Legionnaires' disease outbreak in Rome, Italy

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SUMMARY

Between August and October 2003, 15 cases of Legionnaires' disease were detected in the 9th district of Rome. To identify possible sources of *Legionella* exposure, a matched case-control study was conducted and environmental samples were collected. Hospital discharge records were also retrospectively analysed for the period July–November 2003, and results were compared with the same period during the previous 3 years. The case-control study revealed a significantly increased risk of disease among those frequenting a specific department store in the district (OR 9·8, 95% CI 2·1–46·0), and *Legionella pneumophila* was isolated from the store's cooling tower. Genotypic and phenotypic analysis of human and environmental isolates demonstrated that the cluster was caused by a single strain of *L. pneumophila* serogroup 1, and that the cooling tower of the store was the source of infection. The increased number of hospital admissions for microbiologically undiagnosed pneumonia during the study period may indicate that some legionellosis cases were not identified.

INTRODUCTION

Several community-acquired outbreaks of Legionnaires' disease (LD) have been reported in recent decades, in which the source of infection was an evaporative cooling tower [1–5]. Indeed, a cooling tower was responsible for the largest outbreak of LD ever reported, in which 449 cases occurred in Murcia, Spain, during July 2001.

In Italy, the first extensive outbreak of LD was reported in August 1995 with 34 laboratory-confirmed cases [6]. Using combined molecular phenotypic and genotypic methods, the source of infection was identified as the cooling tower of a public building. During the summer of 1998, an outbreak associated with a hospital cooling tower occurred that involved several patients, some of whom died (G. Lomolino, unpublished data).

More recently, between August and October 2003, a cluster of confirmed cases of LD was detected among residents in the 9th district of Rome, an area of ~ 8 km² with a population estimated at 135 000 inhabitants. Epidemiological and environmental

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investigations were undertaken immediately upon notification of the first cases, all of whom lived in the same area. A case-control study was conducted in order to identify the probable source of *Legionella*.

In addition, to estimate the true magnitude of the outbreak, a retrospective analysis of hospital discharge records for pneumonia was carried out for persons discharged between 14 July and 2 November 2003 from the main district hospital, and results were compared with the same period for the previous 3 years.

In this paper, we describe the epidemiological, microbiological and environmental investigations conducted and the control measures that were implemented.

METHODS

Case-control study

Cases

After the national LD registry at the Istituto Superiore di Sanità had received notifications of seven cases over a 15-day period, the main district hospital and other hospitals in the area were contacted and requested to immediately report any additional case and to collect biological specimens.

A confirmed case of LD was defined as radiologically confirmed pneumonia with laboratory evidence of acute infection with *Legionella* including: (*a*) isolation of any species of *Legionella* from respiratory secretions, lung tissue, or blood; (*b*) a fourfold or higher rise in specific serum antibody titre against *L. pneumophila* serogroup 1 by immunofluorescence or microagglutination in paired acute- and convalescent-phase serum specimens; or (*c*) detection of *L. pneumophila* antigen in urine, in an individual residing in or having visited the 9th district of the city of Rome in the 10 days before the onset of the disease.

A presumptive case of LD was defined as a radiologically confirmed pneumonia with laboratory evidence of acute infection with *Legionella* including: (*a*) a fourfold or higher rise in specific serum antibody titre to *L. pneumophila* other serogroups or other *Legionella* spp. by immunofluorescence or microagglutination in paired acute- and convalescent-phase serum specimens; (*b*) a single high titre ($>1:256$) against *L. pneumophila* serogroup 1; (*c*) the detection of specific *Legionella* antigen in respiratory secretion or direct fluorescent antibody (DFA) staining of the organism in respiratory secretion or lung tissue using evaluated monoclonal reagents in an individual

residing in or having visited the 9th district of the city of Rome in the 10 days before the onset of the disease.

Controls

In Italy, all persons receiving care within the national health system must register with a general practitioner, each of whom has approximately 1500 patients in his or her practice. Each practitioner, as well as the local health unit (ASL), maintains a list of assigned population. To identify appropriate controls, the general practitioner caring for each case was ascertained by interviewing the case, and matched controls were randomly selected from the practitioner's population list at the ASL.

For each case, four paired controls were selected according to the following criteria:

- same gender as the case;
- same age (± 5 years) as the case;
- residence in the 9th district, within a 300 m radius of the house of the case;
- presence in the 9th district during the likely period of exposure of the case (defined as presence for at least 8 of the 12 weeks between 28 July and 19 October);
- absence of respiratory disease symptoms (fever $>38\,^{\circ}\mathrm{C}$, cough, body malaise) during the study period.

A standardized questionnaire to interview cases and controls was developed that addressed health status (presence of chronic diseases, smoking and alcohol behaviours, therapy with corticosteroids and chemotherapy), places visited and routes taken within the district, and usual social activities. The questionnaire also asked about type of residence and domestic water supply and air-conditioning systems, occupational exposure, and travel 2 weeks prior to the onset of illness.

Estimation of the true magnitude of the epidemic

To ascertain whether additional cases may have occurred during the outbreak, the number of hospital discharges from the main district hospital for legionellosis occurring during the period 14 July and 2 November, 2003 was determined and was compared with the number of discharges for the same cause during the same period for each of the previous 3 years (2000–2002). As in ICD9-CM [7] there is no specific code for legionellosis, the code usually

reported is '482.83, Pneumonia due to other specified bacteria'.

The search was further extended by repeating this process for the ICD9-CM codes for acute respiratory infections without aetiological diagnosis (482.9, 485 and 486).

Microbiological and serological diagnosis and molecular biotyping

All laboratory analyses on biological specimens collected from the cases were conducted at the Department of Infectious, Parasitic and Immunomediated Diseases of the Istituto Superiore di Sanità.

Respiratory secretion samples were plated directly onto buffered charcoal–yeast extract (BCYE-α) non-selective and selective agar (GVPC; Oxoid, Basingstoke, UK), following standard procedure for the isolation of *Legionella* spp. [8]. Acute- and convalescent-phase serum specimens were obtained from patients to test for antibody to *Legionella* spp. by indirect fluorescent antibody (IFA).

In addition, a panel of nine monoclonal antibodies (mAbs) (Carl Gustav Carus University, Dresden, Germany) was used for IFA testing to subtype *L. pneumophila* isolates [9]. Pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) and sequence-based typing (SBT) methods were used for genomic typing [10–12].

Molecular analyses were performed with all clinical and environmental isolates using as internal controls *L. pneumophila* Philadelphia 1 strain (ATCC 33152) as well as one clinical *L. pneumophila* Philadelphia 1 strain isolated from a single case of LD that occurred in another area of Rome and one environmental strain isolated from a hospital's water system. Both the controls were unrelated to the outbreak.

For PFGE, the DNA plugs were prepared as previously described [6] and the DNA patterns were generated by electrophoresis on 1% agarose gel. PFGE was conducted using a CHEF-DRII system (Bio-Rad, Hercules, CA, USA) at 14 °C for 24 h, with voltage of 6 V/cm and with pulse time of 5–120 s, with linear gradient. *S. cerevisiae* was used as the molecular-weight standard. AFLP was performed according to Valsangiacomo et al. [11] with some modifications similar to that used in the European Working Group on Legionnaires' Disease (EWGLI) harmonization study [13].

SBT was performed according to the protocols of the EWGLI. Sequence data were obtained for a total of six genes, two genes (*flaA* and *proA*) as described by Gaia et al. [12] and additional gene targets under evaluation by members of the EWGLI as part of a multi-centre SBT proficiency panel [EWGLI (www.ewgli.org), unpublished data].

Purification and DNA sequencing

Amplicons were purified and nucleotide sequences were determined for both strands. Data from forward and reverse primer sequencing primers were combined by Autoassembler software and multiple alignments were obtained by Wisconsin Package, version 10.3 (Accelrys Inc., San Diego, CA, USA).

Environmental investigation

Possible sources of *Legionella* were inspected, and water and biofilm samples were collected from several sites (i.e. stores, patients' homes, and decorative fountains located in a public park). Air samples were taken using the Surface Air System Super 100 (SAS; PBI International, Milan, Italy). All samples were processed according to ISO 11731/1998. Suspected *Legionella* colonies from environmental and clinical isolates, were identified by Latex agglutination test (Oxoid), and confirmed by IFA test using mAbs directed against 15 different *Legionella* serogroups.

Data analysis

Data from the case-control study were entered in a database developed in Microsoft Access 2000 (Microsoft, Redmond, WA, USA) and analysed at the Istituto Superiore di Sanità with Epi-Info, version 3.2 (CDC, Atlanta, GA, USA). A descriptive analysis of cases and of main risk factors among cases and controls was conducted. For purposes of the analyses all exposures related to contacts with stores and streets were categorized in two different ways: as dichotomous variables (yes/no), and by number of contacts, which were subsequently categorized in three groups (never, 1–5, >5 contacts within the exposure period). To evaluate the association between the exposure and the disease, a matched-pair analysis was initially conducted, and χ^2 testing was used to assess the association among different exposures. Subsequently a conditional logistic regression model was constructed including all exposures which were found in the univariate analysis to have a P value of <0·20.

Crude and adjusted odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated to
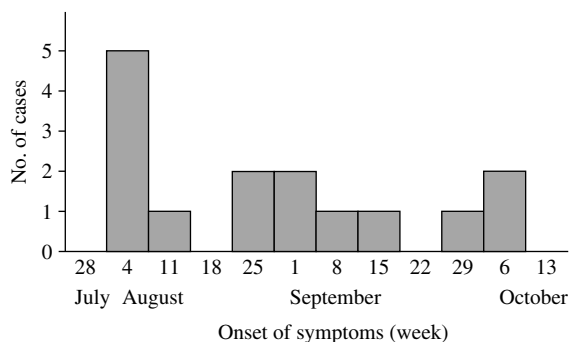
Fig. 1. Cases of Legionnaires' disease by week of onset, among residents of the 9th district of Rome, August–October 2003.

estimate the risk for LD associated with having visited certain stores and streets. Differences have been considered significant at the $P < 0.05$ level.

RESULTS

Descriptive epidemiology

A total of 15 confirmed cases of LD occurring between 8 August and 16 October (Fig. 1) were identified among residents in the 9th district, 13 of whom had been reported by the district hospital and two by other hospitals in Rome. Their mean age was 70 years, ranging from 49 to 89 years; 10 were men, yielding a male:female ratio of 2:1. All cases were hospitalized, and one patient died. Urinary antigen detection performed during hospital admission was positive for all cases.

Two respiratory secretion specimens (sputum and bronchiolar lavage fluid) were available for one patient, and *L. pneumophila* serogroup 1 was isolated from one of the two. Serum samples collected from eight patients were all positive at a low titre for *L. pneumophila* except for one which had a titre of 1:256.

Case-control study

Of the 15 cases, only 14 were included in the case-control study since one patient died before being interviewed. To enrol 56 controls, it was necessary to contact 184 persons, with a response rate among eligible individuals of ~30%. The major reason for non-participation was refusal.

No statistically significant differences among cases and controls were found regarding predisposing factors to the pneumonia and LD, although cases were more likely to report smoking >5 cigarettes per day

Table. *Case-control study. Matched-pair analysis*

Risk factors	Cases ($n=14$) %	Controls ($n=56$) %	OR	95% CI	*P* value
Department store					
A	78·6	28·6	9·8	2·1–46·0	0·004
B	42·9	17·9	4·2	0·9–18·2	n.s.
C	23·1	16·1	1·8	0·3–9·4	n.s.
D	7·1	25·0	0·2	0·1–1·7	n.s.
E	35·7	25·0	2·5	0·4–14·8	n.s.
F	50·0	46·4	1·2	0·3–4·5	n.s.
G	35·7	53·6	0·4	0·1–1·5	n.s.
Street					
H	78·6	64·3	1·9	0·5–7·2	n.s.
I	50·0	39·3	1·7	0·5–6·1	n.s.
L	78·6	64·3	2·6	0·5–13·0	n.s.
M	71·4	51·8	3·2	0·7–15·1	n.s.
N	28·6	23·2	1·4	0·3–5·6	n.s.
O	35·7	41·1	0·8	0·2–2·8	n.s.
P	71·4	39·3	3·8	1·1–13·4	0·04
Q	28·6	46·4	0·4	0·1–1·7	n.s.
Public park	50·0	17·9	4·2	1·2–14·9	0·03

OR, Odds ratio; CI, confidence interval; n.s., not significant.

(42·9% *vs.* 26·8%; OR 2·1, 95% CI 0·6–6·9), and to consume >40 g of alcohol per day (7·1% *vs.* 3·6%; OR 2·1, 95% CI 0·2–24·7). No significant differences were found for presence of chronic diseases (diabetes, chronic bronchitis, pulmonary emphysema, renal failure, transplant and cancer), or for the presence of risk factors in the domestic environment (presence of air-conditioning systems in the house, type of water-heating system, etc). In the matched-pair analysis, visits to department store A were strongly associated with *Legionella* infection (OR 9·8, 95% CI 2·1–46·0), while no association was observed for visits to stores B, C, D, E, F, G. Two other associations, i.e. having walked along a specific street and through the public park, were associated with *Legionella* infection (OR 3·8, 95% CI 1·1–13·4 and OR 4·2, 95% CI 1·2–15·0 respectively) (Table). Overall, 11 out of 14 cases (79%) visited department store A and two (14%) walked along two streets adjacent to department store A.

In conditional logistic regression multivariate analysis, however, department store A was the only exposure that remained strongly associated with *Legionella* infection (OR 9·8, 95% CI 2·1–46·0). Further analysis revealed that individuals who visited department store A more than five times during the likely exposure period were at a higher risk of
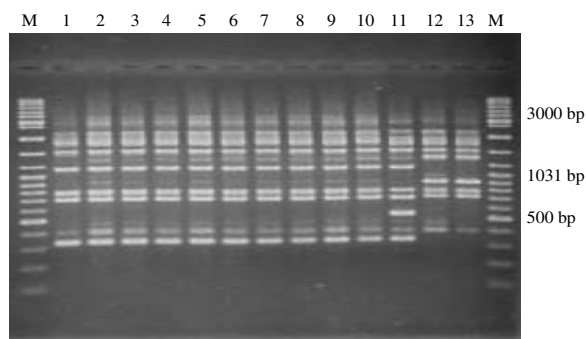
Fig. 2. AFLP analysis: lane 1, clinical strain; lanes 2–10, environmental strains; lanes 11, 12, environmental and clinical unrelated controls strains; lane 13, *Legionella pneumophila* Philadelphia 1 ATCC 33152 strain; M, molecular weight marker in base pairs. GeneRuler DNA ladder (MBI Fermentas, Vilnius, Lithuania).

Legionella infection (OR 22·8, 95% CI 3·6–145·8) compared to visitors who had been there less than five times.

Environmental and microbiological investigation

During the environmental investigation a cooling tower was identified in the basement of department store A. Moist air from the tower was expelled through a grid located on the street near the entry of the store.

L. pneumophila serogroup 1 subgroup Philadelphia was isolated from the storage tank and water system of this cooling tower at a concentration of $1·4 \times 10^6$ and $1·3 \times 10^6$ c.f.u./l respectively. The same *L. pneumophila* mAb subgroup was found at high concentration in the air samples collected on the street grid and in the biofilm samples collected from the storage tank. All cultures of the samples taken in other shopping centres, patients' homes and decorative fountains were negative for *Legionella* isolates. Of the two clinical samples obtained from one patient *L. pneumophila* serogroup 1, subgroup Philadelphia was only isolated from the bronchiolar lavage fluid.

One clinical strain and nine environmental strains isolated from water, air, and biofilm of the cooling tower of store A were analysed by three molecular methods. Both AFLP (Fig. 2) and PFGE analyses showed genomic similarity between clinical and environmental isolates. In addition, using the SBT approach that analyses genes that are selectively neutral and genes under probable selective pressure including *flaA* and *proA*, no nucleotide variation was found amongst the outbreak strains, while the controls showed distinct sequence types.
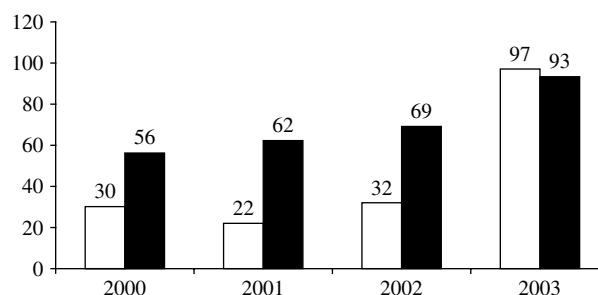

Fig. 3. Hospital admission for pneumonia of non-specified aetiology from 2000 to 2003 (period 14 July–2 November), by place of residence (9th district hospital, Rome). □, Ninth district; ■, other residence.

Estimation of the true magnitude of the epidemic

A total of 16 cases of legionellosis were admitted to the district hospital during the period 14 July to 2 November 2003; of whom 13 resided in the 9th district. During the same period in the previous year, eight individuals had been admitted to hospital for legionellosis, only two of whom were residents of the 9th district; while in 2001 and 2000 the number of hospitalizations was two (none residing in the 9th district) and four (one residing in the 9th district) respectively.

In 2003 in the same period (14 July–2 November), 190 hospital admissions for pneumonia of non-specified aetiology were recorded; of these 97 (51·1%) were among individuals residing in the 9th district. The corresponding values for 2002 were 101 admissions, of whom 32 (31·7%) were residents of the 9th district, while in 2000 and in 2001 the values were 86 (34·9% residents) and 84 (26·2% residents) respectively (Fig. 3). Compared with admissions for 2000–2002 the total number of admissions in 2003 for pneumonia without aetiological diagnosis increased 100% and 200% if only admissions among individuals residing in the 9th district were considered.

The 2003 increase was statistically significant if compared with each the three previous years ($P = 0·0180$, $P = 0·0002$, $P = 0·002$ compared to 2000, 2001 and 2002 respectively). The finding that the number of hospital admissions for pneumonia of non-specified aetiology dropped noticeably after the first disinfection of department store A's cooling tower suggested that some of the cases of non-specific pneumonia were outbreak-related.

Control measures

Following environmental investigation results, emergency control measures were implemented. A first

disinfection was carried out by an experienced company using a mixture of 500 ppm of hydrogen peroxide and silver (shock treatment) at the beginning of October.

The sampling conducted 2 days later on the sites previously found positive, showed a consistent reduction of the c.f.u. ($<10^2$/l) in the storage tank, while in the steam expelled by the cooling tower *Legionella* was still present at a concentration of 31 c.f.u./m³. A second disinfection was conducted at the beginning of December with results completely negative for the presence of *Legionella*.

A further disinfection was conducted by the same method after 15 days and another one a month later both followed by environmental sampling which remained negative for *Legionella*.

DISCUSSION

Epidemiological investigations revealed an increased risk of legionellosis for visitors of department store A, and for people passing nearby and in fact, only one out of 14 cases failed to report having visited or walked along the streets adjacent to the store. Microbiological investigations detected *L. pneumophila* from water and air samples collected from the cooling tower of store A, and the molecular analysis of genomic DNA from human and environmental isolates confirmed that all isolates were identical and belonged to a single strain of *L. pneumophila* serogroup 1, subgroup Philadelphia.

Overall 15 cases of LD among district residents were notified, although this is clearly an underestimation of the magnitude of the outbreak. Less severe clinical forms may have gone unnoticed and, as demonstrated by the increase in hospital admissions for pneumonia of non-specified aetiology, a considerable number of cases may not have been properly identified. The systematic testing of urinary antigen among patients admitted with the diagnosis of pneumonia of unclear aetiology could have allowed the identification of several other cases linked to the same source of infection.

The fact that six cases out of 15 reported the onset of symptoms during the first 2 weeks of August and, in particular, three cases in the same day, suggests that the exposure to *Legionella* in that period was more intense. This was probably due to the fact that, as reported by the store manager, renovation on the cooling system had been taking place during the second half of July and may have contributed to

contamination of the system. In addition, during the summer of 2003, atmospheric temperatures were unusually high. The average July temperature reached 28 °C, almost 3 °C above the average for the period, while during August it reached 29·1 °C, more than 4 °C above the average for the period, making this month the hottest since 1782, when registration of meteorological data began in Rome. These conditions of extreme temperature encouraged an intensive use of the cooling system, which, in association with the renovation, may have facilitated its contamination. Fortuitously, the spread of the outbreak may have been limited because traditionally, in Italy, during the month of August more than half of the population leaves the city to go on vacation, therefore reducing the number of potentially exposed individuals.

The extent of this outbreak highlights three important issues related to legionellosis.

First, it underlines the importance of obtaining cultures from at least some of the cases even though less than half of LD patients produce sputum, the bacteria survive poorly in respiratory secretions and cultures are often of limited diagnostic value because confirmation requires several days [14]. Although detection of urinary antigens permit rapid diagnosis and prompt institution of treatment and represent the most commonly used diagnostic method, it was the availability of a culture in one of the patients that permitted us to make the crucial epidemiological link between the disease and the presumptive source of the exposure.

A second issue is the need to make appropriate, in-depth molecular investigations in order to establish the true cause of infection. Of particular usefulness here was the convergence of data obtained from three different methods and pointing to a single biotype of *L. pneumophila*.

A third issue is the importance of cooling towers as sources of LD and the need for a mandatory registration of all cooling towers and evaporative condensers. Because Italy has thus far not experienced large outbreaks linked to cooling towers or evaporative condensers, registration has not been mandatory. However, the presence of such a register would allow, in case of an outbreak, the immediate identification of all cooling towers present in a certain area and would reduce the time required to conduct an environmental investigation. Furthermore, registration should be coupled with the provision of proper information to the owners on the importance of routine maintenance to avoid preventable diseases.

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