 2 Pulsed-field gel electrophoresis (PFGE) of *Legionella pneumophila* isolates showing ST37 Philadelphia subtype. *Lines 1*, *10*, lambda ladder PFGE marker; *line 3*, outbreak-related isolate; *lines 1*, *2*, *4–9*, other clinical outbreak-unrelated isolates

Discussion

The community outbreak of Legionnaires' disease in Mataró (Spain) in August 2002 was one of the largest reported in terms of number of cases and attack rate. During the epidemiological investigation, molecular analysis to identify the source of the outbreak was carried out by PFGE. A cooling tower from an ice-making factory, located in the middle of the Cerdanyola district, was identified as the direct cause of the outbreak [20]. In this study we further characterized this explosive outbreak comparing MAb, PFGE, and SBT typing for the subtyping of *Legionella pneumophila.*

Using the MAb with the Dresden panel, PFGE, and SBT typing methods it was possible to characterize the strains involved in the outbreak. These methods revealed the common Philadelphia subgroup, ST 37, and PFGE A for the clinical isolates. PFGE and SBT showed a 100 % concordance, allowing seven different genetic profiles to be

obtained by both methods among the clinical and environmental isolates investigated.

We observed a variation in the MAb subtype within the two main genotype clusters with the same PFGE and ST profile (Table 1). Both PFGE profiles B (ST 284) and C (ST 1) showed two main MAb subtypes: Oxford and Olda. Because variable monoclonal antibodies patterns may be found within a stable genetic fingerprinting [25, 26], with studies using MAb typing as a screening technique one should keep in mind that some PFGE and SBT profiles can show different MAb subgroups. More studies are needed to determine the real importance of MAb typing in combination with SBT or PFGE.

In this study, ST1, the profile most frequently reported in Europe and in the rest of the world [9, 11, 27], was found in five environmental isolates collected from a different location during the outbreak. The ST1 represented 13.8 % of the isolates in the EWGLI database whereas the other STs found in this study represented less than 5 %. One epidemiologically unrelated isolate collected during the outbreak investigation (profile E) showed more than 65 % similarity with more than three different bands with the epidemiologically related isolates (profile A) using the PFGE technique. The same isolate showed an allelic profile (3,4,1,1,14,9,43) very similar to the isolates responsible for the outbreak (3,4,1,1,14,9,11) as it only differed in the *neuA* gene using the SBT technique. By sequencing only six of the seven genes, the SBT technique would have shown an identical allelic profile for both the isolates from different environmental sources whereas PFGE showed evident differences in several fragments. These results confirm an increase of the discriminatory power of the consensus sequence-based scheme when the *neuA* gene is added [24] and question previous results by authors using the previous version of the method. Therefore, it is important to always sequence seven genes during an outbreak investigation.

The strain responsible for the outbreak was ST 37 Philadelphia (MAb 3/1+). This strain has been reported worldwide in clinical and environmental isolates [9]. When we consulted the EWGLI database we observed that 89.4 % of the ST37 profiles are associated with the Philadelphia subgroup. Moreover, on consulting our own database, we observed that ST37 is one of the most predominant among the clinical isolates and the Philadelphia subgroup is expressed in 100 % of these ST37 clinical isolates [21]. Moreover, all the ST37 isolates had the virulence-associated epitope recognized by MAb3/1 [22]. Furthermore, on comparing the ST37 from this study with seven other ST37 Philadelphia unrelated isolates using PFGE, we observed five indistinguishable PFGE profiles that could be considered as closely related according to the Tenover criteria [28]. Our results suggest that although both techniques could be applied in outbreak investigations, PFGE shows a higher discriminatory power for studying larger populations of *Legionella* isolates. However, PFGE results may be subject to interpretation. Even though the Tenover criteria have long been used to interpret PFGE results, they are sometimes stringent when applied to large studies. This particular reason often leads investigation groups to set their own criteria a priori in the investigation of an outbreak. Moreover, PFGE is time consuming, requires special equipment, and its interlaboratory reproducibility is somewhat questionable. On the other hand, MLST/SBT has been successfully used to type different strains of bacteria in other studies [29]. SBT is a rapid, easy-to-perform technique providing unambiguous and reproducible results. The SBT technique also allows comparison of global data collection through a web database where information on new outbreaks is constantly updated. However, the cost of performing SBT is much higher than that of PFGE, and SBT usually requires access to a sequence analyzer.

In summary, this study shows how the combination of three typing methodologies was useful to further characterize and obtain additional information on a very explosive outbreak. The ST37 Philadelphia profile may be a virulence marker in our area. The observation of different PFGE patterns in samples showing the same ST in this study suggests that additional studies are needed to evaluate the new version of the SBT protocol in community outbreak investigations. Moreover, our results emphasize the importance of the combination of two molecular methods (PFGE and SBT) in the epidemiological investigation of *Legionella* outbreaks.

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Conflict of interest None.

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