Legionnaires' disease in the Netherlands, 1998-2006

de Boer, J.W.

Citation for published version (APA):

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This thesis was prepared at the Municipal Health Service Kennemerland, Haarlem, the Netherlands.

The research was supported by grants of the Haarlem Tuberculosis Fund and the Public Health Foundation (Fonds ogz).

The printing of the thesis was financially supported by the Public Health Foundation (Fonds ogz), Oxiod BV and Clindia Benelux BV.

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ISBN: 978-90-9022710-8
Photo cover: Maurice Scheltens, Liesbeth Abbenes
Lay-out cover: Baster
Typesetting: TAT zetwerk, Utrecht
Printing: Ecodrukkers, Nieuwkoop

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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
Prof. Dr. D.C. van den Boom

ten overstaan van een door het college voor promoties ingestelde commissie, in het openbaar te verdedigen in de Aula der Universiteit

op woensdag 5 maart 2008, te 14.00 uur

door

Jeroen Willem den Boer
geboren te Eindhoven
Promotie commissie:

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Faculteit Geneeskunde
“Epidemic fever is caused by the air. Because all men inhale the same wind, when the air is infected with such pollutions that are hostile to the human race, the men fall sick.”

Hippocrates, Breaths xi
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CHAPTER ONE

General introduction

HISTORICAL BACKGROUND

Legionnaires’ disease (LD) is an airborne infectious disease named after a point-source outbreak of pneumonia among members of the American Legion. [1] Between 21 and 24 July, 1976, this organisation of military veterans held a convention in the The Bellevue Stratford hotel in Philadelphia. In the two week following the convention, 149 attendees developed pneumonia. Furthermore, 72 visitors and bypassers of the convention hotel attracted LD. Of the 221 LD patients who were part of the outbreak, 34 (15%) died. Five months later, a gram-negative bacillus was shown to be the causative agent and named Legionella pneumophila. [2] The same bacterium was later associated with Pontiac Fever (PF), [3] a flu-like selflimiting illness named after an airborne infectious disease outbreak in 1968 involving 144 persons in a health department facility in Pontiac, Michigan. [4] Pontiac Fever and LD are the only known clinical syndromes associated with Legionella bacteria and are collectively named legionellosis.

CLINICAL FEATURES AND DIAGNOSIS

LD patients initially present with cough, fever and nonspecific symptoms including malaise, myalgia, anorexia and headache, which are later followed by various stages of dyspnoea. Some patients develop shaking chills, chest pain, diarrhea, delirium or other neurologic symptoms. Extrapulmonary involvement is rare. Clinical presentation, [5] physical examination, [6] and radiographic findings [7] do not differ from pneumonias caused by other micro-organisms. Although minor biochemical differences may exist, [8,9] diagnosis of LD is therefore almost exclusively based on microbiology findings. Early diagnosis enables appropriate antimicrobial treatment early in the course of disease which is potentially life-saving. [10,11]

THERAPY

Evaluation of treatment with different antibiotics in the initial outbreak showed beneficial effects for erythromycin. [1] Since then, no clinical trials evaluating LD therapy have been published, but in vitro data and animal models suggest that newer macrolides and fluoroquinolones are superior to erythromycin. In the Netherlands, the Dutch thoracic society has issued guidelines accordingly. [12,13]
MICROBIOLOGY

Description of the pathogen

Legionella is an intracellular parasite of protozoa. So far, 17 different hosts have been described, [14] of which Hartmanella vermiformis is the most frequently observed. [15] The resemblance of human alveolar macrophages to these protozoa is striking and has become a key issue in the understanding of the pathogenesis of LD. Cellular microbiology research has revealed that the focal point of infection by Legionella is the alveolar macrophage. [16]

Culture, serotyping and genotyping

Legionella must be grown on specific media and is tolerable to acid and chloride. [17] It can survive temperatures between 0 and 63°C and grows between 20 and 43°C with an optimum of 37°C. [18,19] The family of Legionellaceae [20] consists of 50 species and 70 serogroups. Nineteen species have been documented as human pathogen. [14] Worldwide, more than 90% of LD is caused by the species Legionella pneumophila, of which 92% is caused by L. pneumophila serogroup 1. [21] Molecular microbiology methods are used to further differentiate beyond the serogroup level. These techniques can be supportive to identify a source of infection, several of which have been used in outbreaks in the Netherlands. [22–27] In recent years the recommended DNA fingerprinting techniques of the European Working Group for Legionella Infections (EWGLI) [28–31] have been introduced in the Netherlands. [32–34] Since 2004, the genomic sequence of the original 1976 Legionella pneumophila isolate is known. [35]

EPIDEMIOLOGY

Case definition

According to the source of infection LD is commonly classified as community-acquired, nosocomial, or travel-related. Also a person-based classification can be used which differentiates single patients (sporadic cases), clusters (geographic or serial), and outbreaks. Furthermore, EWGLI differentiates between confirmed and probable LD patients on the basis of diagnostic criteria. [36]

Incidence

In the Netherlands, LD became a notifiable disease on June 4th, 1987, eleven years after the first Dutch LD patient had been diagnosed. [37] For the 1987–1999 period the mean yearly incidence in the Netherlands was 2.7 per million for an average of 42 notified LD patients per year. Half of these patients suffered from confirmed LD and half from probable LD. [38] The mean incidence in the 1987–1999 period was 50% lower than that of Europe and the US. [39,40]
Several small outbreaks were described during that period, three of which were associated with travel abroad, [41–43] four were hospital associated [24,26,44,45] and two were community-acquired. [25,46] In 1999, a large outbreak involving 188 patients (this thesis, chapter two) [27] marked a sharp rise in notified LD incidence to 11 per million, excluding the outbreak patients. [38] The incidence has continued to rise to 26 per million in 2006, excluding 30 patients who were part of a cooling-tower outbreak in that year. [34]

**Geographic distribution**

LD incidence differs between countries in Europe [47], but also within countries. [48] In the Netherlands, a fivefold difference in LD incidence was observed using province as geographic entity. [38] In this thesis, in chapter four, geographic differences are further explored at the municipal level.

**Temporal influence**

Seasonality has been reported for LD, with peaks in spring and autumn. [49] Since prospective incidence studies did not confirm this pattern, [50] the peaks could also be attributed to ascertainment bias or to unnoticed cooling tower outbreaks. [51] Humidity seems to play a role in increased endemic situations. [52,53]

**Risk factors**

Although most environmental *Legionella* strains are not pathogenic they can infect humans with an impaired immune system, [54] as is seen in nosocomial LD. For community-acquired LD, smoking is the strongest host-related risk factor for sporadic [55] as well as outbreak-related patients. [1]

**Transmission**

**Reservoir**

The natural habitat for *Legionella* is surface water of lakes, rivers as well as thermally polluted water. [56] In these aquatic environments *Legionella* bacteria live as parasites in protozoa. [57] The same observation was made in manmade water systems where biofilm provided a favorable environment sometimes leading to high concentrations of *Legionella*. [58,59]

**Route**

The transmission route from these habitats to the human lung has predominantly been postulated to result from inhalation of infected aerosols. Also, drinking of contaminated water and subsequent aspiration has been described as a route of transmission. [33,60,61]
While the exact mechanism of transmission remains unsolved, several facilities and water systems are associated with LD outbreaks: hospitals, [62] cooling towers, [63–65] saunas, [25] and whirlpool spas. [66] Numerous serosurveys have demonstrated high prevalences of antibody titers against Legionella suggesting that humans are regularly exposed to the bacterium without developing pneumonia. [67,68]

**Aerosols**

It is rather Legionella bacteria than aerosols which are inhaled in the pathogenesis of LD. Aerosols that find their origin in the aquatic environment vary in size between 12–2000 µm. Those measuring 10 µm can float and spread over larger distances. However at 65% air humidity these aerosols are evaporated in milliseconds to seconds. At 100% air humidity they evaporate within 100 seconds. The role of aerosols therefore seems to be Legionella’s escape from the aquatic environment.

**Virulence**

Soon after its discovery it was demonstrated that Legionella lose their pathogenic capacity in artificial media. [69] Contrastingly, they become more virulent after passage in protozoa like amoebae. [70,71] In the interaction with protozoa, lack of nutrition for Legionella accelerates the development of virulent traits like mobility and penetration capacity. Subtyping of L. pneumophila using monoclonal antibodies (MAb) suggests higher virulence for strains reacting with MAb2 (international panel) or MAb3 (Dresden panel) since they are more often associated with outbreaks and less often with nosocomial LD. [72,73] Several outer membrane proteins have been associated with virulence, the macrophage infectivity potentiator (Mip) most convincingly as its absence lead to a 80-fold decrease in infectivity for human macrophages. [74]

**Infective dose**

Environmental research has shown high concentrations of Legionella in aerosol producing water systems and devices as well as in potable water worldwide without the occurrence of LD patients. [75–77] Indeed, the omnipresence of Legionella is in sharp contrast to its low incidence. This has been described as the “infective dose paradox”. [78] Low incidence suggests that the infective dose is very high. Indeed, extrapolation of animal research suggested a high infective dose of 14 million bacteria. At the same time microbiologic research suggests a low infective dose for three reasons. First, to reach the alveoli particles should be smaller than 1 µm, which is the size of a few hundred Legionella bacteria. [79] Second, LD patients who are part of cooling tower related outbreaks are infected a kilometer or more away from the source, [65,80,81] whereas a high concentration of airborne Legionella at such a distance seems highly unlikely. Third, exceptional routes of transmission have been described involving very low calculated numbers of bacteria. [33]
Host

With an intact host defence, inhaled *Legionella* are removed from the bronchial tree by mucociliary clearance and subsequently killed by gastric acid in the stomac. Cell-mediated immunity is the primary host defense against *Legionella* infection. \[82,83\] Human monocytes and alveolar macrophages activated by lymphokines inhibit intracellular multiplication of *Legionella*. Innate and humoral immunity do not seem to play an important role. \[16\]

CONTROL AND PREVENTION

Long term prevention

Removing dead legs and setting the water temperature above 60 degrees Celsius is a widely advised preventive measure. Few of the environmental *Legionella* strains that humans are exposed to on a daily basis are in fact capable of causing LD. With this knowledge, general preventive measures are not very effective. Three observations are ingredients for a potential successful prevention scheme. One is that outbreaks are often preceded by small clusters or solitary LD patients, \[86\] the second that apparently sporadic LD patients are in fact clustered around the same source of infection \[87\] and the third that time clusters of LD patients can span up to seventeen years. \[62\]

Disinfection

Despite very promising results on residual disinfection of municipal drinking water with monochloramine \[88–90\] there is little support for disinfection of potable water in the Netherlands. Although several emergency decontamination methods exist, in the Netherlands the “superheat and flush” method is the most widely used. \[84\] For cooling towers decontamination in the Netherlands, chlorination has been used. \[85\]

Legislation on Legionella

In the Netherlands, the Ministry of Housing, Spatial Planning and the Environment (vrom) is responsible for the quality of drinking water. After the 1999 outbreak, vrom issued a new Drinking Water Law aimed at reducing the level of *Legionella* contamination of drinking water. The Water Law is based on the precaution principle that use of water should lead to less than one death per one million person years. Despite extensive investments to arrive at the reduced contamination, over the last eight years the incidence of LD in the Netherlands has continued to rise.
OUTLINE OF THE THESIS

In part one the epidemiology of LD in the Netherlands is described. A large outbreak involving 188 patients and a prospective case-control study to identify risk factors are presented. Furthermore, geographic differences in LD incidence in the Netherlands are explored.

In part two the transmission of LD is elaborated on. A rare transmission route involving potting soil is shown to exist in the Netherlands and a prospective study comparing patient isolates as well as environmental Legionella strains indicates that current knowledge on transmission is still incomplete.

In part three the laboratory diagnosis of LD is explored and several diagnostic assays are discussed in detail.

In part four control and prevention of LD is discussed. The first results of a newly installed outbreak detection and prevention scheme in the Netherlands are described. Part four ends with a general discussion and conclusion.

INSTITUTES AT WHICH THE STUDIES WERE PERFORMED

The 1999 outbreak investigation was studied at the Epidemiology and Surveillance (epi) department of the National Institute of Public Health and the Environment (rivm) in Bilthoven. First, in 2000 and 2001 three national rivm reports (nrs. 213690006, 213690003 and 213690004) were published that preceded an international publication in 2002 that is presented in part 1 of this thesis. Most other studies were performed at the Municipal Health Service Kennemerland in Haarlem. This institute received several grants, among them three grants from the Haarlem Tuberculosis Foundation that helped realise the two other studies presented in part 1 of this thesis. Also, a four-year grant was provided by the Public Health Foundation (Fonds OGGZ). This grant enabled the formation of a National Legionella Outbreak Detection Programme (bel) which ran from 2002–2006 and has moved since to the Regional Public Health Laboratory (rphl) Kennemerland where it has become part of the rivm. Data for the studies presented in parts 2 and 4 of this thesis were collected by bel. The studies in part 3 of the thesis were performed at the rphl Kennemerland in Haarlem.

REFERENCES


PART I

EPIDEMIOLOGY
CHAPTER TWO

A Large Outbreak of Legionnaires’ Disease at a Flower Show, the Netherlands, 1999

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ABSTRACT

In 1999, an outbreak of Legionnaires’ disease affected many visitors to a flower show in the Netherlands. To identify the source of the outbreak, we performed an environmental investigation, as well as a case-control study among visitors and a serologic cohort study among exhibitors to measure exposure to possible sources. Of 77,061 visitors, 188 became ill (133 confirmed and 55 probable cases), for an attack rate of 0.23% for visitors and 0.61% for exhibitors. Two whirlpool spas in halls 3 and 4 of the exhibition and a sprinkler in hall 8 were culture positive for Legionella pneumophila. One of three genotypes found in both whirlpool spas was identical to the isolates from 28 of 29 culture-positive patients. Persons who paused at the whirlpool spa in hall 3 were at increased risk for becoming ill. This study illustrates that whirlpool spas may be an important health hazard if disinfection fails.

From March 7 to 11, 1999, 10 patients with severe pneumonia were admitted to a hospital in Hoorn, in the northern part of the Netherlands. The clinical condition of these patients deteriorated quickly and unexpectedly, with eight requiring mechanical ventilation. On March 11, six of the eight patients were diagnosed with Legionnaires’ disease on the basis of a positive Legionella urine antigen test. Additional patients with severe pneumonia were sent to another hospital since all the respirators at the Hoorn hospital were in use. Two of these patients were subsequently diagnosed with Legionnaires’ disease by urine antigen test.

An exploratory case-control study using a questionnaire on exposure to potential sources was conducted; six confirmed and four probable cases were included. All patients and 3 of 21 controls had visited the West Frisian Flower Show (p<0.001). Since no other environmental risk factor was identified, the outbreak investigation focused on the flower show. This show is an annual event that includes an agricultural and consumer products exhibition, located

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in the nearby town of Bovenkarspel. Held from February 19 to 28, the flower show attracted 77,061 visitors. After a nationwide warning was issued to ensure detection and appropriate treatment of any additional cases, it became clear that >180 persons had been affected. We report how the source of the outbreak was identified by a combination of an environmental investigation, a case-control study among flower show visitors, and a cohort study among >700 exhibitors.

METHODS

A confirmed case of Legionnaires’ disease was defined as radiologically confirmed pneumonia in a visitor to the exhibition or a member of the exhibition staff, with onset no earlier than February 19, 1999, and no later than March 21, 1999, as well as laboratory evidence of *Legionella pneumophila* infection. Laboratory evidence included isolation of *L. pneumophila* from respiratory secretions, detection of *L. pneumophila* antigens in urine, or a fourfold or higher rise in antibody titers to *L. pneumophila* in paired acute- and convalescent-phase sera, as reported by clinicians. A probable case was defined as radiologically confirmed pneumonia with onset no earlier than February 19, 1999, and no later than March 21, 1999, in an exhibition visitor or a member of the exhibition staff who did not meet laboratory criteria for a confirmed case, but who had no laboratory evidence of infection by other microorganisms. All local health services and hospitals in the Netherlands were informed of these criteria and asked to report cases of pneumonia in persons who had visited the exhibition. Unsolicited case reports from the public were also recorded. Finally, all Dutch clinical medical microbiology laboratories were asked to send clinical *Legionella* isolates from flower show-related Legionnaires’ disease patients to the National Institute of Public Health and the Environment (rivm) for serotyping and genotyping.

Environmental Risk Assessment

A map of the water system at the exhibition site was made to facilitate visual inspection for circulatory dead ends and other potential locations of stagnant water. In addition, we interviewed all exhibitors to compile an inventory of all products using water that had been displayed at the exhibition. Based on these interviews, an 8-point risk-assessment scale was developed to discriminate among the products used during the exhibition. For each of the following items, 1 point was given: use of water; use of water at 20°C to 43°C (the temperature range within which *Legionella* can amplify to dangerous concentrations); [1,2] use of water at 37°C (the optimal temperature for *Legionella* growth); [3] no disinfection of water at 20°C to 43°C; no changing of water at 20°C to 43°C; occasional misting of water at <60°C; continuous misting of water at <60°C; and substantial surface for misting of water at <60°C.

Two weeks after the end of the exhibition, we began to obtain water and swab samples from all potential sources of *Legionella*. The water samples were concentrated by membrane filtration (0.2 µm), and filtered residues were resuspended in 1 mL sterile water. Of this suspension,
100-µL samples were cultured without dilution and after 10- and 100-fold dilutions on buffered charcoal yeast extract agar with α-ketoglutarate (bcye-α) and a selective supplement with dyes and the antibiotics polymyxin, anisomycin, and vancomycin (Legionella MWY Selective Supplement s818, Oxoid Ltd., Hampshire, England). Plates were incubated at 37 °C with increased humidity. In case of bacterial overgrowth, cultures were repeated after pretreatment by heating 30 minutes at 50 °C. Swab samples were dispersed by immersion in 1 mL sterile water and cultured as described. Cultures were examined microscopically daily for 14 days. In case of persistent overgrowth, ceftazidime was added to the media. Colonies suspected of being Legionella were subcultured to blood agar and bcye-α agar. Identification was confirmed by biochemical tests. Legionella isolates were serogrouped by using commercial kits containing antisera against L. pneumophila serogroups 1–14, L. longbeachae 1 and 2, L. bozemanii 1 and 2, L. dumoffii, L. gormanii, L. jordanis, L. micdadei, and L. anisa (Legionella Latex Test, Oxoid Limited, Hampshire, England; Legionella antisera “Seiken,” Denka Seiken Co. Ltd., Tokyo, Japan). Isolates were genotyped by pulsed-field gel electrophoresis and amplified fragment-length polymorphism.

**Case-Control Study**

To measure visitor exposure to possible sources of Legionella, we used a questionnaire, a set of situational drawings, and a floor plan of the exhibition site. The questionnaire addressed health status and details about visits to different parts of the exhibition or displays of devices capable of spreading Legionella. [6–8] As controls, a random sample of 2,500 men and 2,500 women born before 1960 were selected from the municipal population register. A request for participation in the study was sent to all these persons, but they were asked to reply only if they had visited the exhibition. Of the first 469 who replied, all 196 men and a random selection of 203 women were sent the questionnaire. Respondents were excluded as controls if they had symptoms of respiratory infection within 20 days of their exhibition visit (for pneumonia and bronchitis) or within 4 days of their visit (for influenzalike illness). Both cases and controls were asked to indicate the route they had walked and the stands they had visited on a floor plan of the exhibition site and situational drawings of stands at which devices using water were displayed. To avoid bias, drawings of stands at which products were demonstrated that did not use water were also included. Ill persons were interviewed personally or by proxy.

Variables that were significant in univariate analysis were entered in a multiple logistic regression model. With the use of backward elimination, independent predictors of becoming ill, adjusted for age and sex, were established. Variables were retained in the model if the likelihood ratio test was significant (p<0.1).

**Cohort Study**

Letters were sent to exhibition volunteers, staff of the company organizing the exhibition, and all exhibitors (n = 1,616) (with the exception of confirmed and probable Legionnaires’ disease cases), asking them to complete a questionnaire regarding their health status before
and after the exhibition. Questions were included about principal work location during the exhibition. Participants were asked to have paired blood samples drawn and sent to RIVM. The first samples were taken by the end of March and the second by mid-May. Serum immunoglobulin (Ig) M and IgG antibodies against L. pneumophila were detected by indirect enzyme-linked immunosorbent assay (Virion-Serion, Würzburg, Germany). For every 63 cm² area of the exhibition site, the geometric mean IgM and IgG titers of the nearest 35 respondents were determined and plotted after smoothing by using the highest titer in paired sera for each respondent. The correlation of the logs of the antibody titers and distance to aerosol-generating devices was analyzed by linear regression, after the data were adjusted for age and sex of respondents, smoking status, underlying disease, and time worked in each hall. In all analyses, data were included only from persons who had worked at the exhibition after February 22.

**RESULTS**

A total of 188 cases of Legionnaires’ disease (133 confirmed, 55 probable) were reported in visitors (178) and exhibitors, [10] originating from throughout the Netherlands. An additional 21 patients had physician-diagnosed pneumonia but no radiologic studies, and 9 patients had positive urine antigen tests but insufficient clinical data. Dates of onset ranged from February 25 to March 16 (Figure 1). The median age of patients was 66 years (range 20 to 91 years), and the male:female ratio was 1:1.4. Diagnosis was confirmed by culture in 29 cases, urine antigen test in 100 cases, and serologic testing in 53 cases (Table 1). In 54 cases, two or more tests were positive. Among patients for whom data were available and who visited the exhibition once (n = 116), the reported incubation period was 2 to 19 days (median 7 days); in 22 cases (16 %) the time before onset of illness exceeded 10 days. All but one ill person had visited the exhibition after February 22. The exception was a 55-year-old woman with a history of chronic obstructive pulmonary disease and pneumonia who visited the exhibition on February 21. She visited hall 3 for 45 minutes and stopped near the whirlpool spa.

The attack rate for visitors was 178/77,061 or 0.23%. The daily attack rate increased from 0.011 % on February 21 to 0.56 % on February 27 (Figure 2). Ten of the exhibitors, volunteers, and employees became ill, for an attack rate of 0.61 % among staff. Seven of these 10 staff
members worked in the right side of hall 3 (Figures 3a and 3b). The attack rates for staff were 2.7% for hall 3 and 0.4% for hall 4 (Fisher exact test, p = 0.005).

Of the 188 patients, 163 (87%) were hospitalized and 34 (21%) required mechanical ventilation. Seventeen persons with confirmed and 4 with probable LD died, for a case-fatality rate of 11%. The case-fatality rate was highest (17%) in patients >70 years of age.

Clinical isolates of 29 patients were available for genotyping. Twenty-eight were identical to the strain designated B-1, and one was identical to B-2.

Table 1. Positive diagnostic tests results for 188 cases (133 confirmed and 55 probable) of Legionellosis, the Netherlands, 1999

<table>
<thead>
<tr>
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<th>Culture</th>
<th>Urinary antigen</th>
<th>Fourfold rise in titer</th>
<th>Direct immunofluorescence</th>
<th>PCRb</th>
<th>Single high titer</th>
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<tr>
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<td>Fourfold rise in titer</td>
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<td>Direct immunofluorescence</td>
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<td>Single high titer</td>
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<td>0</td>
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</table>

* The table is read as follows: of 29 patients who had positive cultures, 24 were positive by urinary antigen, 3 had a fourfold rise in titer, 2 were positive by direct immunofluorescence, and 11 by PCR, and 4 had single high titers. b PCR = polymerase chain reaction.
The 11 halls of the exhibition site were supplied with water by two separate systems. The flower show was held in halls 2, 5, and 13; the consumer exhibition in halls 3 and 4; and the agricultural exhibition in halls 8 and 9 (Figure 4a). During the 3 months before the exhibition, the right side of hall 3 had been partitioned off to preserve flower bulbs at 30°C. In halls 5 and 13, 11 decorative fountains and a waterfall were installed. The temperature in these two halls was kept under 15°C to preserve the flowers. Our risk assessment of the water-using devices showed (Figure 4b) that a whirlpool spa in hall 3 posed the greatest risk (8 points), followed by a whirlpool spa in hall 4 (6 points), two bubblemats (1) in halls 3 and 4 (4 points), 11 fountains in halls 5 and 13 (4 points), and a sprinkler installation in hall 8 (3 points).

The whirlpool in hall 3 had never been used before, and its disinfection system failed. Ten samples were collected from the municipal water supply and 127 from the water system of the exhibition building (Figure 4a). Of 27 water-using devices that had been on display at the exhibition, 12 (including the whirlpool spas and the sprinkler installation) were available and still contained water (Figure 4c).

1A bubblemat is an inflatable rubber mat that causes a whirlpool-like effect when placed in a normal bathtub.
A total of 145 samples were taken from these 12 devices. All cultures of specimens from the water supply system were negative for _Legionella pneumophila_, but the organism was cultured from paper filters of the whirlpool spa in hall 3 (>100 colonies), the whirlpool spa in hall 4 (2 colonies), and the sprinkler installation in hall 8 (15 colonies). Subsequent sampling of the inner tubing system of both whirlpool spas, 6 weeks after the exhibition ended, yielded abundant growth of _L. pneumophila_ from swabs of the hall 3 whirlpool spa. No growth was found in the remaining water from the whirlpool spa in hall 4, which had a chlorine concentration of 0.64 mg/L.

Serotyping and genotyping of environmental isolates yielded three distinct genotypes: two serogroup 1 isolates (called B-1 and B-2) and one serogroup 6 isolate (called B-3). Genotyping results for pulsed-field gel electrophoresis and amplified fragment-length polymorphism were in agreement (Figure 5).

The filter and the inner tubing of the hall 3 whirlpool spa contained B-1, B-2, and B-3 (semiquantitatively B1>B2>B3), the filter of the hall 4 whirlpool spa contained B-1, and the filter of the sprinkling installation contained B-2 and B-3 (semiquantitatively B-2>B-3). Cultures of the inner tubing of the hall 3 whirlpool spa taken several months after the exhibition were still positive and yielded all three genotypes.
**Figure 5.** Pulsed-field gel electrophoresis (PFGE) and amplified fragment-length polymorphism (AFLP) patterns of a representative selection of clinical and environmental *Legionella pneumophila* isolates; the dendrogram shows clustering in PFGE. The AFLP and PFGE pattern of the isolate of patient 15 (genotype B-1) was found in 28 of the 29 isolates of culture-positive cases; the same pattern was found in isolates cultured from the whirlpool spas in halls 3 and 4. The AFLP and PFGE pattern of the isolate of patient 25 (genotype B-2) was unique among culture-positive cases; the same pattern was found in isolates cultured from the whirlpool spa in hall 3 and the sprinkler.

**CASE-CONTROL STUDY**

The rates of response to the questionnaire and drawings were 85% and 58% for cases and 98% and 65% for controls, respectively. Thirty-six controls who reported symptoms of respiratory infection were excluded from the analysis. Analysis was restricted to data from 71 confirmed and 30 probable cases and 119 controls who visited the exhibition after February 22 (Table 2).

The variables that remained in the multiple regression model were based on 62 confirmed and 21 probable cases and 105 controls (Table 3). Our analysis showed that smoking and length of stay at the exhibition were risk factors for infection. Length of stay exclusively at the consumer products exhibition showed an inverse relation, after data were adjusted for total length of stay. However, cases and controls on average spent the same amount of time at the consumer exhibition. Drinking tap water was not a risk factor. Visiting the whirlpool spa display in hall 3 was a risk factor. Visiting the bubblemat display in hall 3 was also associated with risk, but pausing in the gangway of the bubblemat display showed an inverse relation. Similar but not always statistically significant results were found when the analysis was limited to confirmed cases.
COHORT STUDY

Of the exhibition staff, 880 responded to the cohort study questionnaire (54%), and 714 (44%) provided two analyzable blood samples. Geometric mean IgG and IgM titers were not associated with drinking tap water or contact with potting compost. Geometric mean IgM and IgG titers were significantly increased (p < 0.0002) among exhibitors in hall 3 but not among those in hall 4, compared with exhibitors working in other halls. Respondents who worked in the right side of hall 3 had the highest average antibody titers (Figures 3a and 3b).

Table 2. Results of univariate analysis of data from questionnaires and drawings comparing host factors and visits to specific sites at the exhibition for cases and controls.

<table>
<thead>
<tr>
<th>Study population</th>
<th>Respondents questionnaire and set of drawings; univariate odds ratio (95% CI) (101 cases, 119 controls)</th>
<th>Respondents questionnaire and set of drawings; raw data (101 cases, 119 controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.1 (1.0 – 1.3)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Male sex</td>
<td>2.7 (1.6 – 4.7)</td>
<td>63/45</td>
</tr>
<tr>
<td>Underlying disease</td>
<td>7.2 (3.5 – 15.1)</td>
<td>11/2</td>
</tr>
<tr>
<td>Smoking</td>
<td>2.0 (1.1 – 3.6)</td>
<td>42/31</td>
</tr>
<tr>
<td>Total hours at exhibition</td>
<td>1.7 (1.4 – 2.2)</td>
<td>4/3</td>
</tr>
<tr>
<td>Hours at consumer exhibition</td>
<td>1.0 (0.8 – 1.3)</td>
<td>1/1</td>
</tr>
<tr>
<td>Pausing at whirlpool spa in hall 3</td>
<td>4.1 (1.9 – 9.0)</td>
<td>41/21</td>
</tr>
<tr>
<td>Pausing at bubblemat in hall 3</td>
<td>3.7 (1.6 – 8.2)</td>
<td>37/17</td>
</tr>
<tr>
<td>Pausing in gangway of bubblemat in hall 3</td>
<td>0.4 (0.2 – 1.0)</td>
<td>24/35</td>
</tr>
<tr>
<td>Pausing at electric kettle in hall 3</td>
<td>3.0 (1.3 – 7.0)</td>
<td>26/12</td>
</tr>
<tr>
<td>Pausing at whirlpool in hall 4</td>
<td>2.4 (1.1 – 5.4)</td>
<td>31/20</td>
</tr>
<tr>
<td>Pausing at steam iron in hall 4</td>
<td>5.4 (1.4 – 22.0)</td>
<td>16/3</td>
</tr>
</tbody>
</table>

a OR = odds ratio; CI = 95% confidence intervals; na = not available.

Table 3. Logistic regression model of data from questionnaires and drawings comparing host factors and visits to specific sites at the exhibition for cases and controls.

<table>
<thead>
<tr>
<th>Study population</th>
<th>Respondents to questionnaire and set of drawings; odds ratio (95% CI) (101 cases, 119 controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.1 (1.0 – 1.3)</td>
</tr>
<tr>
<td>Male sex</td>
<td>2.1 (1.0 – 4.5)</td>
</tr>
<tr>
<td>Smoking</td>
<td>6.0 (2.4 – 15.1)</td>
</tr>
<tr>
<td>Total hours at exhibition</td>
<td>2.2 (1.5 – 3.2)</td>
</tr>
<tr>
<td>Hours at consumer exhibition</td>
<td>0.5 (0.3 – 0.8)</td>
</tr>
<tr>
<td>Pausing at whirlpool spa in hall 3</td>
<td>2.6 (1.1 – 6.6)</td>
</tr>
<tr>
<td>Pausing at bubblemat in hall 3</td>
<td>3.0 (1.1 – 8.0)</td>
</tr>
<tr>
<td>Pausing in gangway of bubblemat in hall 3</td>
<td>0.3 (0.1 – 0.8)</td>
</tr>
</tbody>
</table>

a OR = odds ratio; CI = 95% confidence interval. b see figure 3a

29
Multiple linear regression showed that proximity to both the whirlpool spa and the bubblemat in hall 3 was positively associated with increase in antibody titer, but no such relation was found for distance to the bubblemat and whirlpool spa in hall 4. Since the bubblemat and the whirlpool spa in hall 3 were situated close together, exposure to each was highly correlated and risk could not be differentiated. An inverse relation was found between the attack rate for staff with confirmed legionellosis and the distance of their workplace to the whirlpool in hall 3 (p = 0.0009). Staff members who became ill and who worked in the right side of hall 3 differed from their colleagues in the same hall with respect to age, gender, and smoking habit (Table 4).

**DISCUSSION**

A new whirlpool spa, within 4 days after its installation, was the major source of one of the world’s largest outbreaks of Legionnaires’ disease. With 188 (133 laboratory-confirmed) cases, this outbreak is only exceeded by the original 1976 outbreak in Philadelphia (221 cases). Because our case definition for probable cases was broad, the 55 probable cases may include some persons with other, undetected causes of pneumonia. Detailed studies of clinical and laboratory-diagnostic characteristics of the patients are ongoing.

Despite these limitations, this outbreak of *Legionella* pneumonia is certainly the largest to be associated with a contaminated whirlpool spa. Although the overall attack rate (0.24%) was low for this outbreak in comparison with other nonhospital indoor outbreaks (4% to 7%), [9–11] the large number of visitors resulted in a large number of patients. The fact that the outbreak was not detected until 14 days after the first case of pneumonia was diagnosed, when 71 pneumonia patients had already been hospitalized, is remarkable. In hindsight, the

| Table 4. Characteristics of LD cases who worked in the right half of hall 3 compared to staff members who did not get LD and worked in the same hall |
| --- | --- | --- | --- |
| | Cases right half hall 3 (n=7) | Cohort respondents hall 3 (n=153) |
| | n | % | n | % |
| **Age group** |  |  |  |  |
| <30 | 0 | 0 | 23 | 15.7 |
| 30–39 | 0 | 0 | 24 | 16.3 |
| 40–49 | 1 | 14 | 29 | 19.7 |
| 50–59 | 4 | 57 | 46 | 31.3 |
| 60–69 | 2 | 29 | 20 | 13.6 |
| >=70 | 0 | 0 | 5 | 3.4 |
| **Males** | 6 | 86 | 67 | 45.3 |
| **Smokers** | 4 | 67* | 44 | 29.1 |
| **Immunologically compromised** | 0 | 0 | 5 | 3.3 |
| **History of pneumonia** | 1 | 14 | 5 | 3.3 |
| **History of diabetes** | 1 | 14 | 5 | 3.3 |

* data of one case missing
first hospitalized patient could have been diagnosed on February 25, when only 40 to 50 of the eventual 188 patients had been infected. Although immediate diagnosis would have enhanced the possibility of timely public health intervention, the source of the outbreak is unlikely to have been discovered before the end of the exhibition. Late detection may be due partly to small-scale use of the *Legionella* urine antigen test in the Netherlands. Dutch physicians may have considered Legionnaires’ disease a rare event, since over the last 10 years no more than 45 cases per year have been reported and few community-acquired outbreaks have been described. \[12,13\] In 2000, the number of reported cases in the Netherlands was 176, suggesting underdiagnosis in previous years.

No guidelines concerning the use and maintenance of whirlpool spa displays exist in the Netherlands. Our data demonstrate that contaminated spas may remain culture positive for months, perhaps as a result of stagnant water in their extensive inner tubing system. Because *Legionella* is ubiquitous in water systems, prevention of Legionnaires’ disease depends mainly on disinfection. This study shows that whirlpool spas may become a health hazard if their disinfection system fails. Bathing in whirlpool spas has led mainly to outbreaks of Pontiac fever \[14–18\] and, to lesser extent, of Legionnaires’ disease. \[6,19,20\] Our data show that even staying in the vicinity of a whirlpool spa or walking in a hall where an operating whirlpool spa is on display may be important risk factors for Legionnaires’ disease. Considering the popularity of whirlpool spas at home and the number of exhibitions where they are displayed, we suspect that small outbreaks have occurred without detection.

Clearly, strict regulations concerning the use, maintenance, and display of whirlpool spas are needed. The public at large should be informed as to the potential health hazards posed by whirlpools spas in public facilities and at home.

Unique in this outbreak was the circumscribed time of exposure for each individual patient to an identified source of *Legionella* infection. The finding that in 16% of cases the reported incubation period exceeded 10 days has major clinical and public health consequences. This finding contrasts with that of the Philadelphia outbreak, when only two cases had such long incubation times (16 and 26 days, respectively). \[10\]

In this outbreak investigation, a unique combination of three epidemiologic approaches allowed a comprehensive understanding of the chain of events, even as the investigators were confronted with numerous potential sources, three of which were culture positive. Our simple risk assessment of devices capable of spreading *Legionella*-infected aerosols proved to be an effective and timely predictor of the likelihood that a device was a source of the outbreak. The assessment and subsequent cultures revealed that the whirlpool spa in hall 3 was most likely the major source because it had been in continuous operation and its water had not been changed during the exhibition, unlike the whirlpool spa in hall 4. The bubblemats in halls 3 and 4 were demonstrated in room-temperature water, which was changed several times during the exhibition. Both mats had been dried and stored by the time of the environmental sampling.

The results of the case-control study indicate that pausing at this whirlpool spa was the most important consumer-related risk factor. Information bias related to this outcome is probably minimal, since the Dutch news media never mentioned the site of the whirlpool spa when reporting on the origin of the outbreak.
The results of the cohort study show that the average antibody levels were highest in the right side of hall 3, near the whirlpool spa. Plotting the geometric mean IgM and IgG titers of the nearest 35 exhibitors per surface area demonstrated that proximity to the whirlpool spa in hall 3 was associated with an increase in antibody titers, whereas this association was absent in hall 4. These results correlate with the inverse relation between attack rate among staff members and distance to the whirlpool spa in hall 3. The smoothing technique used in our analysis gives an average antibody titer (for the nearest 35 exhibitors) per square meter. The exhibition hall was divided into 63 cm² squares, and for each square the smoothed average antibody titer was calculated and a color was assigned corresponding to a certain titer range. The color pattern gives an idea of the pattern of infected aerosols or movement of exhibitors.

The whirlpool spa in hall 3 had just been purchased; it was filled on February 17 and kept at 37°C throughout the exhibition. The concentration of L. pneumophila must have risen to levels infectious for immunocompromised visitors from February 21 onward and healthy visitors from February 23 onward. Similar growth rates have been reported. [21,22] The increasing attack rate per day indicates that the continued growth of Legionella led to spread of aerosols bearing ever-increasing infectious doses. The lower attack rate on the last day of the exhibition may reflect the different composition of the visitor population on Sundays, when young families with children predominated, compared with weekdays, when elderly visitors predominated.

Although all cultures of specimens from the two separate parts of the local water supply system were negative, it is probable but unproven that the Legionella strains cultured from the whirlpool spas and the sprinkler installation originated from the local system. The finding of identical genotypes in these devices supports this hypothesis.

In conclusion, this large, severe outbreak in the Netherlands shows that diagnosis of Legionella pneumonia should lead to prompt investigation of the source of infection. Our comprehensive epidemiologic investigation identified a new whirlpool spa as the major source of the outbreak. Until strict regulations concerning the operation of whirlpool spas have been developed and issued, public exhibition of these devices in operation should be restricted.

ACKNOWLEDGMENTS

We thank treating physicians, public health doctors, and medical microbiologists for their collaboration in the data collection; Fred Slijkerman and Marc Sprenger for their risk assessment in the first days of the outbreak; Jan K van Wijngaarden and Hans C Tijen for facilitating environmental sampling; and Jacob Bruin, Wim JB Wannet, Anneke van der Zee, and Anneke Bergmans for isolating and genotyping Legionella from environmental and clinical specimens.
REFERENCES


CHAPTER THREE

Risk factors for sporadic community-acquired Legionnaires’ disease. A three-year national case-control study

JW den Boer¹, J Nijhof¹, I Friesema¹,²

ABSTRACT

Objective. Risk factors for sporadic community-acquired Legionnaires’ disease (LD) have been studied in the past, well before the widespread introduction of the urinary antigen test. Our objective was to evaluate the impact of the concomitant decrease in underdiagnosis on established and unknown risk factors for LD.

Study design. Prospective case-control study.

Methods. From 1 July 1998 to 30 June 2001, 228 LD cases and 293 controls were included for a national case-control study. Patients were included upon notification provided that they fulfilled international criteria for confirmed LD.

Methods. A history of diabetes mellitus, current tobacco smoking, travelling abroad, spending one or more nights away from home not leaving the country and being a driver by profession were independent risk factors. LD patients who had travelled abroad during their incubation period differed from LD patients who had not. They appeared healthier than non- or domestic travellers with respect to a history of coronary disease, pulmonary disease, current use of corticosteroids or immunosuppressives and any medication. Also the environmental risk factors differed significantly for the two groups.

Conclusions. The finding of two distinct populations of LD patients calls for a differentiated preventive strategy.

Key words. Legionnaires’ disease; Risk; Community-acquired infections; Pneumonia

INTRODUCTION

Legionnaires’ disease (LD) is an acute pneumonia that accounts for 8–13% of community-acquired pneumonias. [1,2] Although 80% of LD cases are sporadic, the disease was first described after a large outbreak of pneumonia in Philadelphia in 1976 among visitors of a Legionnaires’ convention held in a hotel, and passers-by at the same hotel. [3] The outbreak was shown to be caused by a newly discovered genus: Legionella spp. [4] These gram-negative bacilli live in (manmade) aquatic environments and are capable of infecting humans by

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The combination of the low incidence of LD and the omnipresence of *Legionella* spp. in water makes it worthwhile to identify risk factors for infection to enhance preventive measures. Furthermore, risk factors add to our knowledge of transmission and pathogenesis. Four large studies on host and environmental risk factors for community-acquired LD have been published. Two of these compared incidences of underlying disease in LD patients with US national morbidity data. [5,6] Since these national morbidity data had been collected for a different purpose, it is unknown if they measure disease incidences in the same way as was done for the LD patient in these two studies. Hence, the host risk factors found may have been overestimated or underestimated. Interpretation of the two studies is further hampered by the lack of differentiation between community-acquired LD and nosocomial LD, whereas risk factors for these types of LD seem to differ considerably. [7–10] Also, the morbidity data used were not gender, and agespecific, whereas LD is known to affect men more than women and the relative contribution of *Legionella* as a cause of pneumonia is highest in the 35–49 years age group. [11] In addition to the studies cited above, two US-based case-control studies on community-acquired sporadic LD have been published, [7,10] but their results were incomparable due to differences in aim and study design. Also, data for one of these studies [10] were collected in the 1970s well before urinary antigen tests became widely used. These facts induced us to start a 3-year case-control study in the Netherlands. The study design allowed for application of identical definitions of host and environmental factors for cases and controls. Furthermore, appropriate selection of controls was expected to reduce bias in age, gender, geographic and seasonal variation. The nationwide data collection ensured inclusion of sufficient patients to identify uncommon risk factors.

**METHODS**

Legionnaires’ disease (LD) has been a notifiable disease in the Netherlands since 1987. Diagnosed patients are reported to one of the 40 regional public health services in the country. Public health physicians in order to identify the source of the disease and to control a possible outbreak interview reported patients (or their relatives). Using the interview data, a disease notification form is completed and sent to the Health Inspectorate at the Ministry of Health. Since 1990, all public health physicians use the same national protocol of the National Co-ordination structure for Infectious diseases, which includes diagnostic criteria and a questionnaire. For the purpose of our study, the questionnaire was updated on 1 July 1998 to facilitate a structured interview on potential host and environmental factors, as suggested by published results from epidemiological studies and outbreak reports. From this date until 1 July 2001, all regional public health services in the Netherlands participated in our study. The Health Inspectorate provided questionnaire copies of all confirmed and probable LD patients who had been interviewed. Since the aim of the study was to identify risk factors for sporadic community-acquired LD, nosocomial and outbreak-related LD patients were excluded.
We used the criteria of the European Working Group for Legionella Infections for confirmed cases of LD. A confirmed case was defined as a person presenting with clinical symptoms of pneumonia, with radiological signs of infiltration, and with laboratory evidence of Legionella spp. infection. Laboratory evidence included isolation of Legionella spp. from respiratory secretions or lung tissue, detection of Legionella pneumophila antigens in urine, seroconversion or a four-fold or higher rise in antibody titres to L. pneumophila serogroup 1 in paired acute- and convalescentphase sera, as reported by clinicians. Controls were identified by the family physician of a LD patient. In the Netherlands, citizens are in the care of a family physician who plays a central role in the Dutch health care system (e.g, for referral to hospital care). The family physician of an included LD patient selected, in alphabetical order, the next four patients from his patient register who were of the same gender and year of birth. Controls who were willing to participate were requested to complete a participation form and send it to our institute. Upon receipt of this form, they were interviewed over the telephone using the same questionnaire as for cases. The nine-page questionnaire addressed health status as well as exposure to aerosols originating from any water source (shower, whirlpool, sprinkler, hose, fountain, air conditioning system, heating system) at home, at work, during leisure and during holiday periods. These questions related to the 2-week-period prior to the onset of symptoms for cases and to the 2-week-period prior to the interview for controls. Questions concerning drinking of tap water were not included. Controls who had suffered from pneumonia or who had stayed in hospital in the 2 weeks prior to the interview were excluded. Statistical analysis was performed with version 12.0 of the spss statistical program (Statistical Product and Service Solutions, Chicago, IL, USA). Univariate analysis was used to identify factors associated with LD. After checking for effect modifiers, significant (or borderline significant) host and environmental risk factors were entered in a multiple logistic regression model. Using backward elimination, independent risk factors were established for LD, adjusted for age, gender and season. Variables were retained in the model if the likelihood ratio test was significant (p < 0.1).

RESULTS

From 1 July 1998 to 30 June 2001, 391 non-outbreak LD patients were notified in the Netherlands. Excluded from our study were 118 patients: 113 patients whose microbiologic test results were not in accordance with EWGLI criteria for confirmed LD and five patients who had suffered from nosocomial LD. Of the remaining 273 LD patients, 45 (16%) could not be included because the questionnaire was lacking. These 45 patients did not differ significantly in age, gender, or region of residence (North, Mid, South or West) from the 228 patients who were included. A total of 293 controls were recruited (Fig. 1), whose region of residence (North, Mid, South, or West) did not differ significantly from cases. Although controls were on average slightly older, there was no significant difference in age or gender distribution between cases and controls (see Table 1). The prevalence of respiratory tract diseases, chronic obstructive pulmonary disease (COPD) and of previous episodes of
pneumonia did not differ between cases and controls. Neither was there a difference in prevalence of coronary heart disease, renal disease nor rheumatic disease. The prevalence of minor underlying diseases and the use of any medication was significantly lower for LD patients.

A history of diabetes mellitus and a recent history of non-cutaneous neoplasm were identified as risk factors for LD, whereas no difference was found in the current use of corticosteroids or immunosuppressives. In addition, two lifestyle related risk factors were found: current tobacco smoking and drinking more than four units of alcohol per day. Multiple logistic regression analysis identified diabetes mellitus and smoking as the only two

| Table 1. Age and gender of 228 included Legionnaires’ disease cases and 293 controls |
|-------------------------------------|-------------------------------|-------------------------------|-----------------|-----------------|
|                                    | Confirmed LD cases | Controls |                |                |
|                                    | Men    | Women | Men    | Women |
| Number (percentage)                 | 171 (75%) | 57 (25%) | 211 (72%) | 82 (28%) |
| Mean age in years (range)          | 54.6 (24–82) | 54.2 (26–81) | 57.5 (27–84) | 56.3 (29–83) |

LD = Legionnaires’ disease
independent host risk factors for LD. Analysis of the environmental factors revealed that for many factors no significant differences were observed: having an electrical heater, public hot water distribution or air conditioning system at home, being a plumber by profession, working in a greenhouse, or with a high pressure hose, having visited a sauna, swimming pool, sports facility or a dentist. However, one leisure-related, two work-related and three travel-related risk factors for LD were identified. Furthermore, all three travel related factors appeared to be independent risk factors in the multiple logistic regression model (see Table 2). Of the LD cases, seven were either taxi driver or company chauffeur, five were truck drivers (one international), three were bus drivers, one was tram driver and one was train engineer by profession. Of the controls, three were company chauffeur, two were truck driver, and two were bus driver (one international) by profession.

In a sub-analysis of LD patients we compared 160 international travellers to 68 non- or domestic travellers. The prevalence of five host risk factors was significantly lower in international travellers than in non- or domestic travellers. Also, the environmental risk factors differed significantly between the two groups (see Table 3). We did not find differences between the two groups in age, gender distribution, smoking, drinking of more than four units of alcohol per day, history of pneumonia, COPD, diabetes mellitus, renal failure, non-cutaneous malignant neoplasm, or organ transplantation.

Table 2. Odds ratios with confidence interval for host factors and environmental factors of (borderline) significance in univariate analysis; multiple logistic regression model using backward elimination, corrected for age, gender and season (228 Legionnaires’ disease patients; 293 controls)

<table>
<thead>
<tr>
<th>Host factor</th>
<th>Univariate analysis or (95% CI)*</th>
<th>Patients/controls</th>
<th>Multiple logistic regression model or (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of diabetes mellitus</td>
<td>2.0 (0.9 - 4.3)</td>
<td>17/12</td>
<td>5.4 (1.0 - 29)</td>
</tr>
<tr>
<td>Recent history of noncutaneous malignant neoplasm</td>
<td>4.6 (1.7 - 13)</td>
<td>16/5</td>
<td></td>
</tr>
<tr>
<td>Current tobacco smoking</td>
<td>5.6 (3.8 - 8.2)</td>
<td>137/74</td>
<td>5.5 (2.6 - 12)</td>
</tr>
<tr>
<td>Minor underlying diseases</td>
<td>0.5 (0.4 - 0.8)</td>
<td>96/123</td>
<td></td>
</tr>
<tr>
<td>Current use of any medication</td>
<td>0.5 (0.4 - 0.7)</td>
<td>74/149</td>
<td></td>
</tr>
<tr>
<td>Drinking more than four units of alcohol per day</td>
<td>1.5 (0.9 - 2.6)</td>
<td>31/31</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Environmental factor</th>
<th>Univariate analysis or (95% CI)*</th>
<th>Patients/controls</th>
<th>Multiple logistic regression model or (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Travelling abroad</td>
<td>26 (16–44)</td>
<td>160/24</td>
<td>33 (14–78)</td>
</tr>
<tr>
<td>Spending one or more nights away from home not leaving the country</td>
<td>1.7 (0.9 - 3.2)</td>
<td>23/21</td>
<td>4.0 (1.1 - 15)</td>
</tr>
<tr>
<td>Having a blue-collar job</td>
<td>1.5 (0.9 - 2.7)</td>
<td>36/30</td>
<td></td>
</tr>
<tr>
<td>Being a professional driver</td>
<td>3.1 (1.2 - 7.7)</td>
<td>17/7</td>
<td>3.0 (0.8 - 11)</td>
</tr>
<tr>
<td>Using the shower of a sports facility</td>
<td>0.4 (0.2 - 0.9)</td>
<td>9/29</td>
<td></td>
</tr>
<tr>
<td>Using the shower at a swimming pool</td>
<td>1.5 (0.9 - 2.5)</td>
<td>38/34</td>
<td></td>
</tr>
</tbody>
</table>

* OR (95% CI) = Odds ratio (95% confidence interval)
DISCUSSION

A well designed study to identify risk factors for sporadic community-acquired LD was part of the Ohio population-based pneumonia incidence study. [7,11] In this prospective study inclusion bias was limited by systematically testing all pneumonia patients for Legionella infection. In our study inclusion bias may have influenced the results because only notified cases were included. However, the bias may be limited as a result of antibiotic guidelines for community acquired pneumonia in the Netherlands. Treatment of first choice according to these guidelines does not cover for Legionella infection. [12] When initial drug therapy fails, testing for Legionella infection is recommended. Unfortunately, comparison of our results with the Ohio study is hampered for two reasons: hospital patients who were selected as controls were matched for underlying diseases and the study focus was on domestic transmission only. Our study design is therefore best compared to that of Storch et al., [10] who conducted a 15-month national (US) case-control study in 1976/1977, using self-selected acquaintances as controls. Independent risk factors for sporadic community-acquired LD identified in that study were smoking, drinking of three or more drinks of beer per day, underlying disease and travel. There is a strong resemblance of that results to ours. Our results are more specific in defining underlying disease as a risk factor. Also, they provide more detail on travel as a risk factor. Not only domestic travel with and without overnight stays, but also travel abroad appears to be an independent risk factor. The similarity in study results may indicate that the substantially diminished underdiagnosis of LD as a result of the widespread use of urinary antigen testing has not resulted in a shift in risk factors for sporadic community-acquired LD.

Table 3. Odds ratios with confidence interval for host factors and environmental factors of (borderline) significance in univariate analysis (160 Legionnaires’ disease patients who travelled abroad versus 68 Legionnaires’ disease patients who did not)

<table>
<thead>
<tr>
<th>Host factor</th>
<th>International travellers/Non-travellers or domestic travellers</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of coronary heart disease</td>
<td>0.2 (0.1 – 0.7)</td>
</tr>
<tr>
<td>History of pulmonary disease</td>
<td>0.5 (0.2 – 0.9)</td>
</tr>
<tr>
<td>History of splenectomy</td>
<td>n.a</td>
</tr>
<tr>
<td>Current use of corticosteroids or immunosuppressives</td>
<td>0.1 (0.0 – 0.3)</td>
</tr>
<tr>
<td>Current use of any medication</td>
<td>0.6 (0.3 – 1.1)</td>
</tr>
<tr>
<td>Environmental factor</td>
<td></td>
</tr>
<tr>
<td>Staying in a hotel</td>
<td>2.4 (9.8 – 60)</td>
</tr>
<tr>
<td>Spending one or more nights away from home, not leaving the country</td>
<td>0.4 (0.2 – 1.0)</td>
</tr>
<tr>
<td>Contact with aerosols at work</td>
<td>0.3 (0.1 – 0.8)</td>
</tr>
<tr>
<td>Using the shower at a swimming pool</td>
<td>2.0 (0.8 – 4.8)</td>
</tr>
</tbody>
</table>

* OR (95% CI) = Odds ratio (95% confidence interval)
Given our finding of travelling abroad as a strong risk factor, we were interested to see if part of this could be explained by a different subpopulation of patients, e.g. older patients with a higher incidence of underlying disease. However, to our surprise the travellers were in better health than the nontravelling LD patients. Therefore, our study results suggest that two distinct populations of LD patients exist: relatively healthy international travellers and non- or domestic travellers whose risk profiles resemble that of patients with nosocomial LD. The two case-control studies described above [7,10] did not report such a difference, possibly because fewer patients were included (100 and 146, respectively) or because no distinction was made between short- and long-distance travel.

However, it would be possible that pneumonia patients who had travelled abroad or who presented with underlying disease were selectively tested for Legionella infection, resulting in inclusion bias. Although this cannot explain the finding of two travel-related risk factors in domestic travellers, the finding that LD can be acquired in healthy individuals should urge clinicians to test systematically for Legionella infections in all patients presenting with pneumonia regardless of immune status or presence of underlying diseases. Furthermore, all pneumonia patients above the age of 18 should be tested, because Legionella is more common as a cause of pneumonia in patients under the age of 50 than over the age of 50. [11]

Our study confirms that smoking is the most consistent and strongest independent host-related risk factor for LD. [7,10] The concurrent absence of smoking-related risk factors such as 'COPD' and 'history of pneumonia' in our study as well as three other studies [5,7,10] is remarkable and suggests an additional role for smoking in the pathogenesis of LD. It may be that deep inhalation during smoking facilitates infection with Legionella spp. in addition to the deficient physical defence in smokers' airways. Also, since smoking promotes aspiration, [13] its influence may be indirect. Drinking and subsequent aspiration of contaminated water is a mode of transmission in LD that is often overlooked, [14] especially in the elderly. [15]

Diabetes mellitus was the only underlying disease identified as an independent host risk factor. The impaired macrophage functions in diabetes mellitus patients [16] may account for the underlying mechanism of this risk factor. Diabetes had not earlier been described as a risk factor for community-acquired LD. So far, all host risk factors for LD apart from smoking have been described for nosocomial and not for sporadic community acquired LD. Published prevalences of underlying disease and impaired immune system in community-acquired LD patients have been much lower than in nosocomial LD patients. For example, in a large outbreak in the Netherlands, where all outbreak-related pneumonia patients were systematically tested for Legionella infection, only 11% suffered from any form of underlying disease. [17]

In conclusion, our finding suggests that distinct populations of LD patients exist. However, because inclusion bias cannot be ruled out, ideally our finding should be confirmed in a prospective study of community-acquired pneumonia which systematically includes testing for Legionella infection.
ACKNOWLEDGEMENTS

We want to thank all public health physicians and nurses who have provided the questionnaire data for this study and all general practitioners who helped us to recruit controls. We also thank the hospital doctors and microbiologists for their kind assistance, and the public health personnel at the Health Inspectorate and the National Institute of Public Health and the Environment for their collaboration.

FUNDING

This study was supported by a grant of the Haarlem Tuberculosis Fund. This institute did not play a role in any aspect of the study or in the writing of this paper. All authors are independent of this Fund.

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CHAPTER FOUR

Use of surface water in drinking water production associated with municipal Legionnaires’ disease incidence

Jeroen W den Boer¹, Roel A Coutinho², Ed PF Yzerman,³ Marianne AB van der Sande³

ABSTRACT

Study objectives. Given an observed geographical variation in Legionnaires’ disease (LD) incidence in the Netherlands, the aim of the study was to test the hypothesis that type of drinking water production was an independent determinant of Legionnaires’ disease incidence.

Design. For the 1987–2005 period, the incidence of LD in the Netherlands and the price of water as a proxy for production type was studied at the municipal level. The data on price of water were available at the municipal level.

Methods. For each of the 466 municipalities in the Netherlands a mean standardised incidence rate per 100,000 inhabitants over the 1987–2005 period was calculated, excluding patients with most probable source of infection abroad or in hospital. Logistic regression was used to assess the relation of price of water to the incidence rates. In order to diminish bias, diagnostic and inclusion bias were estimated using questionnaire data collected from all 62 medical microbiology laboratories in the country.

Main results. The incidence of LD varied between municipalities from 0.0 to 5.6 per 100,000 inhabitants per year. In univariate analysis high versus low water price was positively associated with high municipal incidence rate (odds ratio 1.9; 95% confidence interval 1.5–2.6). The association persisted (odds ratio 5.1; confidence interval 3.2–8.0) after correction for diagnostic and inclusion bias.

Conclusions. Price of water as proxy for type of water production was an independent risk factor for high municipal LD incidence in the Netherlands. This can guide future prevention policies.

Key words. Legionnaires’ disease, pneumonia, incidence, public health, environmental exposure

INTRODUCTION

Legionnaires’ disease (LD) is an acute pneumonia of low incidence [1,2] which was first described after a large outbreak in Philadelphia in 1976 among visitors of a legionnaires’ convention held in a hotel, and passers-by at the same hotel. [3] The outbreak was shown to be caused by a newly discovered genus named Legionella. [4] To date, 49 Legionella species have been identified [5] many of which are ubiquitous in (manmade) aquatic environments. Legionella pneumophila which is the predominant species isolated from LD patients consists
of 15 serogroups, of which serogroup 1 is the most common, followed by serogroups 4 and 6. [6] In the USA, 91% of isolates from Legionnaires’ disease patients are typed as *Legionella pneumophila* serogroup 1. [6] The gram-negative bacilli are capable of infecting humans by aerosol inhalation or by drinking and subsequent aspiration of water. Given these modes of transmission, prevention efforts have focused on aerosol-producing plants and devices [7,8,9,10] and the presence of *Legionella* species in water installations in large buildings like hotels [11,12] and hospitals. [13,14]

Less attention has been given to the initial contamination of water installations by the drinking water delivered by water companies and the preceding water production process. We assume that *Legionella* infection of humans follows contamination and subsequent colonisation of water systems as a result of drinking water supplied with low to undetectable concentrations of *Legionella*. In the Netherlands a substantial part of the drinking water is produced from surface water. This raw material is known to be the natural habitat for *Legionella*, [15] and might lead more often to contamination of water installations when compared to production with groundwater. In the Netherlands the drinking water is and has always been distributed without disinfectants. After the 1999 outbreak the Dutch Health Council, asked for an update on *Legionella* prevention, explicitly advised against residual disinfection of municipal drinking water and the Dutch government opted accordingly. [16]

As large differences in LD incidence between provinces in the Netherlands have been observed in the past, [17] we investigated if these differences coincided with geographic differences in the origin of drinking water (ground water or surface water), using the price of water as a proxy. Therefore, we conducted an incidence study at a more detailed level than the province: the municipality. In order to reduce the influence of demographic differences at community level we corrected for age and gender. Furthermore, we assessed potential confounding related to diagnostic and inclusion bias by estimating underdiagnosis and underreporting at laboratory level and subsequently corrected for these factors in a multiple logistic regression model.

**METHODS**

Inclusion of patients

Legionnaires’ disease (LD) is a notifiable disease in the Netherlands since July 1, 1987. Treating physicians report LD patients within 24 hours to one of the 38 Regional Public Health Services (RPHS) in the country. Public health physicians based at the RPFS subsequently report confirmed and probable LD patients within 24 hours to the Ministry of Health. For our study we included all LD patients who had been notified from July 1, 1987 through December 31, 2005, with the exception of 188 patients who were part of a large outbreak in 1999. This outbreak resulted from a contaminated and inappropriately disinfected whirlpool which was on display and functioning for ten days. The water temperature was kept at 37 degrees Celsius and the water was not refreshed. Since the whirlpool was close to the entrance of a large hall a total of 77,000 visitors passed by. [18]
Subsequently, as we aimed to explore geographical difference in the Netherlands, we excluded LD patients who stayed abroad or stayed in a hospital during five or more days of their incubation period. We defined a confirmed case of LD as a patient suffering from symptoms compatible with pneumonia, with radiological signs of infiltration, and with laboratory evidence of *Legionella* spp. infection. Laboratory evidence included isolation of *Legionella* spp. from respiratory secretions or lung tissue, detection of *L. pneumophila* antigens in urine, seroconversion or a fourfold or higher rise in antibody titres to *L. pneumophila* in paired acute- and convalescent-phase sera. We defined a probable case of LD as a patient suffering from symptoms compatible with pneumonia, with radiological signs of infiltration, and with laboratory findings suggestive of *Legionella* spp. infection. These findings included a high antibody titre to *L. pneumophila* in a single serum, direct fluorescent antibody staining of the organism and detection of *Legionella* species DNA by polymerase chain reaction in respiratory secretions or lung tissue.

**Calculation of municipal LD incidence rates**

The incidence was studied previously at the provincial level. [17] The next administrative unit in the Netherlands is the municipality. Based on the LD notification data collected in the 1987–2005 period, for each of the 466 municipalities in the Netherlands a mean yearly LD incidence rate per 100,000 inhabitants was calculated and adjusted for age and gender by direct standardisation using the January 1, 1996 census. In order to minimise bias due to misclassification of the origin of infection, for each LD patient the place of residence was used as a proxy for source of infection, unless:

- A patient was part of a cluster or outbreak with an identified source of infection. Then the case was attributed to the municipality where the source of infection was situated.
- A patient had stayed away from home in a different community for five or more days during the incubation period (2–10 days). Then the case was attributed to the non-residential municipality.

**Diagnostic and inclusion bias**

In order to assess the impact of diagnostic and inclusion bias on differences in LD notifications in August 1998, 167 questionnaires were sent to all registered medical microbiologists working in 62 different hospital-based laboratories in the Netherlands. In February 1999, non-responders were resent the questionnaire. Topics covered in this questionnaire concerned the types of LD diagnostic assays used in the laboratory. Since it was expected that a large outbreak in 1999 in the Netherlands [18] would influence laboratory practice, new questionnaires were sent in August 2002 and for non-responders again in March 2003. Added topics in the new questionnaire concerned the region of adherence and the total number of requests for *Legionella* diagnostics in the years 2000 and 2001. Furthermore, an overview of diagnosed LD patients was asked for the years 2000 and 2001. Several variables were inferred from the questionnaires data. As indicators of diagnostic bias, diagnostic activity was calculated by dividing the number of requested tests by the number of inhabitants of the region of adhe-
rence in the years 2000 and 2001. Also, the ratio of positive tests to requested tests and the ratio of notification after the 1999 outbreak to before 1999 were calculated. As an estimate for inclusion bias, LD underreporting was calculated as a ratio of non-notified to notified LD patients in the years 2000 and 2001. To estimate the effect of the 1999 outbreak, for each province a ratio of notified LD before and after 1999 was calculated. Each of the variables was dichotomised using the mean as discriminating value.

Risk factors

Ideally, as our basic hypothesis was that geographical differences in LD incidence are a result of differences in the initial contamination of drinking water at delivery, we would have used data on Legionella contamination of the drinking water supply. Since 2004, water companies in the Netherlands are obliged to report contamination of Legionella in their distribution system to the Ministry of the Environment. In that year, none of 371 sampling points were reported to be contaminated with Legionella pneumophila and only twelve (3.2%) with other Legionella species. [19] Other than that, systematically collected data on contamination of drinking water with Legionella species in the Netherlands have not been published. A report on the contamination of the water distribution system in the Netherlands with Escherichia coli covering a ten-year period has been published. [20] Unfortunately, since there is no correlation between the presence of E. coli and Legionella species in drinking water, [21] these data could not be used for our study either. Therefore, we hypothesised that differences in raw material used for the production of drinking water would lead to (undetectable) differences in contamination. We used the 2004 price of 1,000 litres of water as a proxy for the raw material used in the production process of the water delivered. [22] In general, higher prices result from more intensive production processes using surface water as raw material. [23] Conversely, use of groundwater leads to lower prices. The variable "price of water" was dichotomised using the mean price of water as the discriminatory value.
Figure 2. Legionnaires’ disease incidence rate standardised for age and gender per 100,000 inhabitants for 466 municipalities in the Netherlands. For a full-color figure, see page 164.

Statistical analysis

Statistical analysis was performed with version 14.0 of the SPSS statistical program (Statistical Product and Service Solutions, Chicago, Illinois, U.S.A.). Univariate analysis was used to estimate the association between municipal incidence of Legionnaires’ disease and price of water. In a multiple logistic regression model using stepwise backward elimination the association was corrected for the factors described above. Variables were retained in the model if the likelihood ratio test was significant (p<0.1).

RESULTS

Patient selection

From 1 July 1987 through 31 December 2005, 2047 LD patients were notified in the Netherlands. Figure 1 shows the number of notified patients for each year of the study period, with the exception of 188 patients who were part of an outbreak in 1999. [18] In total, 45% of LD patients were most probably infected in the Netherlands, 49% abroad, 5% during hospital admission and of 1% the origin was not known. A sharp rise in number of notified LD patients is seen from 1999 onwards.
Demographic description

For our study population we selected the 928 LD patients who most probably attracted their infection in the Netherlands. For 875 of these, table 1 shows age and gender distribution as well as certainty of diagnosis over the years of the study period. The male to female ratio over the entire period was 2.3 to one. For 53 included patients (5.7%) age and gender data were not complete.

Geographic variation in incidences

For 159 of the 466 municipalities (34%), no LD patients were notified in the 1987–2005 period. The standardised mean municipal LD incidence rate for the remaining 307 municipalities was 0.56 per 100,000 per year (standard deviation: 0.48). The incidence rate ranged from 0.04 to 5.6 per 100,000 per year. Figure 2 shows the geographic variation in the Netherlands using three incidence categories.

Table 1. Age and gender of 875 confirmed and probable Legionnaires’ disease patients in the Netherlands, 1987–2005, excluding travel abroad and nosocomial patients.

<table>
<thead>
<tr>
<th>confirmed Legionnaires’ disease patients</th>
<th>probable Legionnaires’ disease patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>men</td>
<td>women</td>
</tr>
<tr>
<td>mean age (range) in years</td>
<td>mean age (range) in years</td>
</tr>
<tr>
<td>1987:</td>
<td>0:</td>
</tr>
<tr>
<td>total</td>
<td>total</td>
</tr>
<tr>
<td>4:10 (%)</td>
<td>0:00 (%)</td>
</tr>
<tr>
<td>1988:</td>
<td>3:00 (%)</td>
</tr>
<tr>
<td>total</td>
<td>total</td>
</tr>
<tr>
<td>4:10 (%)</td>
<td>0:00 (%)</td>
</tr>
<tr>
<td>1989:</td>
<td>5:00 (%)</td>
</tr>
<tr>
<td>total</td>
<td>total</td>
</tr>
<tr>
<td>4:10 (%)</td>
<td>0:00 (%)</td>
</tr>
<tr>
<td>1990:</td>
<td>6:00 (%)</td>
</tr>
<tr>
<td>total</td>
<td>total</td>
</tr>
<tr>
<td>4:10 (%)</td>
<td>0:00 (%)</td>
</tr>
<tr>
<td>1991:</td>
<td>7:00 (%)</td>
</tr>
<tr>
<td>total</td>
<td>total</td>
</tr>
<tr>
<td>4:10 (%)</td>
<td>0:00 (%)</td>
</tr>
<tr>
<td>1992:</td>
<td>8:00 (%)</td>
</tr>
<tr>
<td>total</td>
<td>total</td>
</tr>
<tr>
<td>4:10 (%)</td>
<td>0:00 (%)</td>
</tr>
<tr>
<td>1993:</td>
<td>9:00 (%)</td>
</tr>
<tr>
<td>total</td>
<td>total</td>
</tr>
<tr>
<td>4:10 (%)</td>
<td>0:00 (%)</td>
</tr>
<tr>
<td>1994:</td>
<td>10:00 (%)</td>
</tr>
<tr>
<td>total</td>
<td>total</td>
</tr>
<tr>
<td>4:10 (%)</td>
<td>0:00 (%)</td>
</tr>
</tbody>
</table>
Figure 3. Provincial and national incidence rate of Legionnaires’ disease with origin of infection most probably in the Netherlands, 1987–1998 versus 1999–2005

Diagnostic and inclusion bias

The response for the 1998/99 medical microbiologist questionnaire was 100% and for the 2002/03 questionnaire 82%. The mean number of requested diagnostic LD tests per 100,000 inhabitants of the laboratories’ regions of adherence was 147 (range: 21–465). On average, 2.4% of the requested diagnostic LD tests were positive (range: 0–9%). While the response rate to the second questionnaire was 82%, 353 of 385 (92%) of the notified LD patients of 2000 and 2001 included in our study were reported by the medical laboratories. In addition, they reported 80 non-notified LD patients resulting in an estimate of the overall underreporting rate of 18.5% (80/353 + 80). The mean ratio of notification after the 1999 outbreak to before 1999 was 7.3 (standard deviation: 2.8), ranging from 3.8 to 13. Figure 3 shows the effect of the 1999 outbreak on LD notification at provincial and national level. Comparing the 1987–1998 to the 1999–2005 period, the incidence of notified LD with probable source of infection in the

Table 2. Price of drinking water and mean standardised Legionnaires disease incidence rate with 95% confidence interval for 307 municipalities* of the Netherlands divided into four* groups, using 1987–2005 notification data.

<table>
<thead>
<tr>
<th>price of drinking water</th>
<th>mean standardised LD incidence rate per 100,000 inhabitants (95% confidence interval)</th>
<th>number of municipalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euro 1.01 – 1.22</td>
<td>0.44 (0.37–0.50)</td>
<td>81</td>
</tr>
<tr>
<td>Euro 1.23 – 1.34</td>
<td>0.52 (0.33 – 0.70)</td>
<td>70</td>
</tr>
<tr>
<td>Euro 1.35 – 1.60</td>
<td>0.67 (0.51 – 0.83)</td>
<td>81</td>
</tr>
<tr>
<td>Euro 1.61 – 1.97</td>
<td>0.49 (0.42 – 0.56)</td>
<td>73</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>307</td>
</tr>
</tbody>
</table>

* municipalities with a LD incidence of zero were excluded b while attributing municipalities with equal price of drinking water to the same group an optimum was reached of groups that are approximately the same size c LD = Legionnaires’ disease
Netherlands increased from 0.11 to 0.66 per 100,000 per year. The ratio of the province with the highest incidence to the lowest decreased from 6.0 to 3.1, comparing the 1987–1998 to the 1999–2005 period.

RISK FACTORS

The price of water differed between and within the 16 water companies in the Netherlands resulting in 28 different price regions with a mean water price of Euro 1.46 (range Euro 1.01–1.97). For the 307 communities with reported LD patients, a price above Euro 1.45 was a risk factor (OR 1.9; CI 1.5 – 2.6) for high mean yearly standardised municipal LD incidence (higher than 0.56 per 100,000 per year in the 1987–2005 period). Table 2 shows the mean standardised incidence rate for four water price regions. The incidence rate rises from the first to the second and third price regions, but falls from the third to the highest price region. Despite the absence of a straight dose-effect relation, there is a positive linear association (chi-square for trend=4.2, p=0.02).

Apart from a high water price, three factors on diagnostic activity and underreporting were (borderline) significantly associated with a high LD incidence rate in univariate analysis. In a multiple regression model the association between price of water and incidence was corrected for diagnostic and inclusion bias and remained highly significant (OR=5.1; CI: 3.3 – 8.0; see table 3). For 159 municipalities with no reported LD patients in the 1987–2005 period the incidence was zero, making it impossible to calculate a standardised incidence rate and correct for age, gender, diagnostic and inclusion bias. However, comparing municipalities with high LD incidence to municipalities with no reported LD patients the crude OR for a price of water above Euro 1.45 was 2.5 (CI: 1.5 – 4.3).

Table 3. Crude and adjusted odds ratios with 95% confidence interval for risk factors associated with high standardised municipal Legionnaires’ disease incidence

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Univariate analysis OR (95% CI)</th>
<th>Multivariate analysis * OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High versus low price of 1,000 litres water at municipal level</td>
<td>1.9 (1.5 – 2.6)</td>
<td>5.1 (3.3 – 8.0)</td>
</tr>
<tr>
<td>High versus low number of requested diagnostic LD tests per 100,000</td>
<td>1.7 (1.2 – 2.4)</td>
<td>2.1 (1.4 – 3.3)</td>
</tr>
<tr>
<td>High versus low ratio of positive diagnostic LD tests to requested tests</td>
<td>1.2 (0.9 – 1.7)</td>
<td>1.8 (1.2 – 2.8)</td>
</tr>
<tr>
<td>Ratio of notified LD c in 1999–2005 period to 1987–1988 period</td>
<td>0.8 (0.6 – 1.0)</td>
<td></td>
</tr>
<tr>
<td>High versus low ratio of underreporting</td>
<td>1.4 (1.0 – 1.9)</td>
<td>3.1 (2.0 – 4.9)</td>
</tr>
</tbody>
</table>

* Multiple logistic regression model  b OR (95% CI) = Odds ratio (95% confidence interval)  c LD = Legionnaires’ disease
Discussion

Principle findings

Our results show that a high price for 1,000 litres of water was an independent risk factor for high standardised LD incidence at the municipal level. The association remained highly significant upon correction for differences in diagnostic activity and underreporting.

Strengths and weaknesses of the study

The association between LD incidence and price of water as a proxy for environmental exposure has been corrected for several factors. In general, geographical differences in age and gender standardised LD incidence can be due to differences in environmental exposure, diagnostic activity, underreporting, behavioural differences and differences in the distribution of host susceptibility in the population. The latter two factors were not included in our study and are may therefore limit the generalisability of our conclusions. However, we did include three different measures of diagnostic activity, two of which appeared to be independent factors in the multiple logistic regression model. Furthermore, we showed that underreporting also was an independent factor. We used the price of water in 2004, whereas the price may have developed in an uneven way during the 18.5 years of our study period. However, we know that the changes in the price of water during the 1992–2002 period have been both moderate and general, mostly due to mergers by water companies. [20] If there has been an effect of these mergers, it is likely this diluted any association, implicating that our study underestimated the size of the actual association. The use of price of water as a proxy for the type of raw material used in the production of drinking water in the Netherlands seems justified given the higher operational, depreciation and capital costs for water companies using surface water instead of ground water. [24] Table 2 does not show a straight dose-response relation between higher price and higher incidence, which may be due to merger effects as well (price levelling for adjacent regions of high and low incidence).

Relation to other studies

In the 1990s several studies on geographical differences in LD incidence have been conducted by Bhopal [25,26,27] who points to six sources of bias: [28] data set errors, random fluctuations, variation in time, variation in definition, differences in tests, and diagnostic activity. Our study has taken the latter four sources into account. First, using data from the 1987–2005 period the effect of variation in time has been reduced. Second, our case definition has been applied to all reported LD cases since 1987. The case definition used in our study has been operational in the Netherlands since 2003 and is stricter than notification criteria used in the 1987–2003 period in terms of clinical presentation, radiographic findings and laboratory findings. Third, test variation that is mostly due to serology has not played as dominant a role as in the 1990s after the introduction of the urinary antigen test. In our study population 27% of LD patients was diagnosed by serology. Fourth, as described above we included several measures
of diagnostic activity. With respect to the outcome of our study, Bhopal has emphasised the role of cooling towers as an explanation for geographic differences, whereas our study has focussed on the price of water as a proxy for type of raw material used in water production in a geographical area. Our approach is more general and still leaves room for a possible role of cooling towers or any other aerosol-producing device in the dissemination of Legionella bacteria.

Possible mechanisms and implications
An association between a high price of water and a high LD incidence is plausible since Legionella species are ubiquitous in natural surface water, [15] which in certain parts of the Netherlands is used in the relatively expensive production of drinking water. The alternative raw material is groundwater that is known to be contaminated to a much lesser degree with L. pneumophila, [29] other micro organisms and pollutants. Elimination of these requires less elaborate steps in the drinking water production process, which in turn leads to relatively lower prices. [30] If indeed the use of surface water in the production of drinking water leads more often to (undetectable) initial contamination of water systems that in turn leads to higher incidence of LD, improved disinfection could be an intervention worth considering. Given the increased use of surface water as a raw material for drinking water production in the Netherlands (from 33% to 40% between 1992 and 2002) [20] this could have contributed to the observed increased number of notified LD patients in more recent years, and may give rise to a higher incidence in the future.

Unanswered questions and future research
Since this is the first report of an association between cost of water as a proxy for water production type and level of LD incidence, our findings need to be confirmed. Furthermore, the differential presence of Legionella pneumophila in the specific water distribution systems of the Netherlands needs to be evaluated. Given the high interlaboratory variability in ability to culture Legionella pneumophila [31] this should include molecular microbiology techniques. Also, it might be worthwhile to investigate possible contamination of the water distribution system in circumscribed areas of high LD incidence to identify hitherto unidentified sources of infection (see figure 2).

ACKNOWLEDGEMENTS

We thank all public health physicians and nurses of the 38 regional public health services in the country who helped us with the collection of data. We also thank the medical microbiologists for responding to our questionnaires. We are grateful to the public health personnel at the Health Inspectorate and the National Institute of Public Health and the Environment for their kind collaboration. Especially we thank Dr. AM de Roda Husman for her comments on our use of water price as a proxy for type of water production.
FUNDING

This study was supported by a grant of the Public Health Stimulation Fund (Fonds OGGZ). This institute did not play a role in any aspect of the study or in the writing of this paper. All authors are independent from this Fund.

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PART 2

TRANSMISSION
CHAPTER FIVE

Legionnaires’ disease and gardening

JW den Boer¹,², EPF Yzerman², R Jansen², JP Bruin², LPB Verhoef¹, G Neve³ and K van der Zwaluw⁴

ABSTRACT

Legionella longbeachae was cultured from the sputum of a patient suffering from Legionnaires’ disease. Source identification efforts included analysis of samples of potting soil from the patient’s garden, and a genotypically indistinguishable strain of L. longbeachae was cultured from this material. Following examination of a national collection of Legionella isolates, two more patients with indistinguishable genotypes were identified. One of these patients had visited a garden centre in the same municipality in which the index patient had acquired his potting soil. The study demonstrated the value of systematic collection of identification data and patient isolates over a prolonged period.

Key words. Legionnaires’ disease, pneumonia, public health, environmental exposure, Legionella longbeachae, soil.

Legionnaires’ disease (LD) is a pneumonia caused by Legionella spp., predominantly (>90%) Legionella pneumophila. Worldwide, the second most commonly isolated species is Legionella longbeachae[1]. In Australia, this species is responsible for 30% of reported cases of LD. The transmission of this pathogen has been associated in Australia and the USA with handling potting soil, [2,3] but to our knowledge, there have been no previous reports of such an association in Europe.

In December 2004, the Dutch National Legionella Outbreak Detection Programme (NLODP) was informed of a patient with LD (date of onset, 4 November 2004). L. longbeachae was cultured from the sputum of the patient, who had died 2 weeks following admission. A recent change in the Dutch regulations, abandoning notification of LD caused by species other than L. pneumophila, meant that the patient had not been notified to the Ministry of Health. An interview with the patient’s relatives in December 2004 revealed that the patient, a male who smoked cigarettes, aged 67 years, with no known underlying disease, had been working in his garden with commercial potting soil during the week before his illness. On the basis of this information, workers of the NLODP collected water and biofilm samples from the patient’s home, and potting soil samples from the patient’s garden. All water and biofilm samples were

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negative, but *Legionella* spp. were cultured from the potting soil. Amplified fragment length polymorphism analysis [4] identified two distinct genotypes, one belonging to *L. pneumophila* and one to *L. longbeachae*. The DNA banding pattern of the latter was indistinguishable from that of the *L. longbeachae* isolate from the patient’s sputum (Fig. 1, lanes 1 and 4). The other isolate was typed as *L. pneumophila* serogroup 7–14.

The NLQPD has access to data from all LD patients who have been notified in the Netherlands since 1987, and a collection of 352 patient-derived *Legionella* isolates. Although only five of these isolates are *L. longbeachae*, a patient with LD (date of disease onset, 7 August 2000) caused by *L. longbeachae* had visited a garden centre during his incubation period in the same community where the index patient had obtained his potting soil. The second patient, a non-smoking male aged 81 years, had no other underlying disease apart from type 2 diabetes mellitus, and had not been working in a garden or with potting soil. The DNA banding pattern of the *L. longbeachae* isolate from this patient’s sputum was indistinguishable from that of the isolate from the index patient and the potting soil (Fig. 1, lane 3). Also indistinguishable was a further *L. longbeachae* DNA pattern for an isolate from the sputum of an LD patient (date of disease onset, 27 August 2000) (Fig. 1, lane 2) who had been working with potting soil in his garden during the incubation period. This patient lived 65 km from the index patient, making it unlikely that he had used potting soil from the same garden centre. The third patient was a non-smoking male, aged 69 years, who had suffered for 5 years from chronic lymphocytic leukaemia, for which he did not use medication. The fourth and fifth *L. longbeachae* isolates from the collection both showed distinct DNA banding patterns (Fig. 1, lanes 5 and 6) and were unrelated to the three clustered patient isolates.

Unfortunately, more precise information was not available, since all three clustered patients died following hospital admission. Aspiration of small contaminated potting soil particles seems a rather exotic explanation for transmission of *L. longbeachae* [5]. Aerosol-aided spread following air movement and evaporation of water in potting soil seems more likely, as this resembles waterborne spread, in which aerosols are a vehicle that enables legionellae to escape from the liquid environment. Aerosols that can be inhaled by humans (<5 µm) evaporate in milliseconds at standard environmental air temperature and humidity [6]. This may explain why the second patient, who did not handle potting soil, still became infected.

During the last 3 years, six of the 37 clusters of LD patients identified by the NLQPD have been associated with garden centres, with no satisfactory explanation. Although ten of the
12 patients involved were diagnosed with L. pneumophila infection (four culture-positive for serogroup 1, and one culture-positive for serogroup 3), transmission involving potting soil should not be ruled out in view of the present finding of L. pneumophila in the potting soil used by the index patient. In Australia, [2] but not in Japan, [7] L. pneumophila has been cultured from potting soils. In addition, composted plant material should be considered as a possible source of infection for L. pneumophila. In Australia, all composted plant material from large producers contained L. pneumophila, although these belonged almost exclusively to serogroup 2–14. [5] More data are required to evaluate the possible relevance of this route of transmission in Europe. Two soil surveys in Europe for L. longbeachae and L. pneumophila have both been negative, [2,8] but the present study contradicts the earlier hypothesis that potting soil in Europe is free of Legionella spp. because its major component is peat, whereas potting soils in Australia, Japan and the USA are composed primarily of sawdust and bark. [7] Since August 2002, systematic sampling by the NLODP of potential water sources in relation to patients with LD has not yielded L. longbeachae, but Legionella anisa was isolated from water sources in two of the 22 garden centres sampled.

In Europe, 38 countries collaborating in the European Working Group on Legionella Infections have agreed to use amplified fragment length polymorphism typing for L. pneumophila serogroup 1, [9] later followed by a more sophisticated sequence-based typing scheme. [10] To date, neither technique has been used for typing L. longbeachae, but results with allozyme electrophoresis and restriction fragment length polymorphism analysis suggest that the L. longbeachae isolates were closely related to each other and to most of the Australian environmental strains. [11] Macrestrict digestion with SfiI, followed by pulsed-field gel electrophoresis, distinguished three distinct pulsotypes with >65% similarity, and 11 subgroups with >88% similarity. [12] The amplified fragment length polymorphism assay is preferred to pulsed-field gel electrophoresis [13] by the European Working Group on Legionella Infections for genotyping of L. pneumophila, but the index of diversity for L. longbeachae requires further investigation.

In conclusion, given the low incidence of LD caused by L. longbeachae, the described cluster of isolates is remarkable. It is clear that it is worthwhile sampling potential sources of infection for LD caused by species other than L. pneumophila. Furthermore, the results confirm the value of systematic collection of notification data and patient isolates over a prolonged period. [14]

ACKNOWLEDGEMENTS

This study was supported by a grant from the Public Health Stimulation Fund (Fonds OGG).
REFERENCES

CHAPTER SIX


Jeroen W den Boer¹,², Jacob P Bruins², Linda PB Verhoef¹,
Kim Van der Zwaluw³, Ruud Jansen², Ed PF Yzerman²

ABSTRACT

To evaluate the assumption that the genotype distribution of *Legionella* isolates from sporadic LD patients is different from the genotype distribution of *Legionella* strains in the environment, we compared the genotype distribution in two collections of *Legionella* bacteria.

In our study we prospectively and systematically included *Legionella* isolates and patient related *Legionella* strains. For identification of patient related potential sources a nine-page questionnaire was used. We used an amplified fragment length polymorphism assay (aflp) as recommended by the European Working Group for *Legionella* Infections to genotype patient-derived *Legionella* isolates as well as environmental strains.

We found that the three *Legionella pneumophila* genotypes most frequently isolated from human respiratory secretions were aflp types 004 Lyon, 010 London, and 006 Copenhagen. These genotypes were cultured significantly less frequently from environmental samples (50% versus 4%; p<0.001). The most frequently observed *L. pneumophila* serogroup 1 genotype in patient-derived isolates was 004 Lyon (32%). This type was cultured from only one of 6458 environmental samples. The concentration of *Legionella* in the sample was 1,260,000 colony-forming units per litre. The sample originated from a whirlpool spa that had not been disinfected and was kept at 36 degrees Celsius for several months.

Our conclusion is that the distributions studied differ significantly, reflecting differences in virulence traits. A possible explanation for this is that virulent environmental strains may exist in potential sources at undetectable concentrations.

Key words. Legionnaires’ disease, pneumonia, incidence, public health, disease notification, environmental exposure, laboratory techniques and procedures, *Legionella pneumophila*, distribution

INTRODUCTION

Legionnaires’ disease (LD) is an acute bacterial pneumonia caused by *Legionella* species, which accounts for 8 to 13% of community-acquired pneumonias. [1,2] Worldwide, more than 90% of LD is caused by *Legionella pneumophila*, of which 92% is caused by *L. pneumophila*

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serogroup 1. [3] One major route of transmission of LD is inhalation of the bacterium, [4] whereas drinking and subsequent aspiration of contaminated water has shown to be an alternative transmission route. [5,6] For inhalation to occur the bacterium must be spread into the air as an aerosol from either natural or manmade sources in aquatic environments. Legionella species have been cultured from surface water [7] as well as ground water [8] where it flourishes as a parasite in amoebas and other protozoa. [9] Insufficient filtering and disinfection of these water sources for drinking water production can lead to the introduction and colonisation of water systems by Legionella species. [10] Stagnant water, biofilm formation and favourable growth conditions can subsequently lead to high concentrations of the bacterium. If aerosol producing devices are then connected to such contaminated water systems transmission to humans is possible and patients present with LD. Contaminated water systems and devices associated with LD patients include cooling towers from air conditioning systems, [11] whirlpool spas, [12] shower heads, [13] evaporative condensers, [14] humidifiers [15] and mist making machines. [16] These are known sources discovered from outbreak investigations that include epidemiologic studies and comparison of Legionella genotypes from patient materials to those from the environment. Genotype comparisons have an important role in judging the likelihood of a source of infection being the true source of LD since undistinguishable genotypes have been identified from sources over a period of 17 years. [17] Often, these interpretations are made without a real understanding of the underlying distribution of genotypes in both the patient derived and the environmental population. [18] However, the distribution can differ from one single endemic genotype as in the entire Paris water distribution network in France, [19] to several different environmental genotypes in one hospital in Italy. [20]

The hypothesis tested in our study is that the genotype distribution of Legionella isolates from sporadic LD patients differs from the genotype distribution of Legionella strains in the environment. This reflects virulence factors being more prominent in patient-derived Legionella strains. The aim of our four-year prospective national study is to systematically compare the genotype distribution of Legionella isolates from sporadic cases to the genotype distribution of Legionella strains cultured from environmental sources to which LD patients were exposed during their incubation period. Potential sources of bias and confounding (diagnostic, sampling, seasonal) are evaluated.

**MATERIALS AND METHODS**

**Epidemiological data**

Legionnaires’ disease (LD) is a notifiable disease in the Netherlands since July 1, 1987. Treating physicians are required to report LD patients within 24 hours to a public health physician at one of the 38 Regional Public Health Services (RPHS) in the country. These Public health physicians are required to subsequently report all confirmed and probable LD patients within 24 hours to the Ministry of Health. The international criteria of the European Working Group for Legionella Infections (EWGLI) for confirmed and probable LD [21] are also used in
the Netherlands. For this study we included LD patients who had been notified from August 1, 2002 to October 30, 2006 and whose laboratory diagnosis included isolation of *Legionella* species from sputum or lung secretions. As the aim of this study is to explore differences in the distribution of *Legionella* genotypes in the Netherlands, we excluded LD patients who visited abroad for five or more days of their 2–10 day incubation period. As well, to avoid an overrepresentation of genotypes due to clusters or outbreaks, we randomly selected and included only one LD patient from EWGLI defined clusters or outbreaks. [22] A subgroup of patients from whose pulmonary secretions the most frequently observed AFLP *Legionella* genotype (see below: isolate characteristics) was cultured were compared to a control group of LD patients using a nine-page questionnaire. The questionnaire addressed health status as well as exposure to aerosols originating from any water source (shower, whirlpool, sprinkler, hose, fountain, air conditioning system, heating system) at home, at work, during leisure and during holiday periods. These data had been systematically collected earlier from 68 community-acquired Legionnaires’ disease patients who had stayed in the country for five or more days of the incubation period. [23]

**Bacteria**

After a large outbreak in the Netherlands involving 188 LD patients, [24] a national outbreak detection programme (NOODP) was started on August 1, 2002. [25] This national detection programme is aimed at creating a short response time between the diagnosis of LD patients and the inspection and sampling of potential sources of infection. To identify potential sources in the incubation period medical professionals at the RPHSs specialised in infectious disease control carry out structured interviews (using a questionnaire) with the patient and/or a contact person. The interviews are focused at tracking the patients’ exposure to potential sources of infection. Certain potential sources, e.g. swimming pools, saunas mentioned in the questionnaire are based on previously published results from epidemiological studies and outbreak reports. [25]

Following potential source identification, trained laboratory personnel of the NOODP take water and swab samples from each potential source, which are cultured for the presence of *Legionella* species. The yield of sampling efforts was expressed as the number of included strains divided by the number of samples taken.

Criteria for sampling were revised for budgetary reasons from June 1, 2006 onwards, thereby creating a natural end of the inclusion period for environmental *Legionella* strains in our study.

**Isolate characteristics**

Patient isolates were sent by all 62 medical microbiology laboratories in the Netherlands involved in the diagnosis and treatment of the pneumonia patients to the Regional Public Health Laboratory Kennemerland in Haarlem. After collection isolates were stored at -70°C. *C. L. pneumophila* was cultured on buffered charcoal yeast extract supplemented with α-ketoglutarate (BCYA-α), [26] with dyes and with and without the antibiotics polymyxin B,

Water and swab samples from potential sources that had been collected by environmental sampling were cultured and serotyped as described elsewhere. [25] *L. pneumophila* serogroup 1 patient isolates and environmental strains were genotyped by amplified fragment length polymorphism (AFLP), which is a whole-genome fingerprinting method that relies on the selective polymerase chain reaction amplification of restriction fragments. [27] The European Working Group for *Legionella* Infections (EWGLI) has validated this AFLP [28] for her 35 member counties and defined 31 EWGLI AFLP types which are available in the EWGLI website (www.ewgli.org) for public. The entire NODP dataset, including strains from previous studies contains 42 genotypes that have not yet been designated by EWGLI. Therefore, for the purpose of this study and using the EWGLI AFLP protocol, provisional Not Yet Designated (NYD) numbers have been given starting with NYD01 and ending with NYD42.

**Inclusion bias**

Patient residences were used as a proxy for the place of infection, unless patients had stayed elsewhere for five or more days of the incubation period. The place of infection was subsequently grouped using province of residence as geographic subunit into four regions conform criteria of Statistics Netherlands [29]: North (Friesland, Groningen, Drenthe), East (Overijssel, Gelderland, Flevoland), West (Noord-Holland, Utrecht, Zuid-Holland, Zeeland) and South (Noord-Brabant, Limburg). The geographic origin of environmental strains was grouped accordingly. To explore potential diagnostic bias, incidence rates for culture-proven *LD* were calculated for the four regions and compared to the mean 2002–2006 *LD* incidence rate for these regions. To explore potential inclusion bias for environmental strains the number of identified and sampled potential sources was compared for four regions.

Temperature has an influence on growth of *Legionella* species both in the natural environment [30] as well as in the man-made environment. [31] The level of humidity may also be a determinant in the transmission of *LD*. [32] Therefore, we included data on temperature and humidity as a potential bias and confounding in our analyses. A proxy was used as the day of infection, for which the humidity and temperature was determined. This was calculated by subtracting a median incubation period of five days from the first day of illness. In practice, there is a delay between the day of diagnosis and the day of sampling. If the delay is unevenly distributed over the regions of the country, bias due to seasonal differences can occur. The mean, minimum and maximum temperature (0.1 degrees Celsius) as well as the relative humidity (percentage) was recorded for the calculated day of infection and for the day of sampling. These were compared to those of the other days of the study period.

**Statistical analysis**

Univariate analysis was used to estimate crude regional differences in patient as well as environmental AFLP genotype distribution. Comparison of risk factors for *LD* between two groups of *LD* patients was analysed univariately. Univariate analysis included the Student
Figure 1. Inclusion of patient isolates and environmental *Legionella* strains. 
LD = Legionnaires’ disease

t-test for continuous variables and the Pearson Chi-square test for dichotomous and nominal variables. For continuous variables the distributions were checked for normality. Statistical analysis was performed with version 14.0 of the SPSS statistical program (Statistical Product and Service Solutions, Chicago, Illinois, U.S.A.).

RESULTS

Epidemiological data

Between August 1, 2002 and October 30, 2006 a total of 1133 LD patients were notified to the Ministry of Health (see figure 1). Of these, 691 (61%) fulfilled the definition of five or more days abroad during the incubation period and were therefore excluded. Of the remaining 442 patients, 128 (29%) had their diagnosis confirmed by isolation of *Legionella* species from sputum or lung secretions. For each outbreak all but one patient was randomly excluded, totaling eleven patients. Isolates from the remaining 117 patients were serotyped and genotyped, results of which are given in table 1.
Between August 1, 2002 and May 30, 2006 sampling was required for 442 notified LD patients who had spent five or more days in the Netherlands (see figure 1). Of these, four foreign visitors could not be interviewed because they had already left the country and 35 (8 %)

Clinical and environmental isolates

Table 1. Results of serotyping and genotyping using an amplified fragment length polymorphisms assay (aflp) of 117 Legionella patient isolates and 245 environmental Legionella strains.

<table>
<thead>
<tr>
<th>Patient isolate</th>
<th>Environmental strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Legionella non-pneumophila</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>L. pneumophila non-serogroup 1</td>
<td>12 (10%)</td>
</tr>
<tr>
<td>L. pneumophila serogroup 1, AFLP type 004 Lyon</td>
<td>33 (28%)</td>
</tr>
<tr>
<td>L. pneumophila serogroup 1, AFLP type “not yet designated”</td>
<td>22 (19%)</td>
</tr>
<tr>
<td>L. pneumophila serogroup 1, AFLP type 001 London</td>
<td>11 (9%)</td>
</tr>
<tr>
<td>L. pneumophila serogroup 1, AFLP type 006 Copenhagen</td>
<td>8 (6%)</td>
</tr>
<tr>
<td>L. pneumophila serogroup 1, AFLP type 003 Glasgow</td>
<td>7 (6%)</td>
</tr>
<tr>
<td>L. pneumophila serogroup 1, AFLP type 013 London</td>
<td>7 (6%)</td>
</tr>
<tr>
<td>L. pneumophila serogroup 1, AFLP type 028 Rome</td>
<td>4 (3%)</td>
</tr>
<tr>
<td>L. pneumophila serogroup 1, AFLP type 001 Lugano</td>
<td>3 (3%)</td>
</tr>
<tr>
<td>L. pneumophila serogroup 1, AFLP type 008 Stockholm</td>
<td>3 (3%)</td>
</tr>
<tr>
<td>L. pneumophila serogroup 1, AFLP type 009 London</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>L. pneumophila serogroup 1, AFLP type 017 Lugano</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>L. pneumophila serogroup 1, AFLP type 015 Dresden</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Total</td>
<td>117 (100%)</td>
</tr>
</tbody>
</table>
refused sampling for various reasons, mostly privacy related. For the remaining 403 LD patients 875 potential sources of infection were identified. Workers of the NOIDP sampled all of these. Samples of 185 (21%) potential sources contained one or more different Legionella strains, giving a total of 245 patient-related environmental strains to be serotyped and genotyped. Overall, these 245 strains were cultured from 6,458 samples taken giving a yield of 3.8%. The yield was significantly higher in the Western region compared to the other regions (4.7% versus 3.1%; p = 0.001; Table 2). Also, the yield for Legionella pneumophila serogroup 1 was significantly higher in this region (1.0% versus 0.6%; p = 0.02; Table 2). Details on the origin of the 6,458 samples are shown in table 3. Industry and commerce buildings included factories, office buildings, as well as car wash streets, dentists and hairdressers’ premises. Recreation buildings included public swimming pools, saunas, hotels, restaurants and sports facilities.

Isolate characteristics

Eighteen times a genotype from a clinical isolate was undistinguishable from an environmental related Legionella strain. A total of 13 sources were involved, nine of which were related to sporadic LD patients. Four of the sources were related to clusters involving two to four patients, totalling 15 LD patients. Table 1 shows the sero- and genotyping results for 117 patient isolates.

Table 2. Variation in number of culture-positive Legionnaires disease (LD) patients and number of environmental Legionella strains in four regions in the Netherlands (2002–2006)

<table>
<thead>
<tr>
<th>Region</th>
<th>North</th>
<th>East</th>
<th>West</th>
<th>South</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface in square kilometres</td>
<td>11,367</td>
<td>10,947</td>
<td>12,331</td>
<td>7,314</td>
<td>41,999</td>
</tr>
<tr>
<td>Inhabitants*</td>
<td>1,698,865</td>
<td>3,432,245</td>
<td>7,580,493</td>
<td>3,546,129</td>
<td>16,258,032</td>
</tr>
<tr>
<td>Number of notified LD patients staying in the Netherlands</td>
<td>35</td>
<td>84</td>
<td>189</td>
<td>114</td>
<td>422</td>
</tr>
<tr>
<td>Number of patient isolates</td>
<td>6</td>
<td>18</td>
<td>65</td>
<td>28</td>
<td>117</td>
</tr>
<tr>
<td>Number of environmental samples taken</td>
<td>457 (7%)</td>
<td>1,277 (20%)</td>
<td>2,858</td>
<td>1,866</td>
<td>6,458</td>
</tr>
<tr>
<td>Number of environmental Legionella strains</td>
<td>20</td>
<td>32</td>
<td>133</td>
<td>60</td>
<td>245</td>
</tr>
<tr>
<td>Number of environmental L. pneumophila serogroup 1</td>
<td>2</td>
<td>4</td>
<td>30</td>
<td>14</td>
<td>50</td>
</tr>
<tr>
<td>Number of environmental L. pneumophila serogroup 2-14</td>
<td>2</td>
<td>12</td>
<td>33</td>
<td>8</td>
<td>55</td>
</tr>
<tr>
<td>Number of environmental non-L. pneumophila strains</td>
<td>16</td>
<td>16</td>
<td>70</td>
<td>38</td>
<td>140</td>
</tr>
</tbody>
</table>

* 2004 census; source: Statline, Statistics Netherlands, Voorburg/Heerlen the Netherlands 2006
and 245 environmental *Legionella* strains. Serogroup 1 accounts for 88% of all patient isolates compared to only 20% of all environmental strains, which is a significant difference (p<0.001).

The distribution of *ewgli* Aflp genotypes within *L. pneumophila* serogroup 1 is not congruent at all. The three genotypes most frequently isolated from human sputum or lung secretions are Aflp types 004 Lyon, 010 London, and 006 Copenhagen. These genotypes were cultured significantly less frequently from environmental samples (50% versus 4%; p<0.001). The overrepresentation of LD patients from the Western part of the Netherlands did not influence the overall distribution pattern. Thirty-three patients from whose pulmonary secretions Aflp genotype 004 Lyon was cultured were compared to 68 control LD patients. The mean age of the 33 LD patients was significantly higher than the mean age of the controls (mean difference = 8 years; t-test for equality of means: p = 0.03). The male to female ratio was 3.7 compared to 3.3 for controls (not significant; p = 0.64). None of the other host or environmental risk factors included in the questionnaire differed significantly between the two groups.

Aflp 004 Lyon was cultured from only one of the 6,458 environmental samples. The sample originated from an outdoor whirlpool spa in the Eastern region of the Netherlands. The spa had not been disinfected and was kept at 36 degrees Celsius for several months, circumstances that lead to a concentration of 1,260,000 CFU/l at the time of sampling. Aflp 010 London was not found in any of the environmental samples. Aflp 006 Copenhagen was cultured once from a fire hose that was used for cleaning activities.

The aflp type 'not yet designated' was further divided into subtypes with names ranging from NYD01 to NYD42. Seventeen of the 19 different NYD types were isolated from lung secretions and unique, whereas two types (NYD04 and NYD23) were isolated three and two times, respectively. Types NYD04 was not cultured from environmental samples. Of nine different NYD types cultured from environmental samples only three NYD types were also cultured from lung secretions (see table 1).

**Inclusion bias**

For the 117 patient isolates Table 2 shows the distribution of the patients’ places of residence into four regions. Also, Table 2 shows the geographic distribution of 442 notified LD patients
who stayed in the Netherlands for five or more days of their incubation period. LD patients living in the West were more likely to have their diagnosis confirmed by isolation of *Legionella* species than patients from other regions (34% versus 22%; p = 0.05, Table 2). Therefore, our collection of patient isolates is overrepresented by isolates from the Western part of the country. However, since the genotype distribution of the West did not differ from the rest of the country, the overall genotype distribution was not influenced.

Table 2 shows no significant differences between the percentages of samples taken per region and the percentages of notified LD patients per region. Therefore, no selection bias in sampling procedure can be inferred.

There were no significant differences in the maximum or mean temperatures, relative humidity or hours of sunshine between the day of sampling and the other days of the study period. Neither was such a difference observed for the calculated days of infection as compared to the other days of the study period. However, significant differences were observed on days that two LD patients were presumably infected (see table 4).

**Discussion**

Based on the results of our study we conclude that in the Netherlands the genotype distribution of *Legionella* isolates from sporadic LD patients differs from the genotype distribution of *Legionella* strains in the environment. Most probably, this is a reflection of virulence factors. Earlier studies in different countries have suggested that differences in distribution pattern may exist. [33–36] In contrast to our study, these studies were not based on systematic collection of both patient isolates as well as environmental *Legionella* strains.

Fifty percent of the *L. pneumophila* serogroup 1 strains isolated from LD patients are represented by three *ewgli* AFLP genotypes. These genotypes represent only eight percent of environmental serogroup 1 strains. This finding suggests that these genotypes are more

<table>
<thead>
<tr>
<th>Table 4. Comparison of meteorological parameters on the calculated day of <em>Legionella</em> infection, the day of environmental sampling and control days, 2002–2006.</th>
</tr>
</thead>
<tbody>
<tr>
<td>day with one patient</td>
</tr>
<tr>
<td>averaged maximum day temperature in °C</td>
</tr>
<tr>
<td>averaged mean day temperature in °C</td>
</tr>
<tr>
<td>averaged relative humidity in percent</td>
</tr>
<tr>
<td>averaged number of sunshine in hours</td>
</tr>
</tbody>
</table>

* significant difference
virulent than others. The AFLP genotype 004 Lyon which was isolated most frequently from human lung secretions, does not seem to define a clinical or environmental distinct subgroup of LD patients. The finding of *L. pneumophila* serogroup 1 in the environment appears to be less abundant than expected with only 50 strains cultured from 6,458 patient related environmental samples taken. Comparison to other studies in this respect is difficult since there are no published reports on prospective collections of patient-related environmental strains. It seems that for the Dutch situation AFLP is sufficiently discriminating as can be inferred from the numerous unique NYD AFLP types.

The potential types of bias and confounding we assessed seem to have little effect on the principal findings of the study. Among the weaknesses of our study is the low number (mean: 2.2) of identified potential sources per LD patient. Given that for 91% of patients their own home was one of the potential sources that were sampled, few additional sources were included in the study.

Our study is the first to systematically include patient related environmental *Legionella* strains. A non-systematic study in the United Kingdom involving 401 unrelated LD patient isolates and environmental strains showed that some strains were more likely to cause human infection than would be expected from their distribution in the environment. [33] The same conclusion was drawn by French researchers who investigated 3,387 unrelated patient isolates and environmental strains. [36] Our results are in accordance with these studies, but the differences between the patient isolates and environmental strains identified in our study are more prominent.

There seem to be two possible explanations: One is that our source investigations based on a standardised questionnaire do not identify the true sources of LD. Nevertheless, we included all documented outbreak or cluster related types of LD sources in our questionnaire. Also, it is reasonable to assume that our collection of environmental strains is representative for the country. Still, it is possible that virulent strains should not be looked for in the watery environment but instead in the air because they are spread by various sources at days of increased humidity. [32] Our findings on humidity seem to confirm this, but should be assessed using a more sophisticated analysis. [37] The second explanation is that the most common *Legionella* genotypes isolated from human respiratory secretions are also present in the sources we sampled but at undetectable concentrations. In support of this hypothesis is the finding that the three most common human derived *Legionella* genotypes of our study were also the only genotypes cultured in 1999 from a whirlpool on display that caused a large outbreak in the Netherlands (888 LD patients). Despite substantial effort none of the samples that were taken from the effluent water distribution system contained *Legionella* bacteria at a detectable concentration. However, indirect evidence showed that the whirlpool had been contaminated by the building’s water supply. [24] Our second hypothesis is in accordance with a recent report suggesting that *L. anisa* may be an indicator of water contamination with undetectable *L. pneumophila*. [38] It is also in accordance with the theory that the infectious dose for LD is very low which is mainly based on the observation that humans can become infected hundreds of to thousands meters away from a source. [39]

Our findings need to be confirmed by others, since this is the first study to systematically collect patient isolates as well as patient related environmental samples. Most importantly,
there is a need for systematic collections of environmental *Legionella* strains. Also, there is a need to know the distribution of *Legionella AFLP* genotypes isolated from patients in Europe. So far, only restriction fragment length polymorphism (RFLP) results have been published. The distribution will be valuable in the identification of true sources of infection. It remains to be evaluated whether the quotient of relative frequencies for human and environmental *L. pneumophila* as found in distributions that have been collected systematically is a measure for virulence. Possibly, genotyping of the strains using a virulence-associated epitope recognized by the MAb 3/1 (Dresden Panel) [40] should be included into this evaluation.

**ACKNOWLEDGMENTS**

We thank all public health physicians and nurses of the regional public health services in the country who helped us with the collection of data. We also thank the medical microbiologists for sending us patient isolates. We are grateful to the public health personnel at the National Institute of Public Health and the Environment for their kind collaboration. We thank Max Bencini and Wim Houtenbos who sampled the various potential sources of infection and Peter Handgraaf and Esmeralda van Leeuwen who cultured the environmental samples as well as Stefan Boers who performed the AFLP fingerprinting. We thank Kimberly Boer and Marianne van der Sande for commenting on earlier versions of the manuscript.

**FUNDING**

This study was supported by a grant of the Public Health Stimulation Fund (Fonds OGZ). This institute did not play a role in any aspect of the study or in the writing of this paper. All authors are independent from this Fund.

**REFERENCES**


PART 3

LABORATORY DIAGNOSIS
CHAPTER SEVEN

Diagnosis of Legionella infection in Legionnaires’ disease

JW den Boer¹, EPF Yzerman²

ABSTRACT

Since 1977, the diagnostic tools for Legionnaires’ disease have been culture and serological investigation. Both methods require considerable time to produce results and have low to reasonable sensitivity. Since the introduction of urinary antigen tests in the mid 1990s, underdiagnosis has diminished and mortality has declined, thanks to early diagnosis. To obtain the most accurate diagnosis, culture, serological investigation, and urinary antigen testing should all be performed. In the last decade, much effort has been directed toward the development of assays detecting Legionella nucleic acid. Thus far, only widely varying results with small patient series have been reported. Furthermore, these assays are labour intensive and complicated. As a result, these assays are not yet suitable for the average medical microbiological laboratory.

INTRODUCTION

Legionella pneumophila was discovered as the causative agent of Legionnaires’ disease in 1977. [1] The discovery led to the description of a new genus: Legionella, the sole member of the family Legionellaceae. [2] In later years, it became clear that Legionella spp. were responsible for 2–5% of the cases of community-acquired pneumonia. [3,4] Thus far, 48 species and 70 serogroups have been distinguished. [5,6] Legionella pneumophila consists of 15 serogroups, of which serogroup 1 is the most common, followed by serogroups 4 and 6. In the USA, 91% of isolates from Legionnaires’ disease patients are typed as Legionella pneumophila serogroup 1. [4] This is in contrast to the situation in Australia and New Zealand, where 30% of the cases of Legionnaires’ disease are caused by Legionella longbeachae. [7] In this review, we describe various microbiological methods used in the diagnosis of Legionnaires’ disease: culture, serological investigation, detection of urinary antigen, and detection of Legionella DNA. We chose to describe tests that could be used in an average-sized hospital-based laboratory of medical microbiology. Only tests with a specificity of at least 99% are described, which is a prerequisite, given the relatively low incidence of Legionnaires’ disease. [8] Ideally, the test specificity was studied in a population of patients with pneumonia caused by pathogens other than Legionella spp. We also focus on several clinically relevant aspects of

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these methods: availability of clinical specimens, usefulness of the methods early in the course of disease, lag time to available results, and test sensitivity.

Culture

In 1977, bacilli were isolated from guinea pigs that had been infected with lung specimens of Legionnaires’ disease patients collected on autopsy. In addition, bacilli were isolated by successive passage in embryonated eggs. In both instances, the Giminez method of staining had been used. [1] Subsequently, the bacilli were grown on supplemented Mueller–Hinton agar. [9] Later, a charcoal yeast extract agar supplemented with L-cysteine and ferric pyrophosphate was developed. [10] The addition of N-(2-acetamido)-2-aminoethanesulfonic acid, [11] α-ketoglutarate, and semiselective antibiotics [12] has led to the current type of medium in use, which is enriched with buffered charcoal yeast extract (bcYE) and is available commercially.

Specimens used for culture of Legionella spp. include sputum or bronchoalveolar lavage specimens, bronchial aspirates, lung biopsy specimens, and blood. Several techniques have been developed to enhance the sensitivity of culture. Among them are preheating steps and acid washing procedures, which reduce overgrowth by other microorganisms. [13,14] Isolation of Legionella spp., which has a specificity of 100%, is the gold standard for diagnosis of Legionnaires’ disease. However, according to experts, isolation of Legionella spp. from respiratory secretions is not a very sensitive diagnostic test (25–75%). [8,15] This is mostly due to inexperienced laboratory personnel, as evidenced by the finding that two-thirds of otherwise well-qualified clinical microbiology laboratories in the USA in 1989 were unable to grow a pure Legionella culture. [16]

<table>
<thead>
<tr>
<th>Gold standard</th>
<th>Sensitivity</th>
<th>Patient material cultured</th>
<th>No.</th>
<th>Population</th>
<th>Reference no.</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive DFA test or seroconversion</td>
<td>28%</td>
<td>respiratory secretions, lung tissue</td>
<td>29</td>
<td>prospective inclusion of hospitalized LD patients</td>
<td>[71]</td>
<td>1980</td>
</tr>
<tr>
<td>Positive DFA test</td>
<td>45%</td>
<td>respiratory secretions, lung tissue</td>
<td>56</td>
<td>prospective inclusion of notified sporadic LD patients</td>
<td>[72]</td>
<td>1981</td>
</tr>
<tr>
<td>Fourfold rise in titer or positive UAg or DFA test</td>
<td>11% (compared with rise in titer); 59% (compared with UAg or DFA test)</td>
<td>sputum; sputum or lung autopsy material</td>
<td>27</td>
<td>prospective inclusion of hospitalized LD patients</td>
<td>[46]</td>
<td>1990</td>
</tr>
<tr>
<td>Positive DFA or UAg test</td>
<td>18%</td>
<td>sputum</td>
<td>11</td>
<td>prospective inclusion of hospitalized LD patients</td>
<td>[65]</td>
<td>1994</td>
</tr>
</tbody>
</table>

DFA, direct fluorescent antibody (immunofluorescent microscopy); UAg, urinary antigen
Table 1 shows different sensitivities of culture found in several published studies. Apart from low sensitivity, culture has the disadvantage of delay, because a positive result is not available until after 3 or more days of incubation. Furthermore, 25–78% of patients with Legionnaires’ disease have a nonproductive cough, [16] which excludes a culture-based diagnosis unless invasive methods are used to obtain clinical specimens. Regarding this aspect, it is important to mention that sputum specimens that contain many squamous epithelial cells or few polymorphonuclear leukocytes normally are rejected by microbiological laboratories, even though they may contain culturable legionellae. [17] The abovementioned drawbacks for isolation of *Legionella* spp. have spurred the development and use of easy-to-perform urinary antigen tests, which are now the mainstay of diagnosis in Europe. [18] The decrease in culture-based diagnosis in the last decade, due to the introduction of urinary antigen tests, limits the recognition of non-*Legionella pneumophila* serogroup 1 disease and impairs the investigation of outbreaks because fewer *Legionella* isolates are provided for further examination. [19]

For species other than *Legionella pneumophila*, culture remains, for the time being, the mainstay of microbiological diagnosis. It is important to know that these species may grow at a slower rate and may therefore be detectable only after 10 days. [20] The addition of albumin to the BCYE may be necessary to obtain detectable growth, [21] since commercially available BCYE agars may not support the growth of non-*pneumophila* species. [22]

**Serological investigation**

The first serological test to identify antibodies against *Legionella pneumophila* was the indirect immunofluorescent antibody test (IFAT), developed by the Centers for Disease Control (CDC) in Atlanta in 1977. [1] Live, yolksac grown organisms were used as antigen in the initial test.

<table>
<thead>
<tr>
<th>Type of assay</th>
<th>Antigen preparation</th>
<th>Gold standard</th>
<th>Sensitivity</th>
<th>No. Population</th>
<th>Reference, Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFAT</td>
<td>whole cell</td>
<td>epidemic criteria</td>
<td>46%</td>
<td>136 patients from an outbreak of community-acquired LD</td>
<td>[1], 1977</td>
</tr>
<tr>
<td>IFAT</td>
<td>formalized yolksac antigen</td>
<td>DFE or culture</td>
<td>60%</td>
<td>119 selected sample of LD patients</td>
<td>[24], 1987</td>
</tr>
<tr>
<td>MAT</td>
<td>formalin-killed suspension</td>
<td>DFE or culture</td>
<td>63%</td>
<td>119 selected sample of LD patients</td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>EDTA</td>
<td>IFAT</td>
<td>IgM: 58%; IgG: 41%</td>
<td>11 selected sample of LD patients</td>
<td>[25], 1982</td>
</tr>
<tr>
<td>ELISA</td>
<td>LPS</td>
<td>culture or MAT</td>
<td>IgM: 75%; IgG: 70%</td>
<td>32 selected sample of LD patients</td>
<td>[73], 1994</td>
</tr>
</tbody>
</table>

IFAT, immunofluorescent antibody test; MAT, microagglutination test; ELISA, enzyme-linked immunofluorescent assay; EDTA, ethylene-diamino-tetra-acetate; LPS, lipopolysaccharide
The **CDC** later changed to an ether-killed and, subsequently, to a heat-killed preparation for the **IFA**T antigen. Each of these assays showed different test characteristics and cutoff values.

Several simpler tests were developed as well. Among them was a microagglutination test (**MAT**) developed in 1978, [23] followed by a rapid version in 1982. [24] Additionally, numerous (mostly experimental) **ELISA** assays based on several different antigen-extraction methods have been developed. [23,25,26,27,28]

The reported sensitivities of these assays vary substantially, from 41 to 75% (Table 2). This variation may be due to differences in the antigen preparation, the reference strain used, or the valence (mono- or polyvalent) of the antigen used; cross-reactions; differences in the ability of the assay to detect IgM, [29] IgG, or both classes of antibodies; [30] and differences in the study population.

Apart from these differences, the few studies on the development of antibodies over time show consistent results. Few patients develop antibodies against **Legionella** spp. early in the course of disease. Although 80% of diagnostic titers were seen within 4 weeks after the onset of disease, seroconversion after 2 months or more has been reported. Moreover, antibodies were still detectable 48 months after disease onset in 33% of patients. [31] This observation implies that single high or standing titers detected in sera from patients with pneumonia may be the result of a past infection with **Legionella** spp. Unfortunately, determining the class of antibodies is of no help in differentiating between acute and past infection. In some studies, IgM antibodies are found predominantly early in the course of the disease. [32] In other studies, there is a mix of IgM and IgG. [33] Furthermore, patients have been described in whom only IgG was demonstrated in serum. [34]

Low titers of antibodies against **Legionella** spp. have been found in healthy volunteers, [35–37] blood donors, outpatients, and hospitalized patients. [24,40] Such titers seem to indicate previous exposure to **Legionella** spp. [41] Subclinical seroconversion is known to occur sporadically or during outbreaks. [42]

**Urinary antigen test**

The characteristics of the first urinary antigen tests were published in the late 1970s. [43] Since then, numerous publications have followed, each describing a different antigen detection technique and together providing evidence that confirms the value of urinary antigen detection for the diagnosis of Legionnaires’ disease [44–52]. The urinary antigen test appeared to give positive test results 1–3 days after the onset of disease, while a small proportion of patients remained positive for almost a year. [53] The most important feature of the assay appeared to be its >99% specificity, which is a requirement when testing for a relatively rare disease. Furthermore, a moderate-to-high sensitivity for **Legionella pneumophila** infections was demonstrated, ranging from 56 to 99% (Table 3). All studies showed a higher sensitivity for **Legionella pneumophila** serogroup 1. The differences in sensitivities found were attributed initially to differences in the proportion of clinical material from pneumonia patients with infection caused by **Legionella pneumophila** serogroup 1 versus infection caused by other serogroups. Moreover, variation was attributed to differences in the type of patient material used. This is especially noteworthy since only two prospective studies have been
Table 3. Overview of the sensitivity of the urinary antigen test in patients with Legionnaires’ disease (LD)

<table>
<thead>
<tr>
<th>Test</th>
<th>Gold standard</th>
<th>Serogroups</th>
<th>No.</th>
<th>Study population</th>
<th>Sensitivity</th>
<th>Reference no.</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA</td>
<td>positive culture</td>
<td>1, 4, 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23</td>
<td>retrospective sample of hospitalized LD patients</td>
<td>57%</td>
<td>[50]</td>
<td>1998</td>
</tr>
<tr>
<td>EIA</td>
<td>positive culture or 4-fold rise in IFAT titer</td>
<td>1, 3, 8, 12</td>
<td>120</td>
<td>selected sample of LD patients</td>
<td>77%</td>
<td>[47]</td>
<td>1990</td>
</tr>
<tr>
<td>EIA</td>
<td>culture</td>
<td>1</td>
<td>51</td>
<td>selected sample of LD patients</td>
<td>84%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIA</td>
<td>positive culture or 4-fold rise in IFAT titer or single high titer</td>
<td>1, 4, 6</td>
<td>27</td>
<td>prospectively included hospitalized LD patients</td>
<td>70%</td>
<td>[46]</td>
<td>1990</td>
</tr>
<tr>
<td>EIA</td>
<td>positive culture or 4-fold rise in IFAT titer or single high titer</td>
<td>1</td>
<td>20</td>
<td>prospectively included hospitalized LD patients</td>
<td>84%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>positive culture or 4-fold rise in IFAT titer</td>
<td>1, 3, 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68</td>
<td>prospectively included hospitalized LD patients</td>
<td>56%</td>
<td>[48]</td>
<td>1995</td>
</tr>
<tr>
<td>RIA</td>
<td>positive culture</td>
<td>1</td>
<td>35</td>
<td>prospectively included hospitalized LD patients</td>
<td>80%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIA</td>
<td>positive culture or 4-fold rise in IFAT titer or single high titer</td>
<td>1</td>
<td>59</td>
<td>selected sample of LD patients</td>
<td>78%</td>
<td>[49]</td>
<td>1997</td>
</tr>
<tr>
<td>EIA</td>
<td>positive culture or 4-fold rise in IFAT titer</td>
<td>unknown</td>
<td>65</td>
<td>selected sample of LD patients</td>
<td>64% (89%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>[51]</td>
<td>1998</td>
</tr>
<tr>
<td>EIA</td>
<td>positive culture or 4-fold rise in IFAT titer</td>
<td>unknown</td>
<td>59</td>
<td>selected sample of LD patients</td>
<td>74% (92%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>[52]</td>
<td>2001</td>
</tr>
<tr>
<td>ICT</td>
<td>positive culture or 4-fold rise in IFAT titer</td>
<td>1, 2, 3, 4, 5, 6, 10</td>
<td>187</td>
<td>selected sample of LD patients</td>
<td>80%</td>
<td>[58]</td>
<td>2001</td>
</tr>
<tr>
<td>EIA</td>
<td>positive culture or 4-fold rise in IFAT titer</td>
<td>1, 2, 3, 4, 5, 6, 10</td>
<td>187</td>
<td>selected sample of LD patients</td>
<td>79%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIA</td>
<td>positive culture or 4-fold rise in IFAT titer</td>
<td>1, 2, 3, 4, 5, 6, 10</td>
<td>187</td>
<td>selected sample of LD patients</td>
<td>83%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
When the use of urinary antigen tests was evaluated in an outbreak situation, the sensitivity appeared to be associated with the clinical severity of the disease. While 40–53% of the urinary antigen tests were positive in Legionnaires’ disease patients with mild disease, 88–100% of the tests were positive in patients with severe Legionnaires’ disease. Another association between sensitivity and defined subpopulations was demonstrated in patients with travel-associated, community-acquired, and nosocomial Legionnaires’ disease: sensitivities in these groups were 94%, 76–87%, and 44–46%, respectively. In fact, the observed differences reflect a tendency of the urinary antigen assays to detect mainly monoclonal antibody (Mab)-3/1- and Mab-2-positive strains, which are the predominant strains in travel-associated Legionnaires’ disease. This finding points out one of the limitations of diagnosis of Legionnaires’ disease by urinary antigen testing. Another important limitation is the low sensitivity of urinary antigen tests to detect serogroups other than *Legionella pneumophila* serogroup 1. Different assays have been evaluated, with the sensitivity for detection of other serogroups ranging from 14 to 69%. [56–59]

Table 3. (continued)

<table>
<thead>
<tr>
<th>Test</th>
<th>Gold standard</th>
<th>Serogroups</th>
<th>No.</th>
<th>Study population</th>
<th>Sensitivity</th>
<th>Reference no.</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIA</td>
<td>positive culture or 4-fold rise or single high titer or other UAg</td>
<td>1</td>
<td>167 selected sample of LD patients</td>
<td>99%</td>
<td>[74]</td>
<td>2001</td>
<td></td>
</tr>
<tr>
<td>EIA</td>
<td>positive culture or 4-fold rise in MAT titer or seroconversion in ELISA</td>
<td>1</td>
<td>58 LD patients of 1 outbreak</td>
<td>71% (74%)b</td>
<td>[54]</td>
<td>2002</td>
<td></td>
</tr>
<tr>
<td>EIA</td>
<td>positive culture or 4-fold rise in MAT titer or seroconversion in ELISA</td>
<td>1</td>
<td>58 LD patients of 1 outbreak</td>
<td>69% (79%)b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICT</td>
<td>positive culture or 4-fold rise in MAT titer or seroconversion in ELISA</td>
<td>1</td>
<td>58 LD patients of 1 outbreak</td>
<td>72% (81%)b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIA</td>
<td>positive culture</td>
<td>1, 3, 4, 5, 6, 8, 10, 12, 13</td>
<td>317 selected sample of LD patients</td>
<td>81%</td>
<td>[55]</td>
<td>2003</td>
<td></td>
</tr>
<tr>
<td>EIA</td>
<td>positive culture</td>
<td>1, 3, 4, 5, 6, 8, 10, 12, 13</td>
<td>317 selected sample of LD patients</td>
<td>77%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EIA, enzyme immunoassay; ICT, immunochromatographic assay; IFAT, immunofluorescent antibody test; MAT, microagglutination test; RIA, radioimmunoassay

a Including cases of *non-pneumophila* LD b After concentration of urine
Detection of Legionella Nucleic Acid

Potentially, an assay to detect *Legionella* nucleic acid could detect all known *Legionella* species. Various PCR tests have been developed which aim at different parts of the genome: the macrophage infectivity potentiator (*mip*) gene, the 5S ribosomal DNA gene, the 16S ribosomal DNA gene, and the 23S–5S spacer (Table 4). The *mip* gene is genus specific, whereas the 16S and 5S rRNA genes are specific for *Legionella pneumophila*. Few laboratories use PCR for the diagnosis of Legionnaires’ disease. The sensitivity of the test varies from 11 to 100%, and many publications report specificities of lower than 99%. The PCR assay can be performed on different specimens: BAL samples, pharyngeal swabs, nasopharyngeal swabs, peripheral blood mononuclear cells, urine, and serum. [60–67] The most recent publications on real-time PCR are promising, reporting high sensitivities and specificities. [68–70] However, in order for PCR to become established as a reliable diagnostic tool in Legionnaires’ disease, the reproducible specificity needs to rise to values higher than 99%.

Table 4. Sensitivity of assays detecting *Legionella* nucleic acid in patients with Legionnaires’ disease (LD)

<table>
<thead>
<tr>
<th>Gene targeted</th>
<th>Type of clinical specimen</th>
<th>Gold standard</th>
<th>Sensitivity</th>
<th>No. Study population</th>
<th>Control population</th>
<th>Reference</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>rRNA</td>
<td>respiratory secretions and autopsy tissues</td>
<td>positive culture</td>
<td>71%</td>
<td>64 selected sample of LD patients</td>
<td>pneumonia patients</td>
<td>[75]</td>
<td>1987</td>
</tr>
<tr>
<td>rRNA</td>
<td>respiratory secretions</td>
<td>positive culture</td>
<td>70%</td>
<td>11 prospectively included LD patients</td>
<td>pneumonia patients</td>
<td>[76]</td>
<td>1989</td>
</tr>
<tr>
<td>mip gene</td>
<td>BAL specimens</td>
<td>positive culture</td>
<td>100%</td>
<td>8 selected sample of LD patients</td>
<td>panel of control strains</td>
<td>[60]</td>
<td>1992</td>
</tr>
<tr>
<td>rRNA</td>
<td>respiratory secretions</td>
<td>DFA, positive culture, serology</td>
<td>11%</td>
<td>47 prospectively included LD patients</td>
<td>pneumonia patients</td>
<td>[77]</td>
<td>1993</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>BAL specimens</td>
<td>culture</td>
<td>100%</td>
<td>8 selected sample of LD patients</td>
<td>panel of control strains</td>
<td>[62]</td>
<td>1995</td>
</tr>
<tr>
<td>5S rRNA</td>
<td>serum, urine</td>
<td>culture, serology</td>
<td>64%</td>
<td>28 selected sample of LD patients</td>
<td>patients with pneumonia of other origin</td>
<td>[64]</td>
<td>1996</td>
</tr>
<tr>
<td>5S rRNA</td>
<td>urine</td>
<td>culture, serology, urinary antigen test</td>
<td>72%</td>
<td>58 selected sample of LD patients</td>
<td>[66]</td>
<td>1999</td>
<td></td>
</tr>
</tbody>
</table>
Combining Diagnostic Tools to Achieve Optimal Sensitivity

Clinically, there are no clear clues to differentiate Legionnaires’ disease from other types of pneumonia.

However, early diagnosis of Legionnaires’ disease is associated with lower mortality as a result of timely administration of antibiotics to which Legionella spp. are sensitive. From the discovery of the organism to the mid-1990s, culture and serological investigation were the only diagnostic tools available at the average medical microbiological laboratory. The low sensitivity of culture and the late seroconversion in many patients undoubtedly led to underdiagnosis during that period. Furthermore, both methods required from several days to many weeks before a diagnostic result was obtained. The availability of commercial urinary antigen tests in the 1990s led not only to more Legionnaires’ disease patients being diagnosed but also to a lower mortality, possibly as a result of obtaining a diagnosis earlier in the course of disease.

Table 4. (continued)

<table>
<thead>
<tr>
<th>Gene targeted</th>
<th>Type of clinical specimen</th>
<th>Gold standard</th>
<th>Sensitivity</th>
<th>No. study population</th>
<th>Control population</th>
<th>Reference</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>5S rRNA BAL specimens</td>
<td>culture</td>
<td>100%</td>
<td>9</td>
<td>selected sample of LD patients</td>
<td>panel of control strains</td>
<td>[69]</td>
<td>2001</td>
</tr>
<tr>
<td>5S rRNA biopsy specimens</td>
<td>culture</td>
<td>69%</td>
<td>16</td>
<td>selected sample of LD patients</td>
<td>panel of control strains</td>
<td>[69]</td>
<td>2001</td>
</tr>
<tr>
<td>mip gene BAL specimens</td>
<td>culture</td>
<td>100%</td>
<td>7</td>
<td>selected sample of LD patients</td>
<td>panel of control strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mip gene biopsy specimens</td>
<td>culture</td>
<td>17%</td>
<td>14</td>
<td>selected sample of LD patients</td>
<td>panel of control strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S rDNA BAL specimens</td>
<td>culture</td>
<td>100%</td>
<td>29</td>
<td>selected sample of LD patients</td>
<td>pneumonia patients</td>
<td>[78]</td>
<td>2002</td>
</tr>
<tr>
<td>23S–5S rRNA respiratory secretions</td>
<td>culture, serology, UAg test</td>
<td>94%</td>
<td>17</td>
<td>selected sample of LD patients</td>
<td>patients with pneumonia of other origin</td>
<td>[70]</td>
<td>2003</td>
</tr>
<tr>
<td>mip</td>
<td>sputum</td>
<td>culture</td>
<td>100%</td>
<td>7</td>
<td>selected sample of LD patients</td>
<td>pneumonia patients</td>
<td>[79]</td>
</tr>
</tbody>
</table>

Mip, macrophage infectivity potentiator gene; BAL, bronchoalveolar lavage; DFA, direct fluorescent antibody (immunofluorescent microscopy); UAg, urinary antigen
Still, the overall sensitivity of the urinary antigen test is only 70% for cases of pneumonia caused by *Legionella pneumophila* serogroup 1. For other serogroups, this figure is much lower, which means that, at present, optimal sensitivity for diagnosis of Legionnaires’ disease will be achieved by using a combination of culture, serological investigation, and urinary antigen detection. For public health purposes, both culture and serological investigation remain important diagnostic tools.

In the future, it is likely that an easy-to-perform PCR test with high sensitivity and a specificity above 99% will become available on a wider scale. It would not be surprising if such a test eventually becomes accepted as the new gold standard for diagnosis of Legionnaires’ disease.

REFERENCES


CHAPTER EIGHT

Sensitivity of Three Urinary Antigen Tests Associated with Clinical Severity in a Large Outbreak of Legionnaires’ Disease in the Netherlands

Ed PF Yzerman1,2, Jeroen W den Boer3, Kamilla D Lettinga2, Joop Schellekens4, Jacob Dankert5, and Marcel Peeters5

ABSTRACT

In 1999 an outbreak involving 188 patients with Legionnaires’ disease (LD) occurred among visitors to a flower show in the Netherlands. Two enzyme immunoassays (Binax and Biotest) and one immunochromatographic assay (Binax NOW) were tested, using urine samples from LD patients from the 1999 outbreak. Sensitivity was calculated using positive culture and/or seroconversion as the “gold standard” in outbreak-related patients with radiographically confirmed pneumonia who fulfilled the epidemiological criteria. The Binax EIA, Biotest EIA, and Binax NOW assay showed overall sensitivities of 69, 71, and 72%, respectively. When the tests were performed with concentrated urine samples, the overall sensitivities increased to 79, 74, and 81%, respectively. Using multiple logistic regression analysis with backward elimination, a statistically significant association was found between clinical severity and test sensitivity for all tests. For patients with mild LD, the test sensitivities ranged from 40 to 53%, whereas for patients with severe LD who needed immediate special medical care, the sensitivities reached 88 to 100%. These findings have major implications for the diagnostic process in patients with mild pneumonia and suggest that patients with mild pneumonia may go underdiagnosed if urine antigen tests alone are used.

Legionnaires’ disease (LD) is an acute pneumonia caused by Legionella, a rod-shaped gram-negative bacillus ubiquitous in (man-made) aquatic reservoirs. Currently 43 Legionella species and 65 serogroups have been described. In the United States, over 90% of Legionnaires’ disease cases are caused by Legionella pneumophila, of which 70% of strains belong to serogroup type 1. [16] Legionella spp. are responsible for 1 to 5% of cases of community-acquired pneumonia (CAP). [5] Clinically and radiographically, LD cannot be distinguished from pneumonias caused by other microbial pathogens. Because of the high mortality rate in patients with LD requiring hospitalization, early diagnosis to enable adequate antimicrobial treatment is potentially life-saving. Diagnosis of LD in patients with symptomatic pneumonia is based on culture, serologic

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testing, or antigen detection in urine. Isolation of Legionella from respiratory secretions is not a very sensitive diagnostic test (25 to 75% sensitivity) [15] and has the disadvantage of delay, because a positive result is not available until at least 3 days of incubation. Seroconversion is a diagnostic test with a high sensitivity and a high (serogroup-dependant) specificity, but it is of limited clinical value since it may take up to 9 weeks for patients to develop detectable antibodies. [10,13]

In contrast to the other tests mentioned above, urinary antigen tests combine reasonable sensitivity and high specificity with rapid results. The reported sensitivities of both enzyme immunoassay (EIA) and immunochromatographic test (ICT) show great variation: 50 to 90%. [3,7,8,19] These variations may be explained by differences in patient characteristics, the serogroup with which the patient is infected, the timing of collection the urine sample in the course of illness, and whether the urine is concentrated before testing.

To assess the value of the urinary antigen tests in a large outbreak situation, [6] we used three widely used and commercially available tests with urine specimens from patients with outbreak-related LD: the Biotest EIA, the Binax EIA, and the Binax NOW test.

**MATERIALS AND METHODS**

**Patients**

In February 1999 an outbreak involving 188 cases of LD occurred in Bovenkarspel, the Netherlands. The outbreak investigation indicated that a whirlpool displayed at the consumer product division of an annual flower show was the most likely source of infection. Genotyping revealed that isolates from 27 patients were identical to one of the environmental L. pneumophila serogroup 1 strains. [6]

All 180 hospitalized patients with a confirmed or probable Legionella pneumonia were included in this study after written consent was obtained from patients or their relatives. A confirmed case of LD ("gold standard") was defined as a patient who fulfilled the epidemiological criteria (visitor to the 1999 Bovenkarspel flowershow or member of the exhibition staff) and who suffered from symptoms compatible with pneumonia, who showed radiological signs of infiltration, and who showed laboratory evidence of infection with L. pneumophila. Laboratory evidence included (i) isolation of L. pneumophila from a respiratory sample cultured on buffered charcoal yeast extract supplemented with α-ketoglutarate followed by genotyping and subsequent comparison to the environmental strains of the Bovenkarspel outbreak; or (ii) a fourfold rise in the titer of immunoglobulin M (IgM) antibodies to L. pneumophila in paired acute-phase and convalescent-phase sera, with final titers of 1:32 or higher in accord with the 99% cutoff values found in a serosurvey of healthy volunteers representative of the Dutch population [4] using a microagglutination IgM, serotype 1, antibody assay; [12] or (iii) seroconversion to positive IgM or IgG antibodies to L. pneumophila in paired acute-phase and convalescent-phase sera, with age-specific titers in accord with the 99% cutoff values found in a serosurvey among healthy volunteers representative of the Dutch population, [4] using a commercial enzyme-linked immunosorbent assay to detect IgM and IgG serotype 1.
to 7 antibodies (Serion ELISA; Institut VirionSerion GmbH, Würzburg, Germany). For isolation and genotyping of \textit{L. pneumophila} from sputum samples, the National Institute for Public Health and the Environment was the reference laboratory; for detection of antibodies against \textit{L. pneumophila} in serum, the Regional Laboratory of Public Health Tilburg was the reference laboratory.

\textit{Classification of severity of disease}

To investigate the relation between test sensitivity and severity of disease, the patients were divided into three clinical categories for \textit{CAP}. Clinical data were collected from the hospital chart by using a standardized case report form. Severity of pneumonia was scored according to the minor criteria for severity of \textit{CAP} advised by the American Thoracic Society, \cite{17} using the following clinical criteria at hospital admission: (i) respiratory frequency above 30 breaths per minute, (ii) PaO$_2$ below 60 mm Hg or O$_2$ saturation below 92\%, (iii) bilateral or multilobar infiltration on chest X-ray, and (iv) systolic blood pressure below 90 mm Hg or diastolic blood pressure below 60 mm Hg.

Patients with a radiographically proven unilateral unilobar pneumonia, but without signs or symptoms according to the above-mentioned criteria, were classified as \textit{CAP} category 1 (mild pneumonia). \textit{CAP} category 2 consisted of patients with a proven pneumonia who fulfilled only one of the criteria; according to our definition, these patients were suffering from a moderately severe pneumonia. \textit{CAP} category 3 consisted of patients who presented with two or more criteria; these patients were considered to be suffering from severe pneumonia.

\textit{Collection of urine samples}

All medical microbiologists who had assisted in the diagnosis and treatment of the pneumonia patients in this outbreak were asked to send available urine samples from the patients to the Regional Laboratory of Public Health in Haarlem, the Netherlands. After collection, the urine samples were stored in portions at -70\°C. All but eight available urine samples had been collected during the hospital stay. In four cases, the urine samples had been obtained shortly before admission, and in four cases, they had been obtained after discharge from hospital.

\textit{Urinary antigen tests}

The presence of \textit{L. pneumophila} antigens in urine samples was investigated by using the Binax (Portland, Maine) and Biotest (Biotest AG, Dreieich, Germany) \textit{Legionella} urinary antigen tests, both EIAs, and with the Binax NOW test, a qualitative ICT. All tests were used as specified by the manufacturers. However, to ensure maximum specificity for the ICT, \cite{14} samples giving positive tests were reexamined after 60 min. Urine samples were tested nonconcentrated and, to enhance the intensity of the reaction, after concentration by selective ultrafiltration (Minicon B15; Millipore Corp, Bed Ford, Mass.). This selective ultrafiltration system consists of a permeable membrane that permits the passage of water and substances with molecular weights less than 15,000.
Statistics

Statistical analysis was performed with the statistical program SPSS version 10.0 (Statistical Product and Service Solutions, Chicago, Ill.). Univariate analysis (chi-square test for dichotomous and ordinal variables; independent t-test for discrete variables) was used to calculate the association with positive urinary antigen test results for the following variables: age, gender, clinical severity, number of days between onset of symptoms and collection of first urine sample, and number of times a urine sample was collected. Variables that were (borderline) significant were entered in a multiple logistic regression model.

Using backward elimination, independent predictors for test positivity were established. Variables were retained in the model if the likelihood ratio test was significant ($P < 0.1$).

RESULTS

Patient selection and classification of disease severity

In the 1999 outbreak, 188 LD patients were diagnosed, of whom 133 fulfilled the criteria for a confirmed case. Of the patients with confirmed cases, 132 had been hospitalized and were enrolled in this study. A large number of patients in this outbreak (51 patients) were diagnosed by a urinary antigen test alone, leaving 81 patients for evaluation. For 58 (72%) of these 81 patients, urine samples were available. The microbiological diagnosis in these cases was established by culture alone in 11 cases, by culture and serologic testing in 14 cases, and by serologic testing alone in 33 cases. For 55 of the 58 confirmed cases with available urine samples, data on clinical severity could be collected. Women were overrepresented in CAP category 1 (67%), in contrast to CAP categories 2 (25%) and 3 (39%). The median age for women was lower than for men in categories 1 and 2 (64 and 73 years and 57 and 63 years, respectively), in contrast to category 3 (70 and 62 years, respectively). Mean age did not differ significantly between CAP categories, but the age difference between male and female subgroups in the lowest CAP category was significant (independent t test; $P = 0.04$). All patients who were classified in CAP category 3 needed medical attention in a specialized unit.

Non-concentrated urine samples

Using nonconcentrated urine, the sensitivities calculated for the three tests were 71, 69, and 72% for the Biotest EIA, Binax EIA, and Binax NOW test, respectively. The differences between the test sensitivities were not significant. When the LD patients were divided in three groups according to their CAP category, it appeared that the average sensitivities for the three urinary antigen tests increased from 42% for patients in the lowest CAP category to 75% for those in CAP category 2 and 93% for those in CAP category 3. The individual sensitivities of the three tests when used with nonconcentrated urine samples are shown in Fig. 1. Table 1 shows variables which in univariate analysis were significantly associated with test positivity. Age was not associated with test positivity.
Multiple logistic regression analysis showed that the CAP category remained associated with test positivity in all tests used (Table 2). Separate analysis for male and female patients gave identical results, indicating that the association was not modified by gender.

**Concentrated urine samples**

After concentration of the urine samples for all three tests, a clear but not statistically significant increase in sensitivities was found: to 74, 79, and 81% for the Biotest ELA, Binax ELA, and Binax NOW test, respectively. This increase in sensitivity was small in the Biotest ELA (3%) and more prominent in the Binax ELA (10%) and Binax NOW assay (9%). When test sensitivity

![Figure 1. Sensitivity for three urinary *L. pneumophila* antigen tests in different categories of clinical severity using concentrated and non-concentrated urine](image)

<table>
<thead>
<tr>
<th>Variable</th>
<th>non-concentrated urine</th>
<th></th>
<th></th>
<th>Binax ELA</th>
<th></th>
<th></th>
<th>Binax NOW</th>
<th></th>
<th></th>
<th>Binax ELA</th>
<th></th>
<th>Binax NOW</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP category 2 versus 1a</td>
<td>2.5 (0.6 - 10.9)</td>
<td>4.5 (1.0 - 21.0)</td>
<td>6.5 (1.3 - 33.0)</td>
<td>5.0 (1.2 - 25.0)</td>
<td>6.1 (1.0 - 36.9)</td>
<td>6.1 (1.0 - 36.9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAP category 3 versus 1a</td>
<td>12.6 (2.1 - 74)</td>
<td>10.5 (2.1 - 52)</td>
<td>16.5 (2.8 - 98)</td>
<td>12.6 (2.1 - 74)</td>
<td>20.1 (2.1 - 190)</td>
<td>4.0 (2.2 - 7.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male gender*</td>
<td>2.4 (0.8 - 7.9)</td>
<td>4.2 (1.3 - 13.6)</td>
<td>3.0 (0.9 - 9.9)</td>
<td>2.5 (0.8 - 8.4)</td>
<td>3.4 (0.9 - 13.0)</td>
<td>1.8 (0.5 - 6.6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days before 1a urine sampleb</td>
<td>4.5 (1.3 - 7.6)</td>
<td>3.8 (0.7 - 6.9)</td>
<td>4.9 (1.2 - 8.5)</td>
<td>3.8 (0.4 - 7.2)</td>
<td>4.9 (1.2 - 8.5)</td>
<td>6.7 (3.1 - 10.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of urine samplesb</td>
<td>0.7 (0.0 - 1.6)</td>
<td>0.4 (0.0 - 1.3)</td>
<td>0.7 (0.0 - 1.6)</td>
<td>0.6 (0.0 - 1.5)</td>
<td>0.8 (0.0 - 1.8)</td>
<td>1.0 (0.3 - 1.6)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

* odds ratio  
| mean difference

Table 1. Odds ratios and mean differences with confidence interval for variables associated with urinary *L. pneumophila* antigen test positivity
results were compared for categories with increasing clinical severity, concentration of urine samples yielded higher sensitivities predominantly for patients in CAP categories 1 and 2 (Fig. 1).

CAP category, male gender, number of urine samples, and shorter period between onset of symptoms and collection of the first urine sample were associated with a positive test result, but age was not (individual test results are given in Table 1). Multiple logistic regression analysis showed that the CAP category was the only factor associated with test positivity in all tests used (see Table 2). Separate analysis for male and female patients gave identical results.

**Discussion**

The first urinary antigen tests, based on an ELISA, were described in 1979. [2,21] Since then, numerous publications have followed that confirmed the value of urinary antigen detection for the diagnosis of Legionnaires’ disease, regardless of the technical configuration of the test. [1,3,7,8,18–20] Based on prospective and retrospective studies using data from solitary cases, moderate to high urinary antigen test sensitivities have been described. All reported test sensitivities are based on studies using sporadic LD cases. Most of these studies used a selection of patients or were retrospective (Table 3); they are thereby subject to selection bias. Some of them include patients with LD caused by other serogroups than serogroup 1, which leads to underestimation of the test sensitivity. Furthermore, the clinical conditions of the patients described in these studies were not taken into account, which may explain the range of sensitivity values found by different authors.

To our knowledge, there are no publications on test sensitivities in an outbreak situation. The 1999 outbreak in the Netherlands provided a unique opportunity to evaluate urinary antigen tests in an outbreak caused by an identified *L. pneumophila* serogroup 1 strain. A nationwide alert for LD cases ensured optimal case finding, thereby decreasing patient selection.

<table>
<thead>
<tr>
<th>Variable</th>
<th>non-concentrated urine</th>
<th>concentrated urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biotest EIA</td>
<td>Binax EIA</td>
</tr>
<tr>
<td><strong>CAP category 2</strong></td>
<td>2.0</td>
<td>2.9</td>
</tr>
<tr>
<td>versus 1</td>
<td>(0.4 – 9.7)</td>
<td>(0.5 – 15)</td>
</tr>
<tr>
<td><strong>CAP category 3</strong></td>
<td>10.0</td>
<td>9.1</td>
</tr>
<tr>
<td>versus 1</td>
<td>(1.6 – 63)</td>
<td>(1.8 – 47)</td>
</tr>
<tr>
<td>male gender</td>
<td>–</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>(0.9 – 15.6)</td>
<td></td>
</tr>
<tr>
<td>days before 1st urine sample</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>(0.7 – 1.0)</td>
<td>(0.8 – 1.0)</td>
</tr>
</tbody>
</table>
bias. Furthermore, the conditions for a gold standard were favorable: two national reference laboratories performed all microbiological tests, and Dutch reference titers were calculated using the distribution of antibodies against \textit{L. pneumophila} serogroup 1 in a large sample from a national serum bank. \cite{4} In addition, all available clinical data for the LD patients in this outbreak were recorded centrally.

Assuming that patient inclusion in an outbreak situation resembles a prospective study design, our data are best compared with the published results of two prospective studies on urinary antigen test sensitivity in LD. One study \cite{19} included a single high titer in the gold standard, inherently lowering the test sensitivity to be measured on the basis of misclassification. In this study, however, a higher sensitivity (84\%) was found for patients with LD caused by \textit{L. pneumophila} serogroup 1. The other prospective study \cite{18} included

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Test & Gold standard & serogroup & n & study population & sensitivity & year/country\textsuperscript{ref} \\
\hline
\textit{RIA} & Culture & 1,4,9\textsuperscript{a} & 23 & Retrospective sample of hospitalized LD patients & 57\% & 1988, USA\textsuperscript{1} \\
\hline
\textit{EIA} & Culture or 4-fold rise in IFAT titer & 1,3,8,12 & 120 & Selected sample of LD patients & 77\% & 1990, GB\textsuperscript{3} \\
& Culture & 1 & 51 & Selected sample of LD patients & 84\% & 1990, GB\textsuperscript{3} \\
\hline
\textit{EIA} & Culture or 4-fold rise in IFAT titer or single high titer & 1,4,6 & 27 & Prospective inclusion of hospitalized LD patients & 70\% & 1990, Germ\textsuperscript{19} \\
& Culture or 4-fold rise in IFAT titer or single high titer & 1 & 20 & Prospective inclusion of hospitalized LD patients & 84\% & 1990, Germ\textsuperscript{19} \\
\hline
\textit{RIA} & Culture or 4-fold rise in IFAT titer & 1,3,7\textsuperscript{a} & 68 & Prospective inclusion of hospitalized LD patients & 56\% & 1995, USA\textsuperscript{18} \\
& Culture & 1 & 35 & Prospective inclusion of hospitalized LD patients & 80\% & 1995, USA\textsuperscript{18} \\
\hline
\textit{EIA} & Culture or 4-fold rise in IFAT titer & unknown & 65 & Selected sample of LD patients & 67\% (87\%)\textsuperscript{b} & 1998, Spain\textsuperscript{8} \\
\hline
\textit{EIA} & Culture or 4-fold rise in IFAT titer & unknown & 65 & Selected sample of LD patients & 64\% (89\%)\textsuperscript{b} & 1998, Spain\textsuperscript{8} \\
\hline
\textit{ICT} & Culture or 4-fold rise in IFAT titer & 1,2,3,4,1,6,10 & 187 & Selected sample of LD patients & 80\% & 2001, Germ\textsuperscript{14} \\
\hline
\textit{EIA} & Culture or 4-fold rise in IFAT titer & 1,2,3,4,1,6,10 & 187 & Selected sample of LD patients & 79 & 2001, Germ\textsuperscript{14} \\
\hline
\textit{EIA} & Culture or 4-fold rise in IFAT titer & 1,2,3,4,1,6,10 & 187 & Selected sample of LD patients & 83\% & 2001, Germ\textsuperscript{14} \\
\hline
\end{tabular}
\caption{Overview of urinary antigen test sensitivity for sporadic cases of Legionnaires' disease}
\end{table}

\textit{EIA} = enzyme immunoassay; \textit{ICT} = immunochromatographic assay; \textit{RIA} = radio immunoassay; \textit{IFAT} = immunofluorescence antibody test \textsuperscript{a} including cases of non-pneumophila LD \textsuperscript{b} after concentration of urine
patients with LD caused by *L. pneumophila* serogroups 1, 3, and 7, making a lower sensitivity more likely on the basis of a low urine antigen detection capacity for other serogroups than serogroup 1. Indeed, the reported sensitivity in this study was lower (57%).

Since the 1999 outbreak in the Netherlands was caused by an *L. pneumophila* strain of serogroup 1, high sensitivities for the three tests were to be expected. However, overall test sensitivities found in this study were lower than those reported by Ruf et al. [19] and ranged from 69 to 72% for nonconcentrated urine samples. This lower sensitivity may be explained by a difference in the study populations. Active case finding for LD in this outbreak may have resulted in hospitalization of a higher proportion of patients with relatively mild LD compared to a nonoutbreak situation. As our results show, the sensitivity of urinary antigen tests is relatively low for cases in CAP categories 1 and 2.

We do not know of a published study in which an association between the severity of disease and the test sensitivity for LD has been demonstrated, although one study hinted at such an association. [22] The association between sensitivity and clinical severity demonstrated in the present study has clinical and diagnostic consequences. Because of the high sensitivity in patients with a severe pneumonia, the early recognition of patients with life-threatening LD can prevent delay in initiating adequate antibiotic therapy. However, the urinary antigen test is less reliable in milder cases of LD, indicating that this diagnostic test, despite its rapid interpretation, cannot replace culture and serologic testing. Therefore, in the setting of persistent clinical or epidemiological suspicion of LD and a negative urinary antigen test result in patients in whom no other microorganism is identified, culture and serologic testing are recommended and treatment must include antibiotic coverage for *Legionella*.

When the detection of antigens in urine during an outbreak is used for epidemiological purposes, one has to keep in mind that 50 to 60% of cases of mild pneumonia will stay undiscovered, depending on whether urine is concentrated (concentration improves the chance of detection). This implies that, due to undiagnosed cases, the size of an outbreak will be underestimated unless complementary diagnostic serologic tests using paired sera are performed in all suspected cases. Because seroconversion can take up to 9 weeks after onset of the disease, a prolonged interval between collection of the two sera is advisable for reliable interpretation of serologic results.

Like other researchers, [7,14] we were unable to demonstrate a significant difference in sensitivity between the two EIAs that were tested and the ICT. Since the latter is very easy to perform without special laboratory equipment and the results are available at short notice even after concentration of the urine samples, this test may be preferable in outbreak situations if serogroup 1 is involved. Previous studies [8,9] have also demonstrated that a higher sensitivity of urinary antigen detection was found using concentrated urine, regardless of the test used. Concentration by ultrafiltration is easy to perform and can facilitate an early diagnosis, especially in milder cases.

In conclusion, in outbreak situations the urinary antigen tests are a useful tool for early diagnosis of LD, especially in patients with severe cases. The ICT scored at least equal to the EIAs and has the advantage of ease of performance combined with rapid test results. Concentration of the urine samples increases the sensitivity, particularly in patients with less severe illness,
and is therefore recommended. In outbreak situations, the use of urinary antigen tests alone for evaluation of the incidence rate will lead to underestimation of the actual incidence. Therefore, culture and serologic testing remain necessary diagnostic tools.

ACKNOWLEDGEMENTS

We thank all hospital clinicians and microbiologists in requesting patients’ permission and allowing us to collect clinical data and urine samples. Special thanks to Yvonne Boelens and Jacob P Bruin, who performed the urinary antigen tests.

REFERENCES


CHAPTER NINE

The sensitivity of three serum antibody tests in a large outbreak of Legionnaires' disease in the Netherlands

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ABSTRACT

In 1999, an outbreak involving 188 patients with Legionnaires’ disease (LD) occurred at a flower show in the Netherlands. This large outbreak provided the opportunity to evaluate serum antibody tests to assay anti-Legionella pneumophila, since limited data are available on the sensitivity of these tests. The sensitivities of an indirect serotype 1–6 immunofluorescence antibody test (IFAT), a rapid micro-agglutination test (RMAT) IgM serotype 1 antibody assay, and an ELISA to detect IgM and IgG serotype 1–7 antibodies, were evaluated using serum samples from LD patients related to the 1999 outbreak. Sensitivity was calculated using positive culture and/or a positive urinary antigen test as the gold standard in outbreak-related patients with radiographically confirmed pneumonia who fulfilled the epidemiological criteria. The IFAT, RMAT and ELISA showed sensitivities of 61%, 44% and 64%, respectively. The sensitivity of the three tests combined was 67%. In epidemic situations, however, high standing titres may be included in the laboratory evidence of LD cases. In the study population, high standing titres were found in 16% of cases. If the presence of high standing antibody titres was added to the criteria of a positive test, the sensitivities of IFAT, RMAT and ELISA were 86%, 48% and 75%, respectively. The sensitivity was 91% for all tests combined. The higher sensitivity for the combined use of tests is offset by a reduction in specificity to 97.6%. The results of this study indicate that using a combination of serologic tests in pneumonia patients suspected to have LD does not substantially improve sensitivity. The results suggest that in the microbiological diagnosis of LD, both IFAT and ELISA are reasonably sensitive assays. In an epidemic situation, both tests are highly sensitive, the IFAT more so than the ELISA.

Abbreviations. CAP, community-acquired pneumonia; EWGLI, European Working Group on Legionella Infections; IFAT, immunofluorescence antibody test; LD, Legionnaires’ disease; RMAT, rapid micro-agglutination test.

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INTRODUCTION

Legionnaires’ disease (LD) is an acute pneumonia caused by *Legionella* spp., which are responsible for 2–5% of community-acquired pneumonias (CAPs). [4,18] Patients are infected by inhalation of aerosols containing *Legionella* spp. or by aspiration of water contaminated with *Legionella* spp. Over 90% of LD cases are caused by *Legionella pneumophila*, of which 70–80% belong to serogroup type 1. [18,23] Although over 90% of LD cases are sporadic, much can be learned from outbreak-related LD cases, including their microbiological diagnosis. Diagnosis of LD is mainly based on culture, antigen detection in urine, and antibody detection in serum. Culturing of *Legionella* spp. from respiratory secretions has a sensitivity ranging from 25 to 75%. [17] Urinary antigen tests, both enzyme immunoassay (EIA) and immunochromatographic assay (ICT) combine a 99% or higher specificity with a sensitivity ranging from 50 to 90%. [3,8,9,21] Serological tests to identify antibodies to *L. pneumophila* were developed from 1977 onwards, starting with the indirect immunofluorescence antibody test (IFAT). [19] Several less laborious serological tests followed, among them numerous (mostly experimental) ELISAs and a micro-agglutination test (MAT). The first publication on an ELISA was in 1978. [11] Although commercial ELISA kits have become widely used, few studies of their sensitivity and specificity have been published. The same holds for the MAT, which was also developed in 1978; this was followed by a rapid version, the rapid micro-agglutination test (R-MAT), with identical test characteristics, in 1982. [13] The sensitivity of all three assays has been tested using sporadic cases of LD and not in outbreak situations. A recent outbreak in the Netherlands [6] provided a unique opportunity to evaluate the sensitivity of three serum antibody tests. This outbreak was caused by a *L. pneumophila* serogroup 1 strain. The nationwide alert for LD cases ensured optimal case finding, thereby decreasing patient selection bias. Furthermore, the outbreak situation allowed us to take the clinical spectrum into account, since all available clinical data for the LD patients related to this outbreak were recorded centrally. In addition, the conditions for a gold standard were favourable: two national reference laboratories performed all microbiological tests and a large collection of samples from a national serum bank was available to investigate the distribution of antibodies to *L. pneumophila* serogroup 1 among the Dutch population. To assess the sensitivity of the detection of serum *Legionella* antibodies, we performed three tests on serum specimens from outbreak-related LD cases: a serotype 1–6 antibody IFAT, an IgM serotype 1 antibody R-MAT, and an ELISA to detect IgM and IgG serotype 1–7 antibodies. [5] The aim of our study was to compare the sensitivities of the three tests using confirmative and epidemic LD criteria, to see if combined use of the three tests would be justified, and to compare our results to published sensitivities of IFAT, R-MAT and ELISA in both epidemic and non-epidemic settings.
METHODS

The outbreak

In February 1999, an outbreak involving 133 confirmed and 55 probable cases of LD, according to the European Working Group on Legionella Infections (EWGLI) criteria, [1] occurred in Bovenkarspel in the Netherlands. Of these patients, 89% were hospitalized. The outbreak investigation indicated that a whirlpool displayed at the consumer product division of an annual flower show was the most likely source of infection. Two L. pneumophila serogroup 1 strains were cultured from sputum of 29 patients. One of the strains was of the Allentown/France serogroup 1 subtype [16] and was genotyped 010-London according to the EWGLI criteria. [12] This strain was identified in 28 of the 29 culture-positive patients. Genotyping of the environmental L. pneumophila strains revealed that one L. pneumophila serogroup 1 strain found in the implicated whirlpool was of the 010-London genotype as well. [6]

Patients

In this study, 133 patients with a confirmed Legionella pneumonia according to the EWGLI criteria were included after having obtained written consent from them or from their relatives. Of these patients, 104 fulfilled the criteria of the gold standard used in this study. The gold standard (identical to the EWGLI criteria, but excluding serological evidence of infection) was defined as a patient who had symptoms compatible with pneumonia, with radiological signs of infiltration, who had visited the site of the outbreak during the incubation period, and who had laboratory evidence of infection with L. pneumophila. Laboratory evidence included at least one of the following criteria:

(1) Isolation of L. pneumophila from a respiratory sample cultured on buffered charcoal yeast extract supplemented with 2-ketoglutarate (BCYA-α), followed by genotyping and subsequent comparison to the environmental strain of the Bovenkarspel outbreak.

(2) Presence of L. pneumophila antigens in urine specimens, as shown in an antigen test. The three tests used in our study were two enzyme immunoassays, the Binax (Binax, Portland, ME, USA) and Biotest (Biotest AG, Dreieich, Germany) Legionella urinary antigen tests, and the BinaxNOW test, a qualitative immunochromatographic assay. All tests were used according to the manufacturers’ instructions. Urine was concentrated and reread after 1 h, as recommended. [7,15]

(3) All isolates from sputum samples were sent to the National Institute for Public Health and the Environment (RIVM) for genotyping of L. pneumophila. The Regional Laboratories of Public Health of Tilburg and Haarlem were the reference laboratories for the detection of L. pneumophila antigen in urine.
Classification of severity of disease

To evaluate exclusion bias by differences in severity of disease, the patients were divided into three clinical categories for CAP. Clinical data were collected from the hospital chart using a standardized case record form. Severity of pneumonia was scored on a five-point scale (0–4), according to the minor criteria for severity of CAP, [20] using the following clinical criteria at hospital admission: (1) respiratory frequency above 30 breaths per minute, (2) PaO2 below 60 mm Hg or O2 saturation below 92 %, (3) bilateral or multilobar infiltration on chest x-ray, (4) systolic blood pressure below 90 mm Hg or diastolic below 60 mm Hg.

Patients with a radiographically proven unilateral unilobar pneumonia, but without signs or symptoms according to the above-mentioned criteria, were classified as CAP category 1 (mild pneumonia). CAP category 2 consisted of patients who fulfilled only one of the criteria. According to our definition, these patients were suffering from a moderately severe pneumonia. CAP category 3 consisted of patients who presented themselves with two or more criteria and were considered to suffer from a severe pneumonia.

Serum samples and serum antibody tests

All 62 laboratories of clinical microbiology involved in diagnosis and treatment of the patients related to this outbreak sent all available serum samples to the Regional Laboratory of Public Health in Haarlem. After collection, serum samples were stored in portions at -70°C.

The criteria for a paired serum sample confirmative for LD were defined as:

(1) A fourfold rise in IgG, IgM and/or IgA antibodies to L. pneumophila in paired acute-phase (0–15 days after disease onset) and convalescent-phase sera, with final titre ≥ 1:128, using a commercial serotype 1–6 antibody IFA Test Kit Serogroups 1–6, Meridian Bioscience Europe Srl. [19]

(2) A fourfold rise in IgM antibodies to L. pneumophila in paired acute-phase (0–15 days after disease onset) and convalescent-phase sera, with final titre ≥ 1:32 in accordance with the 99 % cut-off value found in a serosurvey among healthy volunteers representative of the Dutch population, [5] using an IgM, serogroup Philadelphia-I subtype, antibody RMAT. [11]

(3) Seroconversion to positive IgM or IgG antibodies to L. pneumophila in paired acute-phase (0–15 days after disease onset) and convalescent-phase sera, in accordance with the age-specific 99 % cut-off values found in a serosurvey among healthy volunteers representative of the Dutch population, [5] using a commercial ELISA to detect IgM and IgG serotype 1–7 antibodies (Serion classic ELISA, Institut Virion/Serion GmbH, Wurzburg, Germany). [10] The antigens in this test are prepared from cultures of different L. pneumophila serogroups and subtypes: serogroup 1 Knoxville and Philadelphia subtypes, serogroup 2 Togus-I subtype, serogroup 3 Bloomington-2 subtype, serogroup 4 Los Angeles-I subtype, serogroup 5 Dallas-I subtype, serogroup 6 Chicago-2 subtype, and serogroup 7 Chicago-8 subtype.
The criteria for a paired serum sample suggestive for LD used in an epidemic situation were defined as:

1. High standing titres of IgG, IgM and/or IgA antibodies to *L. pneumophila* in paired acute-phase (0–15 days after disease onset) and convalescent-phase sera, with final titre ≥ 1:128 using a serotype 1–6 antibody IFAT, as described above.

2. High standing titres of IgM antibodies to *L. pneumophila* in paired acute-phase (0–15 days after disease onset) and convalescent-phase sera, with final titre ≥ 1:32 using an IgM antibody RMAT, as described above.

3. High standing titres of IgM or IgG antibodies to *L. pneumophila* in paired acute-phase (0–15 days after disease onset) and convalescent-phase sera using an ELISA to detect IgM and IgG serotype 1–7 antibodies, as described above.

**Sensitivity**

Sensitivity was defined as the number of patients with a positive test result divided by the total number of patients tested. Sensitivity for confirmed LD was calculated using the confirmative criteria for LD. Sensitivity for an epidemic situation was calculated using the confirmative and the suggestive criteria for LD.

**Statistics**

Statistical analysis was performed with the statistical program SPSS version 12.0 (Statistical Product and Service Solutions). Univariate analysis was used to identify factors potentially associated with test positivity: age, gender, clinical severity, number of days passed between onset of disease and collection of the first serum sample, and number of serum samples taken.

**RESULTS AND DISCUSSION**

Two or more properly timed serum samples with complete sampling dates were available for evaluation from 45 of the 104 hospitalized patients who fulfilled the criteria of the gold standard used in this study (see Fig. 1 for patient selection). Table 1 shows that excluded patients died significantly more often from LD, although their CAP score distribution was very similar to that of the included patients. Of the 45 included patients, 26 were men, with a mean age of 64 years (range 46–78 years) and 19 were women with a mean age of 60 years (range 21–77 years). The microbiological diagnosis was established by culture alone in one case, by culture and by urinary antigen test in nine cases and by urinary antigen test alone in 35 cases. The first serum to be tested for antibodies against *L. pneumophila* was collected 0–15 days (mean 8 days) after the first day of signs and symptoms of pneumonia. From 22 patients two sera were available, from 13 patients three sera were available and from 10 patients four to
eight sera were available for evaluation. Table 2 shows the timing of the convalescent sera. For one of the patients, the volume of serum was too small to perform all three tests, so only the MAT test was performed. Patients with positive test results on average had their first serum taken 2 days earlier in the acute phase than patients with high standing

Table 1. Differences between 45 included and 59 excluded Legionnaires’ disease patients for factors possibly associated with exclusion bias

<table>
<thead>
<tr>
<th>Factor</th>
<th>Proportion of included patients</th>
<th>Proportion of excluded patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of male gender</td>
<td>60%</td>
<td>68%</td>
</tr>
<tr>
<td>Mean age (range) in years</td>
<td>63 (21–78)</td>
<td>65 (25–84)</td>
</tr>
<tr>
<td>CAP* category 1 b</td>
<td>27%</td>
<td>26%</td>
</tr>
<tr>
<td>CAP category 2 c</td>
<td>32%</td>
<td>31%</td>
</tr>
<tr>
<td>CAP category 2 d</td>
<td>41%</td>
<td>43%</td>
</tr>
<tr>
<td>Admission to intensive care unit</td>
<td>43%</td>
<td>34%</td>
</tr>
<tr>
<td>Fatal outcome</td>
<td>9%</td>
<td>24% *</td>
</tr>
</tbody>
</table>

* CAP = community acquired pneumonia b CAP category 1 = mild pneumonia c CAP category 2 = moderately severe pneumonia d CAP category 3 = severe pneumonia e difference is statistically significant
titres: 7.1 versus 9.4 days after disease onset. This finding may suggest that in LD cases with high standing antibody titres, seroconversion had already occurred. In total, 7/45 (16%) of patients showed high standing titres in all assays. Table 3 shows the sensitivities of the three tests and the sensitivity for the combination of all tests for both the non-epidemic and epidemic criteria. Evaluating the ELISA for its individual components, sera from 48% of the patients showed a seroconversion in IgM ELISA and 50% a seroconversion in IgG ELISA. Using the epidemic criteria, the sensitivities were 61 and 64%, respectively.

It seems that the superior sensitivity of the IFAT and ELISA compared to the RMAT was mostly due to the identification of antibodies other than IgM. In this outbreak, the combination of tests was of limited value.

It has been suggested that in LD, any diagnostic test with a specificity below 99% is unacceptable. [17] Most IFAT sensitivity studies use this criterion and show similar results in non-outbreak patient series. However, the sensitivity for the IFAT in the Philadelphia outbreak was 42% for a fourfold rise in titre, given a specificity of 99.1%. With high standing titres added to the criteria of an LD case, sensitivity was 68%. [19] The IFAT used in our study seems more sensitive, probably due to the use of a polyvalent conjugate directed against IgG, IgM and IgA antibodies.

Table 2. Timing of convalescent serum sample(s) and presence of antibodies against Legionella pneumophila as demonstrated in any of three assays for 45 LD patients for whom two or more properly timed sera were available

<table>
<thead>
<tr>
<th>Time</th>
<th>Number of patients with available sera</th>
<th>Standing titre (\text{a or seroconversion} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–6 weeks</td>
<td>37</td>
<td>6/37</td>
</tr>
<tr>
<td>6–12 weeks</td>
<td>12</td>
<td>0/12</td>
</tr>
<tr>
<td>More than 12 weeks</td>
<td>20</td>
<td>1/20</td>
</tr>
</tbody>
</table>

\(\text{a }\) Acute phase serum was positive

Table 3. Sensitivity of three serum Legionella pneumophila antibody tests using sera from 45 Legionnaires disease patients

<table>
<thead>
<tr>
<th>Test criteria</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fourfold rise in IFAT (\text{a} )</td>
<td>61% (27/44)</td>
</tr>
<tr>
<td>Fourfold rise in RMAT (\text{b} )</td>
<td>44% (20/45)</td>
</tr>
<tr>
<td>Seroconversion in IgM or IgG ELISA</td>
<td>64% (28/44)</td>
</tr>
<tr>
<td>Combination of the three tests above</td>
<td>67% (30/45)</td>
</tr>
<tr>
<td>Fourfold rise or high standing titres in IFAT</td>
<td>86% (38/44)</td>
</tr>
<tr>
<td>Fourfold rise or high standing titres in RMAT</td>
<td>58% (26/45)</td>
</tr>
<tr>
<td>Seroconversion or high standing titres in IgM or IgG ELISA</td>
<td>75% (33/44)</td>
</tr>
<tr>
<td>Combination of the three tests above</td>
<td>91% (41/45)</td>
</tr>
</tbody>
</table>

\(\text{a }\) IFAT = immunofluorescent antibody assay \(\text{b }\) RMAT = rapid micro-agglutination IgM antibody assay
The sensitivity of the rmat was 63% in a study evaluating sera from 119 patients with LD using a positive culture for *L. pneumophila* or its presence shown by direct fluorescence antibody as the gold standard. [14] The substantially higher sensitivity of the rmat assay found in that study (61 versus 44% in our study) is difficult to explain. A different strain of *L. pneumophila* serogroup 1 was used, but the influence of different strains on rmat sensitivity is unknown. There was a difference in the timing of the first serum sample (0–28 days after disease onset in the study of Harrison et al., 1987), but late sampling tends to underestimate the sensitivity. There were differences in study population: higher clinical severity, higher mortality, and over 50% immunocompromised patients in the study of Harrison et al. [14].

To our knowledge, only two reports are available describing data from which an ELISA sensitivity based on a 99% or higher specificity could be inferred. [2,22] Bangsborg et al. [2] studied convalescent sera from 20 culture-proven LD patients and described an ELISA based on a lipopolysaccharide antigen with sensitivities of 70% for IgM and 75% for IgG, respectively. Although in the Bangsborg et al. [2] study the sensitivity of an IgM and IgG ELISA combination is not described, the reported individual sensitivities are 10% higher than those found in our study. This may be due to a difference in the antigen used or a difference in study population. Wreghitt et al. [22] studied sera from 11 patients and found sensitivities of 58 and 41% for IgM and IgG ELISA, respectively. In the Wreghitt et al. [22] study, another serum antibody test was included in the gold standard, and no details were given concerning the timing of serum samples under study, complicating comparison to our data.

In conclusion, to diagnose *L. pneumophila* pneumonia, serology is still a valuable tool. This study is the first to compare the sensitivities of an IFAT, an RMAT and an ELISA in an outbreak situation. The IFAT and ELISA seem to yield similar results using confirmative LD criteria. In an epidemic situation, the IFAT seems superior. Combination of assays in an outbreak situation improves sensitivity only marginally, from 86 to 91%. The data from this study suggest that early sampling may lead to a higher sensitivity for all tests.

ACKNOWLEDGEMENTS

We thank all hospital clinicians and microbiologists for requesting patients’ permission and allowing us to collect clinical data and serum samples. Special thanks to Yvonne Boelens and Jacob P Bruin, who performed the serum antibody tests.

REFERENCES

Legionella urinary antigen enzyme immunoassay (EIA) with the Bio test Legionella Urin Antigen (EIA) for detection of Legionella antigen in both


PART 4

CONTROL AND PREVENTION
CHAPTER TEN

Outbreak detection and secondary prevention of Legionnaires’ disease: A national approach

Jeroen W Den Boer1, Linda Verhoef1, Max A Bencini2, Jacob P Bruin2, R Jansen2, Ed PF Yzerman2

ABSTRACT

Background. To stop a possible outbreak of Legionnaires’ disease (LD) at an early stage an outbreak detection programme was installed in the Netherlands.

Methods. The programme consisted of sampling and controlling of potential sources to which LD patients had been exposed during their incubation period. Potential sources were considered to be true sources of infection if two or more LD patients (cluster) had visited them, or if available patients’ isolates and environmental Legionella spp. were indistinguishable by amplified fragment length polymorphism genotyping.

Results. Rapid sampling and genotyping as well as cluster recognition helped to target control measures. Despite these measures, two small outbreaks were only stopped after renewal of the water system. The combination of genotyping and cluster recognition lead to 29 of 190 (15%) patient-source associations.

Conclusion. Systematic sampling and cluster recognition can contribute to outbreak detection and lead to cost-effective secondary prevention of Legionnaires’ disease.

Key words. Legionnaires’ disease; Public health; Cost-effectiveness; Water supply; Disease outbreaks; Environmental exposure

INTRODUCTION

Legionnaires’ disease (LD) is an acute pneumonia of low incidence [9,22] which was first described after a large outbreak in Philadelphia in 1976 among visitors of a legionnaires’ convention held in a hotel, and passers-by at the same hotel. [13] The outbreak was shown to be caused by a newly discovered genus: Legionella spp. [20] These Gram-negative bacilli are ubiquitous in (manmade) aquatic environments and are capable of infecting humans by aerosol inhalation or by drinking and subsequent aspiration of water. The omnipresence of Legionella spp. in water is in sharp contrast to the low incidence of LD. This contradiction has led to different theories on transmission, prevention and outbreak detection of LD. Outbreak detection became a topic of national interest in the Netherlands after an outbreak in 1999
involving 188 LD patients. [12] An early warning system based on unexpected numbers of LD patients was introduced, leading to an alarm in the summer of 2002 when the incidence rate had doubled. Unfortunately, after thorough evaluation, no cluster or common source of infection could be identified. [8] Shortly thereafter, a national outbreak detection programme was installed, aimed at a short response time between diagnosis and inspection and sampling of potential sources of infection. The programme was based on the observation that outbreaks are often preceded and followed by small clusters or solitary LD cases. [5] Therefore, a source identification unit sampling potential sources was made available to all 39 regional public health services of the country. Officials of the Ministry of the Environment who supervised elimination of potential sources of infection assisted this unit. This action was expected to stop developing outbreaks at an early stage. Furthermore, by eliminating potential sources the outbreak detection programme would contribute to LD incidence reduction. These expectations were based on the observation that apparently sporadic cases of LD that appear over time are in fact clustered around the same source of infection. [7] The longest time span covering such a cluster is 17 years. [17] Source identification and subsequent elimination would thus prevent new LD cases to occur. To our knowledge, a national outbreak detection programme for Legionnaires’ disease has not been tried before. This article describes the results of the first 2 years following instalment in the Netherlands.

MATERIALS AND METHODS

Inclusion of patients

A confirmed case of LD was defined as a patient suffering from symptoms compatible with pneumonia, with radiological signs of infiltration, and with laboratory evidence of Legionella spp. infection. Laboratory evidence included isolation of Legionella spp. from respiratory secretions or lung tissue, detection of L. pneumophila antigens in urine, seroconversion or a four-fold or higher rise in antibody titres to L. pneumophila in paired acute- and convalescent-phase sera. A probable case of LD was defined as a patient suffering from symptoms compatible with pneumonia, with radiological signs of infiltration, and with laboratory findings suggestive of Legionella spp. infection. These findings included a high antibody titre to L. pneumophila in a single serum, direct fluorescent antibody staining of the organism and detection of Legionella species DNA by polymerase chain reaction in respiratory secretions or lung tissue. These definitions are conform to the criteria of the European Working Group for Legionella Infections (EWGLI). [1]

Legionnaires’ disease (LD) has been a notifiable disease in the Netherlands since 1987. Treating physicians report LD patients within 24 h to one of the 39 regional public health services in the country. Public health physicians subsequently report confirmed and probable LD patients within 24 h to the Ministry of Health using an electronic disease notification system. This system was made accessible for this study. Patients were included in the study if the date of disease onset was between August 1, 2002 and July 31, 2004. Given the purpose of the study to identify Dutch sources of infection, all patients who had stayed abroad should
be excluded. However, it is possible that some of these patients got infected with *Legionella* spp. after returning home. Arbitrarily, LD patients were also included if they had fallen ill 6–10 days after their return.

**Identification of potential sources**

The task of the regional public health services is to verify the diagnosis of reported LD patients and subsequently interview these patients (or their relatives) in order to identify potential sources of infection and to control a possible outbreak. Since 1990, all public health physicians use the same national protocol, which includes diagnostic criteria and a questionnaire. The questionnaire facilitates a structured interview focused at individual exposure to potential sources of infection. Inclusion of potential sources in the questionnaire was based on published results from epidemiological studies and outbreak reports. The potential sources of infection in the Netherlands for included LD patients that were identified using the questionnaire were reported to the research group and subsequently scheduled for sampling.

**Sampling of potential sources**

Trained laboratory personnel of the regional public health laboratory of Haarlem sampled potential sources. Both water and swab samples were collected. The water samples were concentrated by filtration and filtered residues were resuspended in 1 ml sterile water. Of this suspension, 100 µl samples were cultured without dilution and after 10- and 100-fold dilution on buffered charcoal yeast extract supplemented with α-ketoglutarate (BCYE-α) agar at 37°C, with increased humidity. In cases of bacterial overgrowth, cultures were repeated after pre-treatment by heating 30 min at 50°C. Swab samples were dispersed by immersion in 1 ml sterile water and cultured as described above. *Legionella* isolates were serotyped using commercially available kits: the *Legionella* Antisera “SEIKEN” Set (Seiken Denka Company Ltd., Tokyo, Japan, cat. nr. 311–701, which contains antisera against *L. pneumophila* serogroup 1, 2, 3, 4, 5, and 6 as well as antisera against *L. dumoffii*, *L. gormanii*, *L. micdadei* and *L. bozemanii*), and the Microscreen Kit (Microgen Bioproducts Ltd., Camberley, UK, cat. nr. M45, which contains antisera against *L. pneumophila* serogroup 1 and against *L. pneumophila* serogroup 2–14). They were genotyped by amplified fragment length polymorphism, which is a whole-genome fingerprinting method that relies on the selective polymerase chain reaction amplification of restriction fragments. [19] AFLP, which until recently [16] has been the method of first choice by EWGLI, [15] is rapid and highly epidemiologically concordant (E = 1.00) but is not highly discriminatory (D = 0.89). [14] All environmental strains were serotyped and only if an available patient isolate was of the same serotype, genotyping was performed. From each environmental sample four colonies were picked for serotyping and subsequent genotyping. In case of an available patient isolate of a different serotype, an additional effort was made by picking up to 20 extra colonies for serotyping and subsequent genotyping.

If potential sources were sampled, priority was given to sources to which clusters could be attributed or to which large numbers of visitors were exposed. Potential sources were visited simultaneously by trained officials of the Ministry of the Environment who verified whether
the water supply system was managed in accordance with legislative requirements. In short, owners of water supply systems are obliged to perform a risk assessment, take control measures and keep a logbook to administer preventive measures. If necessary, installations were put out of order and owners were given time to install control measures. These measures included removal of dead-end piping, regulation of water temperature and heat-flush procedures.

**Interpretation of sampling results**

A source identification result was considered:

1. Negative if *Legionella* spp. were not cultured from the potential sources brought forward by the Regional Public Health Services.
2. Inconclusive if *Legionella* spp. were cultured from one of the potential sources but neither a cluster could be identified nor matching fingerprints (see below) could be established.
3. Positive if a cluster was identified or if matching fingerprints were established.

A cluster defined conforms to EURL criteria as within a 2-year period two or more LD patients who were in their incubation period had visited the same potential source and this has come up in the structured interview. If two or more LD patients had residences less then 1 km apart and no common potential source was mentioned, they were also considered to fit the cluster definition. To indicate the lack of a specific common potential source, this type of cluster was named geographic cluster.

Matching fingerprints were defined as DNA-banding patterns induced by amplified fragment length polymorphism of both patient-derived and source-derived *Legionella* spp. that could not be discriminated.

**Statistical analysis**

Statistical analysis was performed with version 12.0 of the SPSS statistical program (Chicago, IL, USA). Univariate analysis was used to identify factors associated with the presence of *Legionella* spp. in potential sources. The factors considered “possibly associated” were geographic region (north, east, south, west) and season (winter, spring, summer, autumn).

<table>
<thead>
<tr>
<th>confirmed LD patients</th>
<th>probable LD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>men</td>
<td>women</td>
</tr>
<tr>
<td>men</td>
<td>women</td>
</tr>
</tbody>
</table>

**Table 1. Age and gender of 246 Legionnaires’ disease patients included from 1 August 2002 through 31 July 2004 in the Netherlands**

<table>
<thead>
<tr>
<th></th>
<th>confirmed LD patients</th>
<th>probable LD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (percentage)</td>
<td>men 145 (68%)</td>
<td>women 68 (32%)</td>
</tr>
<tr>
<td>Mean age in years (range)</td>
<td>men 57.4 (11–84)</td>
<td>women 58.7 (5–92)</td>
</tr>
<tr>
<td></td>
<td>men 24 (73%)</td>
<td>women 9 (27%)</td>
</tr>
<tr>
<td></td>
<td>men 62.5 (11–88)</td>
<td>women 53.4 (24–88)</td>
</tr>
</tbody>
</table>

LD = Legionnaires’ disease
RESULTS

From 1 August 2002 through 31 July 2004, 446 LD patients were notified in the Netherlands. Of these, 188 patients who had stayed abroad and got ill before their return or within 5 days after their return were excluded. The remaining 258 patients fulfilled the criteria of inclusion, but 12 (5%) of them did not enter the study for various reasons. Apart from organizational constraints, the reasons cited most often were unwanted disclosure of private activities and fear for conflicts at the discovery of *Legionella* spp. in a potential source. The 246 patients who entered the study did not differ significantly in age, gender, fatal outcome, or geographic origin from the non-response patients. Of the patients in our study, 213 (87%) had suffered from confirmed LD, of whom 48 (23%) were diagnosed by culture of *Legionella* spp. from respiratory secretions or lung tissue. The remaining 33 (13%) suffered from probable LD. Table 1 shows their age and gender distribution.

The presence of *Legionella* spp. in environmental samples was significantly associated in univariate analysis with geography and season. An odds ratio of 2.2 (confidence interval (CI): 1.3–3.7) was calculated for the presence of *Legionella* spp. in samples originating from regions south and west versus north and south. Comparison of the presence of *Legionella* spp. in samples taken in winter and spring versus summer and autumn showed an odds ratio of 2.1 (CI: 1.2–3.3).

The source identification result was negative for 129 LD patients (52%). The source identification result was inconclusive for 86 (35%) of the LD patients (see Table 2). The inconclusive results include non-matching environmental *Legionella* spp. for 26 of 48 (54%) culture-positive LD patients. A substantial part (45%) of the environmental *Legionella* spp. belonged to the non-*pneumophila* species.

The source identification result was positive for 31 patients, giving an overall rate of 13% (31/246). The rate was 15% (29/190) for LD patients who had exclusively stayed in the Netherlands during their incubation period. In total, 17 clusters were discovered (sized 2–4 LD

### Table 2. Comparison of diagnostic results and positive environmental sampling results for 86 Legionnaires' disease patients for whom no matching DNA-fingerprint was available.

<table>
<thead>
<tr>
<th>Sampling results</th>
<th>L. pneumophila serogroup 1</th>
<th>L. pneumophila non-serogroup 1</th>
<th>Legionella non-pneumophila</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diagnostic results</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– <em>L. pneumophila</em> serogroup 1</td>
<td>5</td>
<td>5</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td>– <em>L. pneumophila</em> non-serogroup 1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Urinary antigen test</td>
<td>10</td>
<td>8</td>
<td>19</td>
<td>37</td>
</tr>
<tr>
<td>Fourfold rise or seroconversion</td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>2</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Single high titre</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Total number of patients</td>
<td></td>
<td></td>
<td></td>
<td>86</td>
</tr>
</tbody>
</table>

Match = Genotypes of both patient-derived and source-derived *Legionella* spp. could not be discriminated by amplified fragment length polymorphism.

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patients each) involving 29 patients. Twelve of these clusters would not have been identified timely without the outbreak detection programme. To 10 of the 17 clusters LD patients could be attributed who had attracted their disease prior to the study period. This information was reported spontaneously by LD patients or by public health officials and was verified using historical notification data of the Ministry of Health. A matching DNA fingerprint was found for six LD patients (2%). Given that Legionella spp. were cultured from environmental samples related to 32 of 48 (67%) culture-positive LD patients, a matching fingerprint was found in six out of 32 (19%) pairs. Excluding non-pneumophila environmental strains, a matching fingerprint was seen in six out of 20 (30%) pairs. Four of the six patients for whom a matching fingerprint was found had stayed in different hospitals during their incubation period, three of which had been associated with nosocomial LD in the past. One nosocomial LD patient had been using water from a water heater of which the temperature had been set at 35°C to prevent scalding incidents. The second nosocomial LD patient had regularly opened the window of a hospital room for fresh air. This room was situated within 10m of a cooling tower from which a matching L. pneumophila was sampled. The third nosocomial LD had stayed in an intensive care unit and the fourth in a private room at a bone marrow transplantation department. In the latter cases a matching L. pneumophila was isolated from a tap water faucet within 5m distance. Of the two community-acquired LD patients for whom a match was established, the first had stayed in a mobile home during the entire incubation period. In contrast to his spouse this patient had not used the built-in shower. The second patient with community-acquired LD had stayed overnight in the house of his daughter for 4 days. In both cases L. pneumophila was cultured from a showerhead.

Control measures were taken at each potential source of infection as identified by public health officials using structured interviews. Despite these measures, 12 new patients occurred in relation to 10 potential sources. More rigorous measures were taken in order to stop transmission by these cluster-related sources. After these measures, no additional patients were reported in relation to all but two clusters. The first cluster consisted of LD patients who stayed at the same camping site. Eleven days after notification of the first patient L. pneumophila serogroup 1 was cultured from water samples originating from a shower unit on the camping site premises. Despite extensive control measures, two more patients (one with date of onset beyond the study inclusion period) reported after staying at this site. Only after renewal of the water supply system no additional patients were reported.

The second cluster that continued to expand was related to a water faucet in a hospital ward. Control measures were taken after the first patient. Despite these, two more LD patients (with dates of onset beyond the study inclusion period) could be attributed to the same faucet. Only after renewal of the existing water piping system no more LD patients were reported.

**DISCUSSION**

Our study shows that active source identification can be successful on a national scale. Seventeen clusters were discovered of which 12 would not have been detected timely without a national identification system. The overall identification rate was 13% and 15% excluding
international travellers. It seems that the benefits of including patients coming home from travel abroad do not justify the efforts. The identification rate of 15% is high compared to the pre-study period. In the pre-study period no procedures existed for the discovery of clusters involving more than one public health region. Small clusters went unnoticed and larger ones were discovered by chance. For example, a sauna-related LD outbreak of six patients over a period of 6 years involving five public health regions was only discovered when the sixth patient was presented at a regional meeting of public health officials in 1996. [10] The identification rate of 15% is lower than the 25% of EWGLI [21] and this is hardly surprising since the latter group of LD patients all comply to a well-documented environmental risk factor: spending one or more nights away from home. [23,24]

The systematic implementation of control measures at each potential source associated with a LD patient must have had an impact on the elimination of sources of infection. However, despite these measures 10 clusters developed in the course of our study. During the second visit of the source more rigorous preventive measures were advised. Still, on two occasions new LD patients could be attributed to the same source. The final and so far effective measure was complete replacement of the existing water supply system. This experience shows that source elimination not only depends on the speed of source detection. In the camping site outbreak *L. pneumophila* serogroup 1 was cultured from water samples 11 days after notification, whereas the water supply system was replaced 59 days after notification. These findings suggest that potential source elimination measures should be more rigorous and should be implemented within a shorter time period.

A total of six matching fingerprints were established. However, this does not necessarily imply that the thus identified source indeed caused disease. AFLP and other DNA-fingerprinting methods do not always distinguish between *Legionella* strains that have been cultured from sources hundreds of kilometres apart. [4,18] Although not reported so far, in the Netherlands this phenomenon will be present as well. Apart from this restriction, the number of six matches that were established in our study does not seem very high. However, only 48 *Legionella* patient isolates were available meaning that for one in every eight LD patients a source of infection could be attributed with a relatively high degree of certainty (depending on the concomitant epidemiological evidence). The matching fingerprints teaches us about the transmission patterns of LD and are therefore important in public health terms. Isolates were available from only 20% of the included LD patients in our study. Given this low rate, clinicians should be advised to make an effort to diagnose patients based on culture, even if the urinary antigen test or polymerase chain reaction assay are already positive for *Legionella* spp.

The finding the presence of *Legionella* spp. in environmental samples is associated with geographic region is in accordance with reported differences in regional LD incidence rates in the Netherlands in the 1987–2000 period. [11] This confirmation urges more detailed research into the origin of this phenomenon. Possibly, regional differences in drinking water production techniques (surface water versus ground water) can explain the association. The seasonal differences in presence of *Legionella* spp. in environmental samples coincides with documented seasonal variation in LD incidence. [6,11]

With the results of this study some idea can be given about the cost-effectiveness of the Dutch national source identification programme. Given an average hospital stay of 10 days and
an average cost of Euro 700 per day, a prevented LD patient would save Euro 7000. Total costs of the outbreak detection programme are Euro 125,000 per year, meaning that 18 prevented LD patients each year would turn the programme cost-effective. Although information is sparse on the number of clustered cases over time that can be attributed to a single source of infection, it seems reasonable to assume that each single source would on average present five LD patients in 10 years. The identification and elimination of 17 cluster-related sources as during our 2-year study period would then lead to prevention of 46 (85 minus 29 who occurred despite first control measures) LD patients in the next 8 (ten minus two) years, or almost 6 patients each year. If every 2 years 17 new cluster-related sources would continue to be identified, the programme would become cost-effective in a little over 6 years. Probably it would become cost-effective sooner, since the above calculation did not include indirect costs of morbidity and mortality nor indirect benefits resulting from the control measures implemented at all LD patient related sources as identified by public health officials. Let alone the costs of prevented outbreaks.

In conclusion, we have shown that source identification on a national scale can be successful in terms of outbreak detection and secondary prevention of LD, provided that source elimination procedures are effective. Furthermore, we have stressed the need for culture-based diagnosis of LD and made it plausible that a national source identification programme would break even in a little over 6 years.

ACKNOWLEDGEMENTS

We want to thank all public health physicians and nurses of the 39 regional public health services who helped identifying potential sources of infection. We also thank the hospital doctors and microbiologists for making available patient isolates for genotyping. We are grateful to the officials of the Ministry of the Environment for their assistance in source elimination. Furthermore, we thank Anita Warris and Marja Kleinee at the Health Inspectorate for their kind collaboration in data collection. We thank Kim van der Zwaluw at the National Institute of Public Health and the Environment for performing and interpreting the amplified fragment length polymorphism assays. We thank Professor Roel A Coutinho, Director of the Centre for Infectious Diseases Control, for his critical comments on the manuscript.

FUNDING

This study was supported by a grant of the Public Health Stimulation Fund (Fonds OGZ). This institute did not play a role in any aspect of the study or in the writing of this paper. All authors are independent from this fund.
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CHAPTER ELEVEN

General discussion

The central theme of this chapter is that essential, basic knowledge on the reservoir (paragraph one), transmission (paragraph two), epidemiology (paragraph three) and control measures (paragraph four) of Legionnaires' disease (LD) are necessary ingredients for effective and efficient control. In paragraph five the application of the essential, basic knowledge is checked for the situation in the Netherlands after a large outbreak in 1999. Paragraph six gives suggestions for an evidence-based approach of LD control.

I. RESERVOIR

In this paragraph, the habitat of Legionella and the characteristics of the pathogen are discussed.

a. Habitat

Water and soil. Worldwide, Legionella is present in natural waters and soil where it lives as a parasite of protozoa. Legionella can also be found in wet soil. Little is known on the type of soil and combinations of species and soil types. Nor on the survival and capacity of Legionella to infect humans from the soil reservoir. Numerous reports have been published on transmission of Legionella longbeachae from potting soil. First in Australia, later in the United States and since 2006 [1] also in Europe (this thesis; chapter 5). It was assumed that in Europe potting soil was not a problem, as two studies demonstrated that European potting soil did not contain Legionella. After publication of the L. longbeachae cluster the Dutch Food and Consumer Product Safety Authority (VWA) ordered a potting soil survey for growth of Legionella. Also, in Switzerland a survey was performed that found 36% of potting soil samples culture-positive for Legionella species. Recently, a Legionella pneumophila serogroup 1 strain from soil was matched with an isolate from a patient suffering from Legionnaires' disease who had been exposed to soil of a plant nursery. [2] This is in concordance with publications on an excavation site as reservoir of an outbreak involving 27 LD patients [3] and as an independent risk factor for LD. [4]

Despite the omnipresence of Legionella in lakes and rivers, direct transmission from these reservoirs is rare. There has been a case report on LD as a result from near drowning in freshwater. [5] Also, LD associated with natural spas has been reported. [6] The circumstances for transmission are favourable, since the concentration of Legionella in fresh waters seems sufficient to infect humans (10⁵ to 10⁷ CFU/l), [7] aerosol formation occurs at the surface of such
waters, and floating biofilms have been identified which contain *Legionella*. [8] Explanations for the low attack rate may be that the selective pressure of other microorganisms in these environments is too low to enhance development of virulent traits in *Legionella*. Also, temperature and aerosol formation may not be optimal for successful transmission from freshwaters.

*Legionella* can also be cultured from groundwater, in concentrations between $10^2$ and $10^4$ CFU/l. [9] Both freshwater and ground water are raw materials for the production of drinking water. In the production process, physical and chemical substances as well as biological agents are removed and eliminated during extensive filtration and disinfection steps. In the Netherlands, testing for *Legionella* at critical points in the water mains is obligatory. Yet, indirect evidence has implicated the water mains as the source of infection [10] of the Bovenkarspel outbreak (this thesis, chapter two). Despite extensive sampling of the water distribution network of the outbreak premises, no *Legionella* could be cultured from water or biofilm swabs. However, a *Legionella* strain was cultured from three potential water sources two hundred meter apart that could not be distinguished from *Legionella* isolates originating from 28 LD patients. This shows that *Legionella* at undetectable concentrations can be introduced in water systems of end users.

A study comparing LD incidences at municipal level in the Netherlands (this thesis, chapter four) showed that high versus low price of water was a risk factor for high LD incidence in the 1987–2005 period. As higher drinking water prices are associated with the use of surface water as opposed to groundwater as raw material for drinking water production, it can be hypothesised that contamination of drinking water with *Legionella* in the Netherlands is related to the production process.

After delivery of drinking water by the water mains to the water distribution systems of end-users, *Legionella* has been shown to grow to detectable concentrations in household drinking water. Substantial differences in contamination of water with *Legionella* have been reported. These regional differences occur in private households [11] as well as in hospitals. [12] For example, in Chicago 30% of household water systems tested positive for *L. pneumophila* serogroup 1. [13] There is an association between contamination with *Legionella* and the size of a water distribution system. In a large survey 68% of hospital water systems were contaminated, some of them having over 30% of water samples tested positive for *Legionella*. [14]

*Legionella* is also cultured from water sampled from cooling towers. Surveys of cooling towers consistently report a high percentage of contamination. [15]

**Amoebae and biofilm.** As early as 1980, Rowbotham described that *Legionella* infects amoebae and he suggested that these protozoa play a role in the transmission of LD. [16] Later, he hypothesised that entire amoebae sizing 10–60 µm packed with 365–1,483 bacteria could be the inoculum of LD. [17] Others confirmed that *L. pneumophila* serogroup 1 can infect, multiply and cause lysis within *Hartmanella*, *Acanthamoeba* and *Naegleria* species. More than five genera have been described since. Cianciotto discovered a 24kDa surface protein (Mip = macrophage infectivity potentiator) required for optimal intracellular infection and involved in resistance to intracellular killing [18] both in the amoebae as in the human macrophages. [19] *Legionella* multiplies within amoebal cysts that are freed when the amoeba ruptures at the end of a parasite cycle. Amoebal cysts protect *Legionella* from harmful environments [20] and
possibly amoebal vesicles packed with *Legionella* act as vectors for the direct transmission of *L. pneumophila* to the human host. It has been calculated that these vesicles contain 20–200 *Legionella* bacteria [21]. Spread by amoebae and infection by amoebal vesicles is in accordance with findings on infectious dose and pathogenesis. It has been shown that amoebal passage of *Legionella*, enhances their pathogenic features by incorporating molecules of amoebal origin. [22] The amoebal vesicles seem quite robust and could protect *Legionella* from harmful environments. In addition, amoebae have been shown to resuscitate viable nonculturables *L. pneumophila* after disinfection by biocides, which may account for the re-emergence of *Legionella* in water systems after disinfection. [23]

In man-made environments, *Legionella* is not only protected by protozoa inclusion, but also by a micro layer of organic material, microorganisms and nutrients, called the biofilm. The biofilm protects against heat and disinfectants. [24]

b. Characteristics of the pathogen

In the next section, the physical characteristics of *Legionella* in water and in air are discussed. Furthermore, *Ld* pathogenesis and virulence traits of *Legionella* are elaborated on.

*Physical characteristics.* In water *Legionella* can survive 139 days in distilled water and 369 days in tap water. [25] Also, *Legionella* can survive low pH as well as temperatures up to 55 degrees Celsius thanks to protection by hosting protozoa and biofilm. [24, 26, 27] Acanthamoebae produce vesicles that protect *Legionella* against biocide exposure and freeze thawing. [28]

In vitro experiments studying airborne *Legionella* have indicated that the bacterium survives best in aerosols at a relative humidity of 65%. [29] Also in vitro, virulent strains of *Legionella* survived in aerosols for a longer time than avirulent strains. [30] The latter may be explained by assuming that the virulent strains originated from airborne amoebae or amoebal vesicles. Their higher resistance to evaporisation and capacity to containing hundreds of *Legionella* make them relevant infectious particles. Apart from that, there may be a role for humid weather in the transmission of *Ld* [31], although other reports contradict seasonality in the presence of airborne *Legionella*. [32]

*Pathogenesis.* In the discovery of *Legionella*, guinea pigs played an essential role. Guinea pigs injected intra-peritoneally with *Ld* patient material died of pneumonia. [33] As they also developed pneumonia when exposed to contaminated aerosols, airborne transmission was proved.

After being inhaled by humans, *Legionella* must reach the alveoli in order to find an appropriate host, which is the alveolar macrophage. Particles larger than five micrometer are retained in the nose. Only particles between one and five micrometer can reach the lung. The smallest of these can reach the alveoli (one micrometer). [34] This is the size of a few *Legionella*, which measure 0.3 to 0.9 in width and 2 to 20 micrometer in length. For amoebal vesicles to reach the lung alveoli, they will have to be ruptured in the bronchioli. Probably, this does not happen in the intact human host, whose mucocillairy defence operating for particle sizes between one and five micrometer will clear such vesicles. However, in smokers’
airways with damaged mucociliated defence, amoebal vesicles may get stuck in the bronchioli and rupture while releasing hundreds of *Legionella* bacteria.

**Virulence traits.** As demonstrated with guinea pig models in the 1980s, some *L. pneumophila* serogroup 1 subtypes are more virulent than others. [35] At the same time, a role of protozoa was suggested. [36] Since then, the interaction between *Legionella* and its protozoan hosts has been studied extensively in vitro. Protozoan hosts are necessary for replication of *Legionella*. [37] It has been shown that temperature is one of the triggers for intracellular replication. [38] Intracellular *Legionella* in the growth phase was shown to be more virulent in a guinea pig model. [39] Tissue culture models confirmed that *L. pneumophila* is more invasive after growth within amoebae. [40] Generally, the *Legionella* virulence traits identified so far have been divided into motility, invasiveness and the capacity to replicate inside hosts and kill them. The difference in virulence between species is mostly due to replicating and apoptosis skills that are better developed in most *L. pneumophila* strains. [41]

Molecular epidemiology has also provided some clues on virulence. Genotyping efforts using monoclonal antibodies revealed that certain types are associated with community-acquired LD and others with nosocomial LD. Nosocomial infections are believed to result from infection with *Legionella* strains of low virulence. Furthermore, the different frequency distribution of genotypes in patients and in environmental sources hints that virulence differences with a genetic base do exist amongst *L. pneumophila* serogroup 1 strains. [42] In chapter six of this thesis, a study using systematically collected strains shows large distribution differences between the two. This finding leads to the hypothesis that yet unexplained selective environmental pressure at an environmental site can lead to *Legionella* strains at undetectable concentrations that are virulent for humans. These strains are at the same time dominated by genotypes that have a competitive advantage in this particular environmental niche. An alternative way to explain the distribution difference is by the existence of viable but nonculturable (vbnc) *Legionella*. [43] It remains to be demonstrated that vbncs are truly infectious agents for humans.

2. **Transmission**

In this paragraph, waterborne transmission of LD is described briefly. The discussion is focussed on several aspects of airborne transmission of LD. First, because of its central role in transmission of LD the concept of aerosols and droplet nuclei is explained. Then, air sampling, and aerosol producing sources are discussed. At the end of this paragraph, susceptibility to LD is elaborated on.

a. **Waterborne transmission**

Although airborne transmission is the dominant infection route, drinking of contaminated water and subsequent aspiration has been described as transmission mode for LD. [44] These case reports mostly involve nosocomial LD patients with predisposing factors like head and neck surgery.
b. Airborne transmission

There has been extensive discussion on the strength of proof that certain implicated sources are indeed the cause of LD, be it in an outbreak of sporadic patient situation. Ideally, air-sampling results would be supportive in such cases. Some published results on this matter are presented in this section. First, a brief history of air as the source of human disease is given.

Aerosols and droplet nuclei. In the age of bacteriology the idea of airborne diseases was abandoned for quite some time. It came back in 1934, when Wells [45] proposed the idea of the droplet nuclei in airborne disease which he defined as a particle smaller than 10 micrometer in diameter resulting from evaporation of (bio)aerosols. In fact, droplet nuclei are dried residua of larger (respiratory) droplets. Later trials on experimental animals showed that small particles are capable of travelling considerable distances. This spurred research on the spread of bacteria [46] and virus (polio, influenza) [47] and showed that microorganisms stayed viable in the air for hours to days in the form of droplet nuclei of varying sizes. The most convincing studies [48,49] involved an experimental tuberculosis ward in Baltimore where guinea pigs were directly exposed to the air breathed out by hospitalised smear-positive tuberculosis patients. Later studies have shown that droplets between 60 and 100 micrometer can travel 1–6 meters before evaporating to droplet nuclei containing *Mycobacterium tuberculosis* measuring 2 to 3 micrometer. [50] It was shown that for deep deposition in the human alveoli particles should measure between 0.5 and 2 micrometer. [51]

Air sampling. Several air sampling devices were used from the 1950s onwards, among them the Andersen sampler, which differentiates on size of droplet nuclei varying from 9.2 micrometer and larger to 1 micrometer and smaller. [53] These devices have been used to study several types of airborne transmission [52], but not for LD. It was guinea pigs that demonstrated airborne transmission of *Legionella* initially. [54] In the Netherlands it was thus shown that nosocomial LD was transmitted with tap water as the source. [55] Later, genotyping techniques, combined with epidemiology results replaced guinea pigs and air sampling as proof for LD transmission. Only recently, a renewed interest in air sampling has occurred. [56,57] Several different techniques have been described, some commercially available [58,59], some by own design. [60–62] *Legionella* have on a variety of occasions been demonstrated in air samples. In a comparative study, air samplers base on the liquid impingement method appeared superior. [63] Some findings indicate that repetitive sampling provides the best results. [64]

Aerosol producing sources. Initially, a correlation between the concentration of *Legionella* in a water distribution system and the risk of LD was suggested. [65] Later it became clear that there is no correlation between *Legionella* in water and *Legionella* in the air. [66] Thanks to air sampling, now slowly knowledge on the spread of airborne *Legionella* is growing. Below, results of air sampling near the site of a proven or suspected source of LD infection are described for showers, cooling towers, whirlpools, faucets, toilets and other aerosol producing devices. On numerous occasions aerosols have been sampled and found contaminated with *Legionella* within one-meter distance but not further from a showerhead. [67–71]
The longest distance away from a cooling tower that *Legionella* has been cultured by air sampling is 300 metres. The genotype was indistinguishable from that of a *Legionella* strain originating from the cooling tower that was suspected as the source of an outbreak of LD. However, this genotype was found in various other potential sources in the neighbourhood of the cooling tower, including private household water distribution systems. [72] Apart from this, *Legionella* has been cultured by air sampling at 30 centimetres from a cooling tower [73] and at the exhaust vent of an evaporative condenser [74] at which occasions the water was contaminated with *Legionella* in a concentration of $10^6$ cfu/l. Most evidence of airborne transmission is indirect and varies from having been at the tower for cleaning activities in the incubation period, [75] to being up to seven kilometres away from a cooling tower. [76–79] Most outbreak reports describe that the LD attack-rate is reciprocal to the distance from the cooling tower. [80] A large proportion of cooling towers is contaminated with *L. pneumophila* serogroup 1 at concentrations of $10^5$ or higher. [81,82] This concentration has been associated with solitary cases and small clusters of LD patients. [83] Concentrations of $10^6$ or higher are reported typical for cooling tower related outbreaks. [84] One study showed that amoebae in cooling towers are sixteen times more often infected with *Legionella* than amoebae in tap water. This may explain the strong association of LD with cooling towers and the relative high frequency of cooling tower related outbreaks as compared to other potential sources. [85]

Whirlpool spas on display have been implicated as the source of several outbreaks. [86–91] In this thesis, a large whirlpool associated outbreak is presented in chapter two. In this outbreak, air-sampling results were not available because the implicated source had been removed at the time of the outbreak investigation. Although two whirlpool spas contained a *L. pneumophila* serogroup 1 genotype that could not be distinguished from that isolated from sputum derived from 28 LD patients, two epidemiological studies implicated one of them as the source. Reports on LD associated with bathing in a whirlpool are rare, [92] possibly indicating that transmission is only noticed when the exposed population is substantial. No studies involving air sampling in the vicinity of a whirlpool have been published. Since most whirlpool spas are drained and displaced after an outbreak, no data are available on the concentration of *Legionella* in the water at the time of such outbreaks.

Faucets have been suggested as the source of sporadic LD [93] as well as outbreaks of LD. [94,95] In chapter ten of this thesis, one of the described LD clusters was genotypically linked to a faucet in a hospital. Rarely, air sampling has been used to substantiate the risk for LD transmission from water faucets. In one publication aerosols within one-meter distance from bathtub faucets were shown to contain *Legionella*. [96]

Toilets have not been implicated as a source of infection in LD. However, experiments using seeded water indicated that toilet flushing could spread bacteria and viruses, which can stay airborne for prolonged periods. [97]

Furthermore, numerous unique outbreaks have shown that LD transmission can involve any aerosol-producing device. Examples include a small decorative fountain in a restaurant, [98] a grocery mist machine [99], a footbath in a sauna [100] and a defect pump that unintentionally produced aerosols in a crawling space of a flooded bar. [101] In the latter example, air sampling and genotyping results revealed an undistinguishable *L. pneumophila* serogroup 1 genotype in
a sewage pump, in the air of the crawling space and of the bar as well as in sputum of LD patients. Apart from the above examples, many outbreaks have been published in which the source could not be identified. This includes an outbreak at a car parking. [102]

c. Susceptibility

In the whirlpool associated outbreak presented in chapter two of this thesis, the attack-rate increased linearly on a daily basis, from 0.011% to 0.56% six days later, probably due to larger amounts of airborne amoebae or vesicles corresponding to an increase in Legionella concentration in the whirlpool water. [103] This increasing attack-rate is a strong argument for general susceptibility of humans for Legionella infection. Furthermore, the whirlpool associated outbreak presented in chapter two and an outbreak of community-acquired LD due to a grocery mist machine [104] show similar host risk factor patterns. In the first, 29% had chronic underlying disease (smoking, alcohol intake, and immunosuppressive medication excluded), whereas in the other 62% had at least one current underlying disease (smoking, alcohol intake, and immunosuppressive medication included). Case-control studies for community-acquired LD that were matched for age and gender indicate that underlying disease is not an independent risk factor. [105] In chapter three of this thesis it is shown that probably travelling abroad is a confounder for this relation. [106]

Smoking is the only strong and independent risk factor for LD that is consistently reported from the original outbreak onwards. Furthermore, the risk was shown to increase significantly with the number of cigarettes smoked. [107] The prevalence of chronic bronchitis and emphysema is reported to be higher among LD patients than expected, based on US national morbidity data, [108] although not in case-control studies. Still, both findings strongly support the hypothesised pathogenesis of LD in which Legionella packed amoebae or vesicles are not removed by the mucociliary defence mechanism.

Numerous outbreaks of LD in hospitals show that health personnel are not affected, although they have been exposed to Legionella as shown by elevated titres of antibodies against Legionella in serum. [109] Especially transplant patients are vulnerable to infection with Legionella. [110] But also, other underlying diseases and immunosuppressive medication have been shown to increase the risk of nosocomial LD. Nosocomial infection is predominantly not due to Legionella serogroup 1, but to other serogroups [111] and other species [112] that are known to be less virulent.

Outbreaks of community-acquired LD are the best proof of general susceptibility. However, several publications have suggested that certain polymorphisms in the gene regulated innate host immune response may enhance susceptibility to LD as demonstrated in LD patients. [113,114]
3. Epidemiology

In this paragraph, the role of epidemiology in source identification and its role in understanding the transmission of LD are discussed. Different types of studies have implicated potential sources of infection with Legionella. Molecular epidemiology is becoming more prominent as shown by our recent discovery of a mismatch in Legionella genotype distribution (chapter six of this thesis). First, in this paragraph some typical LD transmission patterns are discussed: sporadic cases, serial cluster, clusters in time and space and outbreaks.

a. LD transmission patterns

Sporadic LD patients are single LD patients with no association to other LD patients in time and space. In the literature any LD patient who is not part of an outbreak or cluster is called sporadic. These LD patients are rarely linked to a source of infection. A study including 203 non-outbreak, non-travel community-acquired LD showed that many apparently sporadic cases are in fact serial clusters (LD patients spread over time, linked to one source) or clusters in time and place. [115] Sporadic LD patients may precede a cluster of cases (e.g. one patient six weeks before an outbreak of 16). [116] These findings made Bhopal in 1992 suggest that control of LD should include epidemiological and environmental investigation of sporadic LD patients, instead of waiting for two LD patients who could be linked to a common source. [117] One of the reasons that sources for sporadic LD patients are rarely identified, may be that risk factors for sporadic LD are not identical risk factors for outbreak related LD.

The finding of serial clusters of LD leads to the hypothesis that many apparently sporadic cases result from low intensity, intermittent exposure to a common source and are hence part of mini-outbreaks. [118] Examples of such undetected clusters include a group of 25 nosocomial LD patients in a period of 17 years in a university hospital, [119] and a cluster of six community acquired LD patients in a period of six years who visited the same sauna. [120]

The long period during which clusters are caused by the same environmental Legionella genotype in the same environmental niche has been used in public health intervention strategies. The European Working Group for Legionella Infections (EWGLI) implicates hotels which have been visited by two or more LD patients during their incubation period as a source of LD and stimulated participating governments to implement surveillance schemas. This strategy has lead to a successful decrease of hotel-related outbreaks. [121] The EWGLI strategy has been tried in the Netherlands on a national scale as described in chapter ten of this thesis, with positive and cost-effective results. [122] Based on a two-year evaluation, it was calculated that the programme would break even after six years. Despite the programme, small clusters may go undetected because of underdiagnosis and unawareness, or because detailed information on exposure in the incubation period is insufficient (e.g. postcodes).

There are several prerequisites for typical outbreaks of LD to occur: a large number of exposed individuals, among them male smokers of middle age, who inhale Legionella loaded (vesicles of) amoebae, which originate from an aerosol producing environmental niche. The Legionella are of the L. pneumophila species and serogroup 1 serotype, are MAb-3 positive as defined by monoclonal antibody typing, possess the Mip-gene, have been under selective
pressure from protozoa (most probably *Hartmanella vermiformis*) which potentiated their virulence, have grown at an optimal temperature of 37 degrees Celsius for a number of days to weeks, with sufficient nutrients available in the biofilm. The amount of aerosols produced, the ambient temperature and humidity as well as other climate conditions that favour spread and prevent dehydration of the airborne amoebae or vesicles further determine the size of the outbreak. The reason that outbreaks are rare events seems to be that the prerequisites are many and mostly do not all occur at the same time.

b. *Epidemiological studies to implicate sources of LD*

Ideally, the source of an outbreak is demonstrated by culture of the same *Legionella* genotype in LD patients’ sputum culture, air samples and in water from an aerosol-producing source. In most descriptions of LD outbreaks air sampling is missing. Transmission evidence in that case is further supported by epidemiological studies (cohort studies, case-control studies, sero-surveys, and molecular epidemiology).

In chapter two of this thesis a cohort study is described involving 742 healthy individuals who were exposed to *Legionella*. The cohort study was part of an investigation of an indoor community-acquired LD outbreak due to a whirlpool. [123] The geometric mean serum antibody titres against *Legionella* rose reciprocal to the distance to the source of the outbreak. [124] Using the same data, it was later demonstrated that sub-clinical infection with *Legionella* is very common (40% of workers within a 40 meter distance), [125,126] suggesting that a shorter distance to the source of infection was required for LD to occur.

A case-control study comparing 107 solitary LD patients to lung cancer patients revealed that the population living within 0.5 kilometre of a cooling tower had a three times higher risk of LD than people living more than 1 kilometre away. [127] A large outbreak involving 85 LD patients showed an association with living downwind of a cooling tower. [128] Visiting a retail shop next to an excavation and construction site was shown to be an independent risk factor in an outbreak of LD involving 27 patients. [129] Residing near an excavation site has been described as an independent risk factor as well as in a large case-control study. [130] In chapter three of this thesis a case-control study for risk factors of LD is described. The results of this study suggest that there are two distinct LD populations based on foreign travel. LD patients who travelled abroad were of better health than those who attracted their disease in the Netherlands.

Sero-surveys have been published suggesting the potential of certain sources to transmit LD. Among them several surveys aimed at quantifying the risk of dentist equipment. [131] However, numerous studies have failed to indicate this type of aerosol spread as a true source of LD. Results described in chapter three of this thesis confirm that visiting a dentist is not a risk factor for LD. In outbreaks of nosocomial LD, sero-surveys among hospital personnel showed elevated antibody titres and seroconversion in the absence of disease.

Different molecular biology techniques have been used to genotype *Legionella* isolates and environmental strains in order to implicate the source of an outbreak. Still, finding indistinguishable genotypes is no proof of transmission as they may be found in large geographical areas. [132] Essential in the interpretation of genotyping results is knowledge...
on the background distribution of both clinical isolates as well as environmental *Legionella* strains, preferably based on prospective studies. Unfortunately, few such studies have been published, one of them is discussed below. \[133\]

c. Distribution mismatch

In a large prospective study in the Netherlands presented in chapter six of this thesis, patient isolates and environmental *Legionella* strains were collected and genotyped. The distribution of genotypes of the two groups appeared to differ substantially. Most prominent was the finding that the most common clinical isolate (*ewgli aflp* type 004 Lyon) was only found once in 6,500 environmental samples.

Several explanations are possible. The first is a laboratory contamination in the patient collection. This is not logic, since all 62 laboratories in the country have made clinical isolates available. The second possible explanation is that the environmental samples that were tested for *Legionella* had a low a priori chance to contain 004 Lyon. This is not the case, since only samples were taken that originated from potential sources to which LD patients had been exposed during their incubation period. Only types of sources with a documented association with LD had been included in the sampling procedures. The third possible explanation is that 004 Lyon is mostly found in cooling towers. As locations of cooling towers are generally not known in the Netherlands, they are rarely sampled. A fourth explanation is the choice of sampling sources that contain water per se. Instead, air as well as soil samples should have been taken in order to identify potential sources.

The most likely explanation is that strains of 004 Lyon were present at undetectable levels.

4. Intervention

Control of LD is possible at different levels. To describe these levels, in the section below the chosen direction is from the patients towards the environmental sources. First the role of diagnosis and treatment, notification, outbreak detection and source identification is discussed, followed by a summary of preventive measures. A differentiation is made between general measures and those aimed at the hospital situation.

a. Diagnosis and treatment

Without diagnosis, source identification and prevention are impossible. Since the use of urinary antigen testing, under-diagnosis of LD in the Netherlands has dramatically diminished, resulting in ever-rising incidences. Thanks to a more timely diagnosis and concurrent timely treatment, at the same time the case fatality rate has decreased from 15% to 8% in the Netherlands. \[134\] These data correspond to an even larger reduction of mortality in the USA, from 34% to 12%. \[135\]
b. Notification, outbreak detection and source identification

Since a large outbreak of LD in 1999, the notification process in the Netherlands has been speeded up by the introduction of a web-based notification system for all notifiable infectious diseases (OSIRIS). As described in chapter ten of this thesis, an LD outbreak detection programme was installed in the Netherlands in 2002, aimed at identification of serial and geographical clusters as well as a short response time between notification and sampling of a potential source of LD. The response time has been significantly reduced as compared by the 1999 outbreak. This was evident in 2006, when an outbreak involving 30 LD patients was related by genotyping to a cooling tower in Amsterdam ten days after the first LD patient was notified. [136]

In the first two years, the outbreak detection programme identified seventeen clusters, twelve of which would not have been identified timely without its existence. Between 2002 and 2006, the programme was able to detect a potential source of infection for 25% of LD patients in the Netherlands, excluding patients who were part of the Amsterdam outbreak and those who had visited abroad in their incubation period. However, for most of the clusters in space and time, no specific source could be identified. A recent grant of the Netherlands Organisation for Health Research and Development (Zon-Mw) is being used to clarify these clusters.

c. Temporary disinfection

As soon as a source is identified, it should be controlled or eliminated. In most circumstances, checking of the water distribution system will reveal imperfections concerning stagnant water and inappropriate temperature, which can easily be corrected. If temperature levels cannot be adjusted, disinfection with heat or chemical substances may be needed. Unfortunately, these measures are not sufficient. As described in chapter ten of this thesis, 12 new LD patients occurred in a two-year period due to failing disinfection of identified LD patient-related potential sources. Even after a more rigorous second disinfection, two more LD patients occurred. Ultimately, removal and renewal of the entire water distribution system was required. [137] In case of a cooling tower as the source, mechanical cleaning and chemical disinfection are more appropriate. Chemical disinfection of cooling towers has shown to be difficult as well. [138]

d. Preventive measures

All of the above are interventions in the presence of LD patients. However, legislation on Legionella requires that preventive measures be taken in the absence of LD patients. In that case, installations that are notoriously contaminated with Legionella may become part of a Legionella control programme.

General preventive measures. The World Health Organisation (WHO) in 1990 stated in a memorandum from a WHO-meeting on epidemiology, prevention and control of legionellosis that general measures to control LD are ineffective. [139] In a 2007 update, [140] the WHO seems to have adapted its view since it now advises to apply water safety measures to potable water.
and in-building distribution systems, cooling towers and evaporative condensers, health-care facilities, hotels and ships, natural spas, hot tubs and swimming pools.

The preventive measure most applied to control LD is temperature regulation. It has been shown that gas heaters are less often associated with growth of Legionella than electrical heaters. [141] Monitoring of the temperature in a water distribution system can be a sufficient control measure, provided that water in the entire system is held above 55 degrees Celsius. In practice, it is advised to keep the temperature of heaters above 60 degrees. Culture is the ultimate test for LD control of water distribution systems. Demonstration of Legionella by culture has a sensitivity of only 50% as measured by seeded tap water as golden standard. [142] Several conventional as well as real-time-PCR-based methods for detection of Legionella in water samples have been described. Joly recently reported that the type of water sample and inter-laboratory differences influences these assays. [143]

If temperature control cannot be achieved throughout a water distribution system, disinfection is warranted. Unfortunately, few controlled studies on Legionella disinfection have been published. Serial clusters can occur if temperature niches exist that create a stable microenvironment for growth of Legionella. Such niches are difficult to disinfect. Due to the protection of amoebae, Legionella is resistant to several disinfectants. [144] Also, amoebae can adapt to disinfectants. [145] In cooling towers, Legionella has shown to be highly resistant to disinfectants. [146]

Preventive measures in hospitals. All hospitals should be aware that immuno-compromised patients are at risk for LD caused by strains that are not pathogenic for healthy individuals. Furthermore, a virulent strain of Legionella can be introduced in the water distribution system at any time. Therefore, nosocomial pneumonia patients should be tested for LD.

Temperature monitoring and sanitation of the water distribution system are prerequisites for LD control in hospitals. Dead legs have been shown to contain Legionella concentrations up to $10^8$ CFU/l and should therefore be removed. [147] Removal of faucet aerators has been shown to reduce the risk of transmission. [148,149] Sampling and culturing of water two to four times per year will provide information on the effectiveness of LD control measures in place. Legionella is protected by amoebae [150] and biofilm [151] which makes disinfection problematic. Several strategies have been tried, the most common one being the so-called superheat-and-flush method. This method is based on a hospital outbreak in the early 1980s, involving 100 LD cases that was eventually stopped by heating of the water temperature above 60 degrees Celsius. [152] Later studies showed that the method gives only temporary results. [153,154] The use of copper-silver-ionisation, monochloramine, ultraviolet radiation and filters all has been described in uncontrolled studies. [155-157]

5. LD CONTROL IN THE NETHERLANDS

In this paragraph, LD control in the Netherlands after 1999 will be discussed. That is, as it took shape in the period following the large whirlpool-associated outbreak in Bovenkarspel in 1999 that was described in chapter two of this thesis.
a. Bovenkarspel and vrom

In 1999, the Ministry of Housing, Spatial planning and the Environment (vrom) in the Netherlands, responsible for the drinking water quality immediately took the initiative in Legionella control. vrom applied its own safety benchmark for environmental policies to the drinking water quality. The vrom safety benchmark allows for one death in a million exposed.

b. Drinking water legislation and public health actors

On October 15, 2000 a temporary legislation was issued aimed at reducing the overall level of Legionella in drinking water below 50 CFU/l. This general control strategy was consolidated two years later in the new Drinking Water Law, issued by vrom. Initially, all owners of a drinking water system to which third parties were exposed, were obliged to assess the risk of the system, create a control plan to contain growth of Legionella, and use a logbook to document control measures. If Legionella concentrations above 1000 CFU/l were cultured from water samples, water systems were closed and municipal health services consulted to assess the risk for LD transmission. In the new Drinking Water Law, the normative level was raised to 100 CFU/l and certain categories were excluded decreasing the impact from 600.000 to 10.000 owners of installations. The reduction was a direct consequence of intensive interaction of vrom with public health actors in the preceding three years. The public health actors consisted of the Municipal Health Services of the four biggest cities (Amsterdam, Rotterdam, Utrecht and The Hague), the Preparedness and Response Unit (LCE) and the National Centre for Hygiene and Safety (LCHV) of the National Institute of Health and the Environment (RIVM), the LD outbreak detection programme (BEL) and the National Society of Municipal Health Services in the Netherlands (GGD Nederland). The actors all pointed to the lack of evidence for the proposed Legionella control interventions to be effective and to the very high costs involved.

Since 2000, control of LD in hospitals falls under the jurisdiction of vrom. Control of LD related to cooling towers falls under the Ministry of Social Affairs and Employment.

c. A nine year evaluation

Nine years have passed since the 1999 outbreak. During this period vrom has actively stimulated LD control efforts and has actively pursued compliance to the Drinking Water Law. Below the impact of these efforts is discussed in terms of LD incidence and costs involved.

Incidence. The incidence of LD in the Netherlands was relatively stable at 2.7 per million in the 1987–1998 period. After a sharp increase in 1999 to 11 per million, the incidence has steadily continued to rise to 26 per million in 2006.

Costs. Most of the costs involved in control of Legionella in water distribution systems have not been made by the government, but by owners of collective water distribution system divided over several sectors in society. They in their turn asked higher prices for their services
to cover the investment in improved water distribution systems and installations. So, the general public as the end-user of these services paid for the investments made by businesses like hotels, saunas, camping sites, and sport facilities. The municipal health services of the biggest Dutch cities calculated that in the 1999–2002 period a one-time investment of ten billion Euros was spent, followed by a yearly investment of one billion Euros. The yearly costs were reduced to 500 million Euros per year in the post 2002 period. \[158\]

In the Netherlands over a hundred hospitals exist all of which have complied with the Drinking Water Law, making large investments in safe water distribution systems. These investments were made using the normal budget for the health care sector. Given the financing structure in the Netherlands, this means that every citizen contributed to these costs. There are no data available on the height of the costs.

National and local government as owners of buildings like military training camps and municipal indoor swimming pools have invested in Legionella control measures. The Association of Netherlands Municipalities (vng) calculated that its members spent 48 million Euros on general measures in the first years after 1999. The Ministry of Defence spent 16 million Euros. \[158\]

There has not been a large investment in fundamental Legionella research. However, the drinking water companies and vrom have invested in applied Legionella research by kiwa. Also, 500,000 Euro was invested by a Ministry of Health Fund (Fonds Openbare Gezondheid) in an outbreak detection programme. This implementation programme had some research off-spin, which has been presented in parts two and four of this thesis.

The cost of Legionella prevention as expressed by quality of life adjusted life years (QALYs), was estimated between Euro 100,000 and Euro 1,000,000 per QALY by the National Institute of Public Health and the Environment. In comparison, in 2000 the minister for Health considered that Euro 26,400 per QALY was not cost-effective enough to justify introduction of hepatitis B vaccination in the National Vaccination Programme.

As QALYs are often used in calculating costs and benefits of public health interventions it allows for comparisons to alternative interventions. Comparing this way, one QALY gained by the Legionella prevention activities in the Netherlands was as expensive as 100 QALYs gained by open-heart surgery. \[159\] When the Drinking Water Law was adjusted, limiting its range to 10,000 water installations, the Legionella prevention was estimated to cost ten times lower.

6. AN EVIDENCE BASED APPROACH OF LD CONTROL

As discussed in chapter five of this thesis, in the last nine years general LD control measures in the Netherlands have not coincided with a decrease in LD incidence. In this paragraph a more targeted approach is presented. The approach is based on the identification of several circumscribed LD transmission situations for which different programmes can be developed: outbreaks, hospitals, and cooling towers. Furthermore, two important contributions to prevention are acknowledged for which a programme is suggested: a contamination database and a cluster evaluation and disinfection expertise team. This paragraph finishes with recommendations to practical research, results of which will enhance LD control measures.
a. Outbreak detection programme

Since 2002, an outbreak detection programme is operational in the Netherlands. Although it has been more successful in identification than in elimination of sources, its effectiveness could be enhanced by adding several tools: a geographic information system (GIS), air and soil sampling, and a culture procedure for amoebae.

Geographical information system. In outbreak situations as well as during surveillance efforts to identify clusters in time and space, GIS is a powerful tool as a visual aid to integrate complex data. In one view and at any desired level of geographical detail, LD patients as well as potential sources can be seen. Hypothesis can be generated and immediately tested for probability, as the GIS system can provide distances, sampling results, and seasonal information as desired. The GIS should contain detailed information on patients like their home address as a proxy for the site of infection, gender, date of birth, first day of illness, microbiology diagnosis, isolated genotype for culture-positive patients, smoking habit and presence of underlying diseases. The GIS should contain detailed information on cooling towers like the address and exact location on or at the building, the addresses and telephone numbers of the owner, the user and the maintenance company, the type of cooling tower and its capacity, a log with maintenance data, sampling results including the genotype(s) cultured from the water, the concentration in CFU/l and the disinfection regime. Also, address, type of use and maintenance details of potential sources of infection should be part of the GIS as identified during interviews with LD patients on activities during their incubation period. Available sampling results (genotype, concentration, disinfection) should be added. With informed consent of the owners, sampling results of contaminated water systems should also be part of the GIS. For certain types of installations, sampling is required by law. At present no central registration of contamination exists, whereas thousands of installations are sampled each year.

Air and soil sampling and culture for amoebae. Given that LD is an airborne disease and given the amount of controversy when implicating an installation as a potential source of infection, air sampling should be part of sampling procedures. For example, a cooling tower in Pas-de-Calais is considered to have causes LD patients at seven kilometres distance [160], whereas in a comparable outbreak in Amsterdam culture-positive patients have been within only 400 metres of the cooling tower implicated as the source. In both instances, positive air sampling results would have strengthened the microbiological and epidemiological findings. The same is true for the whirlpool-associated outbreak of chapter two of this thesis. In order to measure the spread of bacteria by aerosols, a study using an identical whirlpool was performed at the outbreak premises with *Serratia marcescens* as a marker, which was cultured on plates at varying heights and distances from the whirlpool. Unfortunately, air sampling was not part of this study.

Garden centres are among the premises most frequently visited by LD patients in their incubation period (this thesis, chapter five). Yet, rarely an environmental *Legionella* strain has been cultured from water samples during source identification efforts. It would be
worthwhile to take additional soil samples as soil has been implicated as a potential source of LD transmission.

Existing sampling procedures and culture techniques should be extended to enable demonstration of amoebae and other protozoa that play a crucial role in LD transmission.

b. Hospital control programme

Hospital associated LD comprises 15% of all LD patients who did not travel abroad in their incubation period. As these patients are clearly located, nosocomial LD is easier to prevent. Given that nosocomial LD is different from community-acquired LD with respect to the susceptible population and the virulence of the environmental Legionella strains involved, control should be more rigorous, especially for wards for transplantation patients. To date, no specific hospital control programme in the Netherlands exists. Recent experiences with a nosocomial outbreak involving nine LD patients in the Netherlands indicate that substantial progress can still be achieved here. A hospital control programme should be build around hospital microbiologists and hygienists who have experienced and successfully controlled nosocomial outbreaks.

c. Cooling tower control programme

In neighbouring countries up to 30% of all LD patients have been linked to cooling towers. After a cooling tower related outbreak in 2006 [161] these installations have received much attention. Presently in the Netherlands, municipalities are responsible for the prevention of cooling tower related outbreaks of LD. The have been advised by the Ministry of Housing, Spatial planning and the Environment (vrom) to explore their territory for the existence of cooling towers and urge owners to maintain them properly. For adequate outbreak detection, information on cooling towers is essential. A national register containing detailed information should fill this gap. Ideally, all known cooling towers should be sampled regularly. Sampling is an ultimate control tool and has the additional advantage that environmental strains can be genotyped, fluctuations in the concentration of Legionella can be monitored and disinfection efforts checked for effectiveness. Municipalities should be helped to identify cooling towers. Extensive experience in Rotterdam, Amsterdam and Haarlem has learned that these efforts consume substantial time and resources.

d. Cluster evaluation and disinfection expertise team

Outbreaks and clusters are rare events originating from rare environmental niches. Why of all saunas, hotels, camping sites, and shopping malls do patients get infected in this particular one? This is the central question that a cluster evaluation team should answer. During an outbreak or cluster this multidisciplinary team should be able to identify the most likely transmission route and find ways to control it. A lot can be learned from these unique situations. In the past, several clusters in the Netherlands have occurred that would have been controlled more rapidly had such a cluster evaluation team existed. Still, elimination
of the source of a cluster can take up to 59 days (this thesis, chapter ten). In the absence of an experienced team, precious time during outbreaks can be wasted due to lack of authority and coordination. Maybe the biggest problem in LD control is disinfection of contaminated water distribution systems and installations. In the Netherlands an authority in this domain is lacking, leading to a wide range of disinfection methods marketed and sold, without proper knowledge of is effect.

e. Contamination database

Water from thousands of installations in the Netherlands is being sampled and cultured for *Legionella* on a yearly basis. Workers in this branch indicate that up to five percent of cultures are positive for *L. pneumophila* serogroup 1. The sampling results are feedback to the owners of the installations and the positive cultures thrown away. If installation owners can be persuaded to hand over sampling results to a central database and if environmental *Legionella* strains were to be stored centrally instead of thrown away, useful information for *Legionella* control programmes would become available with little extra costs. Sampling results could be incorporated into the proposed GIS system (see paragraph 6a), speeding up response time in outbreaks and increasing the source identification percentage.

f. Research efforts

Essential information in LD control is still lacking as was described in the previous paragraphs. Research efforts should focus on transmission, virulence development, disinfection and amoebae.

A mismatch in distribution of patient-derived and environmental strains in the Netherlands has been described in chapter six of this thesis. Several studies could help identify the cause of the mismatch. After several years of data collection, the outbreak detection programme has identified 60 geographical clusters without a specific source. In cooperation with municipal health services air sampling at various spots within the boundaries of the identified clusters could pin down sources of LD infection. Apart from that, in the vicinity of different types of implicated sources of infection an air sampling study should give insight in the spreading pattern. Air sampling should include efforts to detect amoebae and other protozoa in the air.

The *ewgli AFLP* genotype 004 Lyon is common in patients, but not in environmental samples. As molecular biology tools are theoretically more sensitive than culture, a PCR for 004 Lyon should be tested on water samples gathered during source identification efforts. Positive PCR results may indicate concentrations below the culture detection level or may confirm the existence of non-culturable but viable (NCBV) *Legionella* of this genotype.

If the mismatch finding is repeated in other countries, conclusions on virulence based on relative frequency can be drawn. Apart from specific PCRs, typing based on DNA micro-array and interaction with amoebae are promising study fields.

Preliminary typing results based on DNA micro-array using a patient-derived and environmental *Legionella* strain collection indicate that both groups differ in genetic material. [162] This finding could form the basis of a virulence test, which would be useful in LD control
settings outside hospitals where decisions to disinfect or not may have substantial economic impact.

As amoebae and amoebal vesicles probably are the inoculum of LD, research effort should be directed to this field. Questions to be answered include: What amoebae are found in proven sources of LD transmission? Which types of protozoa render Legionella avirulent for human macrophages? Can disinfection strategies be build on host competition in water distribution systems? To which type of disinfectants are amoebae sensitive? Can amoebae be used as a marker for risk of transmission of LD?

Controlled studies in public health settings to evaluate the effect of disinfection are rare. However, there is a strong need for evidence-based disinfection practices in hospitals, hotels and other places where people gather.

It has been suggested that LD is a travellers disease paralleling E. coli infection, meaning that humans are exposed and naturally immunised to Legionella at home and only fall ill when exposed to Legionella in premises other than one’s own house. Such a relation is suggested in several studies involving workers in dental practices who are exposed on a daily basis to Legionella contaminated aerosols from dentist equipment inducing high antibody titres in the absence of LD. A study that would test the hypothesis would be a sero-survey in an exposed population using the environmental strain antigen to prepare a serum Legionella antibody test.

7. Conclusion

Fresh water and ground water are natural habitats for Legionella. As these raw materials are used for drinking water production, Legionella is introduced in end-users’ water distribution systems by the water mains. General measures aimed at reducing this contamination have been tried in the Netherlands in a period of nine year. During this period, the incidence of LD has risen from 11 to 26 per million inhabitants. Between 2002 and 2006, for 25% of LD patients a potential source of infection was identified by the national LD outbreak detection programme (BEL). Elimination of these patient-related sources has so far been moderately successful. To improve elimination and increase the percentage of identified potential sources, a geographical information system, additional sampling of air and soil, a cluster evaluation and disinfection expert team, and a contamination database will be supportive. Hospitals and cooling towers represent successful niches for LD transmission. These niches are well suited for specific LD control programmes, which must be rigorously executed to be effective. Implementing these measures will improve the efficiency of current LD control. However, LD will continue to be a public health problem as Legionella is ubiquitous in water, the exact mode of LD transmission is far from clarified and disinfection remains difficult to implement.

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Summary

Chapter one gives an introduction to Legionnaires’ disease (LD) covering its history, diagnosis and treatment, epidemiology, transmission and control.

In chapter two, a large outbreak of LD in Bovenkarspel, the Netherlands, involving 188 patients is described. The combination of microbiology and epidemiology studies revealed that a whirlpool spa on display was the major source of the outbreak. The outbreak spurred LD control measures by the Ministry of Housing, Spatial Planning and the Environment (VROM).

In chapter three, a national case-control study for sporadic community-acquired LD is presented. It suggests that two distinct populations of LD patients exist, based on their ability to travel long distances.

In chapter four, regional differences in LD incidence are explored on the municipal level. An association was found between high LD incidence and high price of water. The latter could be an indicator for the use of groundwater as raw material for drinking water production.

Chapter five describes the first documented cluster of LD associated with potting soil in Europe. Two of the three clustered LD patients visited a garden centre in the same municipality in the Netherlands. The finding contradicts earlier reports of Legionella free potting soil in Europe and is in line with earlier documentation of such transmission in Australia and in the United States.

In chapter six, the distributions of clinical Legionella isolates in the Netherlands and patient-related environmental Legionella strains are compared. As these distributions differ substantially suggesting virulence factors, the implications for the knowledge on LD transmission is discussed.

Chapter seven gives a review of the literature on LD diagnosis. Specifically, the place of polymerase chain reaction is elaborated on. Suggestions for future research are made.

In chapter eight it is shown that the urinary Legionella antigen test has different sensitivities for different patient categories based on the population of the Bovenkarspel outbreak. The more severe the clinical picture, the higher the sensitivity of the test.

In chapter nine three assays demonstrating antibodies against Legionella in serum are compared to the gold standard of culture or positive urinary antigen test.

Chapter ten describes the benefits of the first two years of a national LD outbreak detection program. The programme was shown to break even after six years.

In chapter eleven different aspects of LD control are discussed. In the first four paragraphs the findings of chapters two to ten are incorporated in the body of knowledge on reservoir, transmission, epidemiology and intervention of LD. The fifth paragraph describes nine years of Legionella control in the Netherlands. In paragraph six an evidence based LD control approach is presented. Central in this approach is the outbreak detection programme that can be
strengthened by adding several components: a geographical information system, sampling of air and soil, a cluster evaluation and disinfection expert team, and a contamination database. Specific LD control for hospitals and cooling towers programmes can be effective provided that they be rigorously executed. Implementation of these components will increase the efficiency of LD control. However, LD will continue to be a public health problem.
Samenvatting

Hoofdstuk één geeft een introductie over Legionella-longontsteking of Veteranen-ziekte (Legionnaires’ disease, LD), waarin de geschiedenis, diagnose en behandeling, epidemiologie, transmissie en controle besproken worden.

In hoofdstuk twee wordt een grote epidemic van LD beschreven die in Bovenkarspel ontstond en waarbij 188 patiënten betrokken waren. De combinatie van microbiologische en epidemiologische studies liet zien dat een tentoongestelde whirlpool de belangrijkste bron van de epidemic was. De epidemic leidde tot controle maatregelen door het Ministerie van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer (VROM).

In hoofdstuk drie wordt een landelijk patiënt controle onderzoek gepresenteerd naar in de open bevolking opgelopen LD. De resultaten suggereren dat twee verschillende patiënten populaties bestaan, gebaseerd op het lichamelijk vermogen om grote afstanden te reizen.

In hoofdstuk vier worden regionale verschillen in LD incidentie geexplocreerd op gemeentelijk niveau. Een associatie werd gevonden tussen hoge LD incidentie en een hoge prijs van drinkwater. Een hoge prijs zou een indicator kunnen zijn voor het gebruik van grondwater als grondstof voor waterproductie.

Hoofdstuk vijf beschrijft het eerste gedocumenteerde cluster in Europa van LD geassocieerd met potgrond. T wee van de drie geclusterde LD patiënten bezochten een tuincentrum in dezelfde Nederlandse gemeente. Deze bevinding is in tegenspraak met eerder publicaties over de afwezigheid van Legionella in Europese potgrond en is in overeenstemming met eerder beschreven soortgelijke transmissie in Australië en de Verenigde Staten.

In hoofdstuk zes worden de verdelingen van klinische Legionella isolaten in Nederland en patiënt gerelateerde Legionella stammen in de omgeving vergeleken. Deze verdelingen verschillen sterk, wat een aanwijzing is voor virulentie factoren en implicaties heeft voor de kennis van LD transmissie.

Hoofdstuk zeven geeft een overzicht van de literatuur over LD diagnostiek. Specifiek wordt stilgestaan bij de plaats van de polymerase chain reaction. Suggesties voor toekomstig onderzoek worden gemaakt.

In hoofdstuk acht wordt aangetoond dat de Legionella urine antigen test een verschillende sensitiviteit heeft voor diverse patiënten groepen gebaseerd op de populatie van de Bovenkarspel epidemic. Hoe ernstiger het klinische beeld, hoe hoger de sensitiviteit van de test.

In hoofdstuk elf worden verschillende aspecten van LD bestrijding besproken. In de eerste vier paragrafen worden de bevindingen van de hoofdstukken twee tot tien geplaatst in de LD kennis over reservoir, transmissie, epidemiologie en interventie. De vijfde paragraaf beschrijft negen jaar *Legionella* bestrijding in Nederland. In paragraaf zes wordt een evidence-based benadering van LD bestrijding gepresenteerd. Centraal in die benadering is bel, dat versterkt kan worden door er verschillende componenten aan toe te voegen: een geografisch informatie system, bemonstering van aarde en lucht, een cluster evaluatie en desinfectie team, en een besmettings database. Speciale LD bestrijdingsprogramma's voor ziekenhuizen en koeltorens kunnen effectief zijn op voorwaarde dat ze rigoureus geïmplementeerd worden. Al deze componenten samen zullen de efficiëntie van de LD bestrijding verhogen.

Echter, LD zal een openbare gezondheidsprobleem blijven.
Acknowledgments

I want to thank my supervisor Roel Coutinho for his stimulating and challenging role throughout the research period. We tried embedding my research in existing groups at RIVM, AMC, and GGD Amsterdam, but time spent on travel and meetings soon exceeded my available research hours. Although this certainly slowed the learning and writing process, it also allowed me to develop my own research group in Haarlem. Roel, thank you very much for giving appropriate advice all the time.

I am indebted to Peter Köhne and Hans Groenendijk who gave me room to develop the scientific side of my work as a public health physician at the Municipal Health Service Kennemerland. Peter, I hope our recently acquired Zon-Mw grant for psittacosis control will further put Haarlem on the public health map.

I am grateful to Ed IJzerman of the Regional Public Health Laboratory Kennemerland who is my “partner in crime” since 1996 when we first got involved in LD control and research. Ed, it has been a continuous pleasure working with you.

This thesis would not exist without the support, criticism and contributions of my colleagues at the Dutch Municipal Health Services. In 1998, you agreed to co-operate in a national case-control study to determine LD risk factors. It meant using a nine-page questionnaire ever since. I hope our newsletters, National Legionella Symposia, sampling efforts and web-based GIS provided you with sufficient feedback. Thank you very much, public health nurses and doctors, for your help over the last ten years.

A crucial role was played by pulmonologists and other clinicians who treated and diagnosed LD patients. Extra efforts were made to obtain patient material to enable culture of Legionella. I thank you for this important contribution to public health and LD control.

I owe a lot to the medical microbiologists working in hospital-based laboratories in the Netherlands. They contributed to the content of this thesis not only by sending patient materials over the last ten years, but also by responding to questionnaires on LD diagnosis. Thank you for your kind assistance throughout the study period.

I am still grateful for the privilege of working as a member of the 1999 LD outbreak investigation team at the National Institute for Public Health and the Environment (RIVM). I enjoyed the collegial atmosphere and professionalism at the epidemiology and surveillance unit (epi), where I was greatly helped by Arnold Bosman, Susan van den Hof, Hendrik Boshuizen and Marina Conyn-Van Spaendonck. Especially I want to thank Joop Schellekens, who enthusiastically guided me through the process of writing three RIVM reports and my first international article.

Also, I would like to thank Peter Speelman, Kamilla Lettinga, Annelies Verbon and Jan Prins for an inspiring post-Bovenkarspel period of research exchange and development. 
Kamilla, thank you for a pleasant collaboration; sorry Ed and I could not keep up with your pace.

Over the years, we developed an important database on LD patients and their environmental exposure. I am grateful to Wim Wannet and Kim van der Zwaluw of the Laboratory for Infectious Diseases and Perinatal Screening (lis) for their collaboration over the years to build a national Legionella strain collection. Wim and Kim, I hope together we will continue to generate interesting information from this collection.

The database on LD patients was developed over the years with the help of Jan van Wijngaarden, Anita Warris and Marja Kleinee of the Health Inspectorate, and Marianne van der Sande, Petra Brandsema and Vivian Reeskamp of the RIVM epidemiology and surveillance unit (epi). Thank you for the tedious work of collecting and verifying these data.

Last but not least I want to thank the (ex) members of the Haarlem LD study group: Anne de Vries, Ingrid Friesema, Linda Verhoef, Maarten Nijhof, Kimberly Boer, Sacha Bleeker, Sander van Kuijk, Jacob Bruin, Ruud Jansen, Stefan Boers, Max Bencini and Angela Puts who helped to collect and clean the data for this thesis. Thank you for your efforts and pleasant collaboration.
Curriculum vitae

Jeroen den Boer was born in 1961 in Eindhoven, the Netherlands. In 1980 he went to study Medicine at the Maastricht University. After his graduation, he studied Public Health at the Institute of Public Health (TNO/NIPO) in Leiden from 1988–1990, and Infectious disease control in Utrecht (SSG) from 1991–1992. The latter two degrees were obtained while working as an epidemiologist at Leiden University and as a department head at the Den Helder Municipal Health Service, respectively. From 1992–1994, he worked as a health economist for the Ministry of Foreign Affairs at the WHO country office in Nairobi, Kenya. In 1993, he co-operated with experts of the U.S. Centers of Disease Control (CDC) on the detection and control of a Yellow Fever outbreak in West-Kenya. In the evenings, he studied at the United States International University in Nairobi. Having assisted in small business development (furniture workshop and art trade) and armed with an MBA, a career-switch was explored from 1994–1996, as a marketing trainee for Astra Zeneca in Zoetermeer. In 1996, a switch-back to public health was made at the Haarlem Municipal Health Service, where he still works. In the evenings he started studying fine arts at the Sculpture department of the Gerrit Rietveld Academie in Amsterdam where he graduated in 2001. In 1999, he was asked to become principal investigator of the Bovenkarspel LD outbreak and worked for six months at the National Institute for Public Health and the Environment. After having obtained a Master degree in Epidemiology at the VU University of Amsterdam, he started working for the Public Health Laboratory Kennemerland in 2001, where he still works.
Publications

1. Yzerman EPF, Geskus RB, Lettinga KD, Schellekens JF, Peeters MF, Den Boer JW. Estimated time of seroconversion or fourfold rise in serum antibody titre in the diagnosis of Legionnaires’ disease. (submitted)

2. Verhoef LPB, Yzerman EPF, Bruin JP, Den Boer JW. Four years of Legionnaires’ disease source tracing at national level: a tool for public health. (submitted)


18. Boshuizen HC, Nagelkerke NJ, Den Boer JW, De Melker H, Schellekens JF, Peeters MF, Van Vliet H,


Figure 3a. Smoothed mean geometric immunoglobulin (Ig) M antibody titers to Legionella pneumophila of nearest 35 exhibitors in hall 3 and 4 per 63 cm² of exhibition area; confirmed and probable cases among exhibitors in halls 3 and 4. ● = confirmed case in exhibitor; ○ = probable case in exhibitor; Bu = bubblemat; W = whirlpool spa.

Figure 3b. Smoothed mean geometric IgG antibody titers to L. pneumophila of 35 exhibitors nearest to whirlpool in halls 3 and 4 per 63 cm² of exhibition area; exhibitors ill with confirmed and probable cases in halls 3 and 4. ● = confirmed case in exhibitor; ○ = probable case in exhibitor; Bu = bubblemat; W = whirlpool spa.
Figure 4a. Exhibition hall, West Frisian Flower Show, Bovenkarspeld, the Netherlands, 1999. Circles indicate locations in water-supply system where water samples were taken. PE = polyethylene.
Figure 4b. Exhibition hall, West Frisian Flower Show, Bovenkarspel, the Netherlands, 1999. Assessment of risk for *Legionella pneumophila* infection.

Figure 4c. Exhibition hall, West Frisian Flower Show, Bovenkarspel, the Netherlands, 1999. Water samples taken and culture status.
Figure 2. Legionnaires’ disease incidence rate standardised for age and gender per 100,000 inhabitants for 466 municipalities in the Netherlands.