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Detection and Epidemiologic Subtyping of *Legionella pneumophila* Using DNA-based Molecular Methods

by

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Two probverbs, one in Greek and the other in Kiswahili:

”Ο χρονος διδασκαλος των ανθρωπων εστιν” and

”Haraka, haraka haina baraka”

To my wife, Marie-Louise

”Time is the teacher of men” in Greek.

”Haste, haste is no blessing” in Kiswahili.

Image on front cover: Burst of a phagosome from an *Acanthamoeba polyphaga*, congested with *L. pneumophila*. Short rods of legionella cells possessing one polar flagellum are released from the amoeba. Staining: FITC-conjugated monclonal. By courtesy of T. Rowbotham, Leeds, UK. Photo of micrograph, 1989. Enlargement x5,800.

Abstract
Legionella pneumophila causes 2 – 6 % of hospitalised pneumonia cases. Several community- and hospital-acquired outbreaks have been reported over the years. Legionnaires´ disease (LD) or legionellosis is also a frequent cause of pneumonia amongst travellers. Conventional methods for diagnosing legionella infection include culture, detection of antibodies (serology) and urinary antigen. These methods, however, either lack full sensitivity or specificity. *Legionellae* are found in water distribution systems, especially hot water, and are able to multiply intracellularly in free living protozoa. Thus, the bacteria are transmitted to humans either by aerosols or by micro-aspiration. It is therefore important to link the patient isolate to possible environmental sources. In this thesis a nested format for polymerase chain reaction (PCR) was constructed for detecting *L. pneumophila* in clinical specimens. The method, which was highly sensitive and specific, was found to be more rapid than earlier PCR methods. In a multicentre study, conducted in collaboration with the European Working Group on Legionella Infections (EWGLI), 14 different DNA-based methods were evaluated for typing 114 related and unrelated isolates of *L. pneumophila* serogroup (sg) 1. The group favoured amplified fragment length polymorphism analysis (AFLP) because of superior reproducibility and epidemiological concordance. In a second phase of the study the use of AFLP was assessed using a standard protocol. In a third phase, 31 AFLP genotypes were designated from a European culture collection of 130 isolates and a proficiency panel was distributed among the participating laboratories. An international database of defined *L. pneumophila* genotypes was established for the first time. AFLP and to some extent macro-restriction followed by pulsed-field gel electrophoresis (PFGE) was used for the investigation of the molecular epidemiology in four Swedish nosocomial clusters. The genotypes of the causative *L. pneumophila* strains were different, but the three sg 1 genotypes were found to be widely distributed geographically in Sweden. In these studies, monoclonal antibody (MAb) 3/1-positive and negative phenotypes were found inside the same genotypic clusters. For the first time this was proven to be due to a genetic event, which was not reflected in the fingerprinting patterns. The (MAb) 3/1-negative strains thus lacked the lipopolysaccharide associated gene (*lag*-1). Also, for the first time a legionella outbreak was shown to be caused by strains belonging to two different serogroups, sg 4 and 10, that possessed a common fingerprinting pattern. MAb subtyping and genotyping methods should be used together and in conjunction with clinical and epidemiological data in investigations of Legionnaires´ disease.

Sammanfattning

Legionella pneumophila orsakar mellan 2 och 6 % av sjukhusvårdade pneumonier. Flera samhällsförvärvade och vårdrelaterade utbrott har beskrivits under åren. Legionärsjuka eller legionellos är också en vanlig orsak till pneumonier som inträffat under resa. Konventionella metoder för diagnostik omfattar odling, antikroppspåvisning (serologi) och påvisning av urinantigen. Dessa metoder saknar dock full känslighet och specificitet. Legionellabakterier återfinns i renvattensystem, särskilt i varmvatten, och kan föröka sig intracellulärt i protozoer. Smittan överförs till människor antingen genom inandade aerosoler eller genom mikroaspiration. Det är därför viktigt att kunna jämföra ett patientisolat med legionellaisolat från en möjlig smittkälla. I denna avhandling utvärderades en nestad variant av polymeraskedjereaktion (PCR) för påvisning av *L. pneumophila* i kliniska prov. Metoden var både känslig och specifik och kunde utföras på kortare tid än tidigare publicerade PCR-metoder. I en multicenterstudie med European Working Group on Legionella Infections (EWGLI) utvärderades 14 varianter av DNA-baserade metoder för typning av 114 epidemiologiskt relaterade och icke relaterade isolat. Gruppen valde att gå vidare med AFLP (amplified fragment length polymorphism) på grund av metodens goda reproducerbarhet och epidemiologiska konkordans. I en andra fas validerades AFLP-metoden med användande av en standardiserad metodbeskrivning. I en tredje fas definierades 31 AFLP-typer från en europeisk stamkollektion med 130 isolat och en kontrollpanel sändes ut till de deltagande laboratorierna. En internationell databas av definierade AFLP-typer skapades för första gången. AFLP och i viss mån pulsfältselktrofores (PFGE) användes i de fortsatta studierna av den molekylära epidemiologin vid fyra svenska vårdrelaterade anhopningar av legionellainfektioner. Genotyperna hos de skyldiga stammarna av *L. pneumophila* var olika vid de olika tillfällena men 3 serogrupp1-stammar visade stor geografisk utbredning. Utmärkande var också att isolat med olika reaktionsmönster för monoklonala antikroppar (MAb) hade samma DNA-profil. För första gången kunde det visas att detta berodde på en genetisk förändring som inte återspeglades i genotypen. Således saknades hos MAb 3/1-negativa isolat *lag*-1-genen (lipopolysaccharide associated gene). För första gången beskrevs också ett utbrott av legionärsjuka orsakad av en genotyp som kunde tillhöra två olika serogrupper, *L. pneumophila* sg 4 och 10. Både MAb- typning och genotypning bör användas vid epidemiologiska utredningar av legionärsjuka. Hänsyn måste tas till kliniska och epidemiologiska data vid bedömning av resultatet.

ORIGINAL PAPERS

This thesis is based on the following original papers:

I. S Bernander, H-S Hanson, B Johansson, and L-V von Stedingk. 1997. A nested polymerase chain reaction for detection of *Legionella pneumophila* in clinical specimens. Clin. Microbiol. Infect. 3: 95–102.

II. NK Fry, S. Alexiou-Daniel, JM Bangsborg, S. Bernander, M Castellani Pastoris, J Etienne, B Forsblom, V. Gaia, JH Helbig, D Lindsay, PC Lück, C Pelaz, SA Uldum, and TG Harrison. 1999. A multicenter evaluation of genotypic methods for the epidemiologic typing of *Legionella pneumophila* serogroup 1: Results of a pan-European study. Clin. Microbiol. Infect. 5: 462–477.

III. NK Fry, JM Bangsborg, S Bernander, J Etienne, B. Forsblom, V Gaia, P Hasenberger, D Lindsay, A Papoutsi, C Pelaz, M Struelens, SA Uldum, P Visca, and TG Harrison. 2000. Assessment of intercentre reproducibility and epidemiological concordance of *Legionella pneumophila* serogroup 1 genotyping by amplified fragment length polymorphism analysis. Eur. J. Clin. Microbiol. Infect. Dis. 19: 773–780.

IV. NK Fry, JM Bangsborg, A Bergmans, S Bernander, J. Etienne, B Franzin, V Gaia, P Hasenberger, B Baladron Jimenez, D Jonas, D Lindsay, S Mentula, A Papoutsi, M Struelens, SA Uldum, P Visca, W Wannet, and TG Harrison. 2002. Designation of the European working group on *Legionella* infection (EWGLI) amplified fragment length polymorphism types of *Legionella pneumophila* serogroup 1 and results of intercentre proficiency testing using a standard protocol. Eur. J. Clin. Microbiol. Infect. Dis. 21: 722–728.

V. S. Bernander, K. Jacobson, JH Helbig, PC Lück, and M. Lundholm. 2003. A hospital associated outbreak of Legionnaires´ disease caused by *Legionella pneumophila* serogroup 1 is characterized by stable genetic fingerprinting but variable monoclonal antibody patterns. J. Clin. Microbiol. 41: 2503–2508.

VI. J Darelid, S. Bernander, K. Jacobson, and S. Löfgren. 2003. The presence of a specific genotype of *Legionella pneumophila* serogroup 1 in a hospital and municipal water distribution system over a 12-year period. Manuscript. Submitted.

VII. S. Bernander, K. Jacobson, and M. Lundholm. 2003. A hospital associated outbreak of Legionnaires´ disease caused by *Legionella pneumophila* serogroups 4 & 10 with a common genetic fingerprinting pattern. Manuscript. Submitted.

Abbreviations

AFLP	Amplified fragment-length polymorphism analysis
BCYEα	Buffered charcoal yeast extract medium containing α-ketoglutaric acid
Cfu	Colony forming units
IFAT	Indirect fluorescent antibody test
Lag-1	Lipopolysaccharide associated gene
LD	Legionnaires´ disease
MAb	Monoclonal antibody used for serogrouping and subgrouping (subtyping) of *Legionella pneumophila*, here represented by the Dresden panel
*Not*I	A macro-restriction endonuclease, used in conjunction with PFGE
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis, performed subsequent to macro-restriction using endonucleases with infrequent cutting of the genome
*Pst*I	Restriction enzyme used in AFLP
REA	Restriction endonuclease analysis, conventional method
RFLP	Restriction fragment length polymorphism
*Sfi*I	A macro-restriction endonuclease, used in conjunction with PFGE
Sg	serogroup
SMI	Smittskyddsinstitutet (The Swedish Institute for Infectious Disease Control)
Spp	species (plural)

CONTENTS

INTRODUCTION

Background. A major outbreak of pneumonia among participants in an American Legion convention occurred in 1976 in Philadelphia, USA. (1). The subsequent investigation revealed the causative microbe to be a bacterium, which had not been identified earlier (2). This bacterium was named *Legionella pneumophila* after the Legionnaires who contracted the disease, and the infection was generally called Legionnaires´ Disease (LD), which refers to the pneumonic form of legionellosis. The source of infection was believed to be the air conditioning system at the conference hotel. However, no clear proof of the mode of transmission was obtained. Twenty-nine of 182 patients (16%) died and it was obvious that this new type of pneumonia did not respond to treatment with beta-lactam antibiotics. Subsequently, by isolating the causative bacterium, it was also possible to conduct seroepidemiological studies, which resulted in the recognition of earlier outbreaks of the same pneumonic infection (2). Over the years several new serogroups of *L. pneumophila* and other *Legionella* species (spp) have been discovered. Currently, there are 48 known species comprising 70 distinct serogroups (3). Fifteen serogroups of *L. pneumophila* have been described.

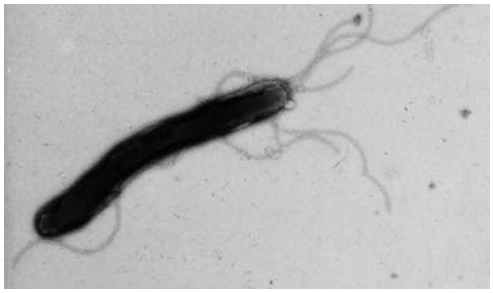


Fig. 1. Electron micrograph of *L. pneumophila* sg 1 from a culture on BCYEα. Note the presence of flagellae. Photo: K-O. Hedlund 1992.

Bacterial characteristics. *Legionellae* are gramnegative aerobic and motile rods belonging to the gamma subdivision of proteobacteria (4). They are not related to other bacteria except for *Coxiella burnetii*. The cells are 0.5 μm by 1-3 μm when cultured in the laboratory, normally having one polar flagellum in the stationary phase (Fig.1) (5). The cell wall contains long, branched hydroxy fatty acids that are unique for the family (6). The polysaccharide epitopes of the lipopolysaccharide in the cell wall are specific and can be used for serological grouping (7). *Legionellae* have a special requirement for cysteine and iron. Several amino acids are metabolised, especially serine and threonine, which are the main sources of energy (8, 9). Sodium chloride (NaCl) is inhibitory to planktonic *Legionellae* in water and stationary phase cells recovered from samples (5).

Buffered charcoal yeast extract medium (BCYEα), pH 6.9, is the medium of choice for culturing *L. pneumophila*, although it might not be the most favourable for some non-*L. pneumophila* species (10, 11). The bacterium is a strict aerobe. In the laboratory it grows equally well on BCYEα in air and in a microaerophilic environment. Reduced oxygen tension has however been shown to be favourable under certain conditions (12). The addition of CO_2 in the laboratory does not stimulate growth, except possibly for some non-*L. pneumophila* species (11). The temperature optimum is 35 - 37ºC, but some growth can also be observed in the laboratory at 42°- 43ºC (13, 14, 15). Since *Legionellae* are able to survive and retain metabolic activity in temperatures over 50ºC, it is conceivable that they might also multiply at temperatures higher than 43ºC in natural environments, e.g. in biofilms and intracellularly in amoebae (15).

Ecology. *L. pneumophila* was isolated from water already in 1979 (16). *Legionellae* are found in freshwater lakes, streams and municipal water distribution systems (17, 18, 19, 20, 21). *L. longbeachae* has also been detected in soil and potting mixes (22, 23, 24). In 1983 Rowbotham reported that *Legionellae* could multiply inside cells of Acanthamoeba (25). One amoebic cell could thus host >1000 legionella bacteria. At least 16 species of protozoa have been found to harbour or host *Legionellae* spp (3). They can also persist in amoebal cysts, which might be important for survival and dissemination in adverse environmental conditions. Although *Legionellae* grow on laboratory media, it has not been shown that they can multiply outside amoebae in natural environments (3, 26). This would otherwise seem plausible as they obviously can persist outside amoebae in biofilms.

Legionellae can be found in water distribution systems, especially in hot water plumbing and calorifiers, when temperatures are below 50°C (3, 18, 19, 20, 21). They are found growing in the biofilm that lines the inside of pipes (3, 26, 27, 28). Some parts of water distribution systems are especially prone to contain *Legionellae*, i.e. blind loops, plumbing fixtures, showers, whirlpool spas, and cooling towers (Fig. 2).

In a study conducted by the Swedish Institute of Infectious Disease Control in 1992, *L. pneumophila* was cultured from 57% of samples obtained from the hot water system in hospitals, and from 23% of similarly obtained samples in large municipal buildings (29).

Pathogenicity. *Legionellae* have developed mechanisms for invasion and multiplication in protozoan hosts in the course of evolution. The same mechanisms of intracellular invasion that they use when multiplying in amoebae seem to operate in alveolar macrophages (3, 5). One of the first pathogenicity factors to be characterised was the macrophage infectivity potentiator protein (mip) which is encoded by the *mip* gene. The protein is an enzyme, peptidyl prolyl *cis-trans* isomerase, which is exported to the bacterial surface, where it seems to influence intracellular establishment (3, 5, 30, 31). The exact mechanism and role in pathogenicity are unknown.

When amoebae or macrophages have ingested virulent legionella cells, a phagosome is established; this is surrounded by endoplasmic reticulum and becomes completely isolated from the endosomal pathway (3, 5). Initially, a merge with lysosomes is inhibited. It has been postulated that *L. pneumophila* converts to a replicative form in this protected environment, at which time it no longer expresses virulence traits but becomes acid and sodium chloride (NaCl) tolerant (5). As a consequence endosomes containing the pathogen are able to fuse with lysosomes enabling the intracellular bacteria to make use of a nutrient-rich niche which in ordinary circumstances would kill other bacteria. When the amino acid supply is depleted, the cells convert to a stationary phase form, simultaneously developing features that are needed for transmission to a new phagocyte. Legionella cells released from eucaryotic cells are short, thick, and highly motile (Fig 3 and image on front cover). They thus exist in nature in two phases. In this system a number of factors are implicated including type II and IV secretion, acquisition of iron, pore-forming toxins, and induction of apoptosis in the host cell. The intracellular establishment and trafficking of

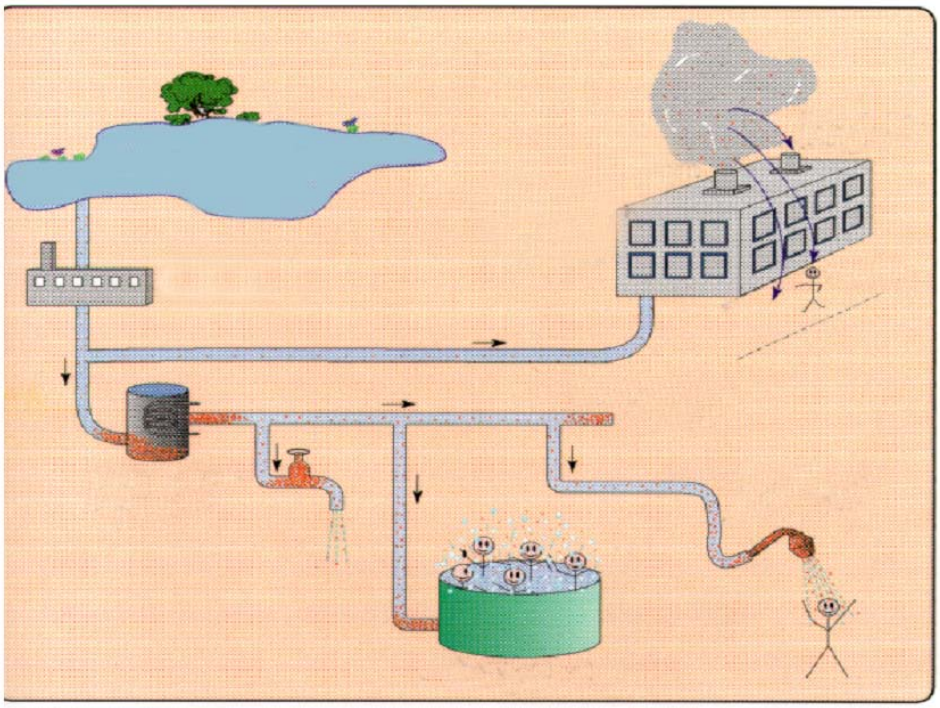


Fig. 2. Sites of possible legionella multiplication in a water distribution system. Areas having an increased density of red dots, such as calorifiers, whirlpools, plumbing fixtures and cooling towers are liable to support growth of *Legionellae.* Note the possibility of aerosols reaching a passer-by on the street from a cooling tower.

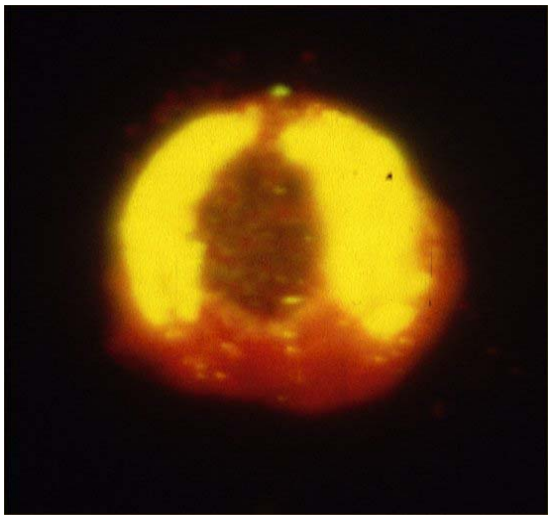


Fig. 3. Two phagosomes inside a cell of *Acanthamoeba polyphaga* congested with *L. pneumophila*, stained by FITC-conjugated monoclonal antibodies. The front cover image shows such a phagosome bursting, releasing motile legionella cells. By courtesy of T. Rowbotham, Leeds, UK. Photo 1989.

Legionellae are believed to be regulated by the *dot/icm* (defective organelle trafficking/intracellular multiplication) gene complex, which encodes the substances involved in type IV secretion. *Legionellae* also produce extracellular cytotoxins. Experimental work indicates that virulence is significantly reduced when the incubation temperature of a cultured inoculum is reduced from 37°C to 24°C (32).

Clinical features. Legionellosis usually presents as pneumonia (Legionnaires´ Disease, LD), but opportunistic infections in other organs occur in rare cases (1, 33, 34, 35, 36, 37). The attack rate is low, not exceeding 1 %, even though large numbers of legionella bacteria might contaminate the involved water system (38, 39). For instance, this rate was 0.6% in the Stafford outbreak in 1985 and between 0.11 and 0.56% in the flower show outbreak in the Netherlands 1999. An incubation period of 2 to12 days (median time 5 days) was reported in connection with the Stafford outbreak (38) and between 2 and 19 days (median time 7 days) at the flower show. Sixteen percent had an incubation period exceeding 10 days in the latter outbreak (39).

Symptoms and signs of legionella pneumonia are often, though not always, severe, including fever >39ºC, hyponatremia, dyspnoea, alveolar filling infiltrates, diarrhoea, myalgia, and confusion (33, 34, 40). There is no single clinical sign or symptom that is specific for legionellosis; a combination of them may however indicate possible LD, especially in outbreak situations (3, 33, 34). Some investigators have proposed a subset of factors that might predict legionella pneumonia, such as fever >39ºC, previous β-lactam therapy, male sex, gastrointestinal symptoms, alcohol abuse, heavy smoking and myalgia (34). Cavitation in the lung in immunocompromised patients, especially those treated with glucocorticosteroids, is apparently often linked to legionella pneumonia (33). Concomitant infection with other pulmonary pathogens occur and may add to diagnostic confusion (33, 41).

Mild disease undoubtedly occurs. However, the incidence of legionella infections with few or no symptoms is difficult to assess. Physicians usually only test for legionella in severe cases, especially in connection with outbreak scenarios, travel-associated disease, or lack of response to previous antibiotic therapy (34). Some serologic surveys indicate a significantly raised level of antibody titres against legionella antigens in populations that have only mild symptoms or no symptoms of respiratory disease during an

ongoing overt outbreak (42, 43, 44). A seroepidemiological study related to the Västerås outbreak in Sweden 1979 showed that antibody titres for *L. pneumophila* sg 1 amongst blood donors were significantly higher in the outbreak city than in neighbouring towns and in Sweden in general (unpublished results, 45, 46).

Legionella spp also cause a self-limited disease called Pontiac fever (non-pneumonic legionellosis) (47, 48). This illness is flue-like and symptoms usually subside without antibiotic therapy in a few days. Non-pneumonic legionellosis is associated with a high attack rate amongst individuals who have been in contact with the infectious source, which is contrary to LD pneumonia. Several legionella species can cause the disease, including non-*L. pneumophila* species such as *L. micdadei* (49, 50).

Diagnostic tools. Traditionally, the diagnosis of legionella infection is based on culture, antigen detection and detection of legionella antibody in serum (serology) (3, 33, 51). Although *culture* is 100% specific, only 10 - 20% of patients are sampled in surveillance studies (51, 52, 53). There are several reasons for this, among them the erroneous belief that culture is very slow and insensitive. Furthermore, fewer than 50% of LD patients do not produce sputum, and specimens are thus difficult to obtain (54). However, a well-controlled culture method for *Legionella* is still a "golden" standard and should be used if samples from the lower respiratory tract are possible to obtain (3). Experienced laboratories may attain a sensitivity of 80% (3, 33, 54, 55). Bronchoscopic samples are likely to give a greater diagnostic yield than do sputum samples; these, however, should not be rejected because of low numbers of leukocytes. Obtaining clinical isolates by culture is important since other laboratory methods, especially urinary antigen detection, mainly detect *L. pneumophila* sg 1 infections while the non-sg 1 infections are missed (51, 56). A further reason for culture is obtaining a clinical isolate that might be compared to isolates in the environment of the patient.

L. pneumophila, being a fastidious organism, must be cultured on specially designed media. Furthermore, aliquots of clinical specimens, e.g. sputum and bronchoscopic material, should be subject to acid or heat treatment (or both) in order to suppress commensal flora that is always present in this area (11, 57, 58). The standard basic medium used for culturing is BCYEα, supplemented with α-ketoglutarate (59). Making the medium selective by the addition of antimicrobial agents is necessary for a good yield in clinical

testing (11). Most *L. pneumophila* produce colonies on these media within 3 – 5 days at 35 - 37°C, though some non-*L. pneumophila* spp might need a longer incubation period. Plates are screened in a low power microscope in order to visualise the cut glass appearance of legionella colonies, which are then tested for cysteine requirement. By using specific antibody reagents, species identification can be performed either by agglutination or by fluorescent antibody technique (FA). Sequencing of the *mip* gene, which is found in all *Legionella* spp, is a novel approach for identification at the species level (60). A positive culture for *Legionellae* is considered sufficient for a confirmed diagnosis of legionella infection (3, 61).

Antigen detection of *Legionella* by direct staining on slides using a fluorescent antibody technique (DFA) has been used for rapid diagnosis of LD. Reagents based on monoclonal antibodies are preferred for the sake of specificity (>95%). Further, the use of this method requires much experience (3, 33, 54). Even in the hands of experienced staff, sensitivity ranges between 25% and 70%, usually about 50%. Positive DFA results are only considered presumptive for a diagnosis of LD (61).

About 80% of patients with *L. pneumophila* sg 1 infection excrete a polysaccharide *urinary antigen*, which may be detected by enzyme-linked immunoassay, and more recently, an immunochromatographic method (62, 63, 64). Both tests are rapid and can provide results within 15 min. to 2-3 h. However, these commercially available tests are only reliable for the detection of *L. pneumophila* sg1, although some cross-reactions in cases of non-sg 1 infections may occur (56, 65, 66). There are also some concerns about the sensitivity of urinary antigen test in the early stage of disease (67). Legionella urinary antigen testing should be repeated in suspected cases, if an initial assay is negative. The overall sensitivity for *L. pneumophila* sg 1 infections is 70 – 90% and specificity >99% (54). Sensitivity might be greater in patients infected by *L. pneumophila* sg 1 belonging to the Pontiac subgroups (MAb 3/1-positive strains tested by the Dresden panel, 56) than in patients infected by MAb 3/1-negative strains. A positive test for legionella urinary antigen is considered to be sufficient for a confirmed diagnosis of LD (61, 67, 68), and is thus a valuable tool for rapid diagnosis.

Serology has been the main diagnostic tool in earlier studies (51). Detection of antibodies against *Legionella spp* is a valuable epidemiological tool but has little influence on urgent clinical decisions (54). The standard reference test is indirect immunofluorescence. Only a significant fourfold titre change

or seroconversion between two samples can be relied upon. A rise in titre may however take several weeks and is in some cases not detected at all. A proportion of patients with proven legionella infection do not have detectable seroconversion (69). A rise in IgM antibody titre might be observed at an early stage subsequent to onset of symptoms. However, since these antibodies can persist for long periods, such a finding is unreliable. The sensitivity of serological testing is 60 – 80%, and the specificity in *L. pneumphila* sg 1 infections is approximately 95% (54). Serology in *L. pneumophila* non-sg1 infections is generally considered to be less reliable than in sg 1 infections. Notably, a diagnosis of non-pneumonic legionellosis (Pontiac fever) relies only on clinical picture and serological testing, since the causative organism cannot be cultured. A confirmed case of LD, based on serology alone, requires a significant, fourfold rise in antibody titre against *L. pneumophila* (61). Single high titres may however be of value in outbreak investigations.

Table 1. Target genes used in PCR for *Legionellae.* References are given in the introductory chapter and in paper I. Also see reference 3.

Gene	**Lengths of amplified fragments, bp**	**Specificity for Legionella spp.**
16S rDNA	106, 245, 375, 386	Variable, usually broad
5S rDNA	100 - 110	All species
mip	124, 131, 168, 600, 630, 649	Species specific e.g. *L. pneumophila*
Other	800	

DNA amplification techniques make it possible to detect minute amounts of legionella DNA and also provide a possibility of rapid diagnosis of legionella pneumonia (3, 54). The polymerase chain reaction (PCR) has repeatedly been shown to have a sensitivity equal to or greater than culture (3, 54). The commonest target genes are the *mip*, 5S rDNA, and 16S rDNA (Table 1) (3). Thus, PCR-based testing is potentially the test of choice for respiratory tract specimens. In some other studies DNA has been detected in serum and urine (70, 71, 72, 73, 7 4). However, the role of such testing is not clear and further evaluation is needed (54). Problems seem to arise with regard to specificity in testing urine specimens. PCR for *Legionella* is currently only available in a limited number of laboratories that use a variety of in-house assays. Real-time PCR methods have been evaluated recently by several groups showing promising results (75, 76, 77). Apart

from being more rapid, these methods have the potential of decreasing workload by automation at the laboratory. Positive results using DNA amplification methods are currently only considered to be presumptive for LD diagnosis (61).

Use of diagnostic methods. The utilisation of available diagnostic tests has changed considerably over time. In one study, the increased use of the urinary antigen test (0% - 69%) and the diminishing use of culture and/or serology changed significantly between 1980 and 1998 (51). During the same period the case fatality rate declined significantly from 34% to 12%. This change in the pattern of diagnosis and mortality may be interpreted in different ways. Firstly, using urinary antigen makes diagnosis of LD more rapid; secondly, mild cases of the disease might be diagnosed more frequently because of higher test sensitivity. Third, independently of the diagnostic methods used, therapy has become more effective over time due to more frequent use of new antimicrobial agents such as azalides and quinolones.

The frequent use of urinary antigen tests has resulted in the preferential diagnosis of *L. pneumophila* sg 1 infections, which are therefore more pronounced in surveillance studies (51, 52, 56). Infections caused by *L. pneumophila* non-sg 1 and other *Legionellae* are missed since only culture and serology may diagnose them. In suspected urgent LD cases the diagnostic strategy should accordingly include both urinary antigen test and culture from the lower respiratory tract (3, 51, 54).

Treatment. Only antibiotics that penetrate into macrophages may be used for treating legionella pneumonia, since the causative bacterium multiplies intracellularly (78, 79, 80). Thus β-lactams and aminoglycosides are ineffective. Historically, since the first described outbreak, a macrolide, erythromycin, has been the drug of choice in treatment of legionella pneumonia. However, the case fatality in immunocompromised patients with legionellosis has remained high despite this treatment (79). While erythromycin is still widely used, experimental studies show that azithromycin, an azalide, and new quinolones are more effective in-vitro and in eradicating *Legionellae* from intracellular compartments (79, 81, 82, 83, 84).

Mode of transmission. It is generally believed that *Legionellae* infect humans as a result of inhalation of aerosols (5, 85, 86, 87). This concept is supported by the fact that patients in case-control studies have been infected by merely passing outside buildings, where the source of the causative legionella strain has been found (85, 86, 87). Some individuals have been at a distance of several 100 m from the source. A whirlpool spa, a shower or possibly a humidifier may also create an infectious aerosol (88, 89, 90). In the large outbreak at a flower show in the Netherlands, the proximity of individual persons to the source, a whirlpool spa, was correlated to the risk of acquiring LD (39, 42).

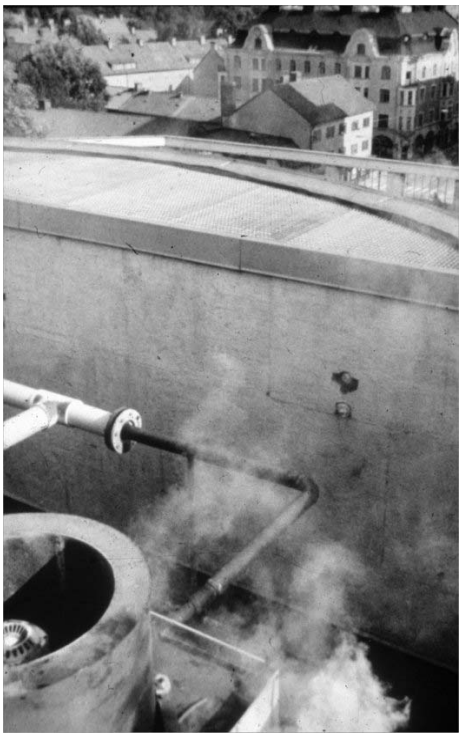


Fig. 4. A cooling tower associated with the 1979 outbreak in Västerås. Note the proximity of the air intake on the roof of the department store. Smoke simulating an aerosol is immediately drawn down into the intake. Photograph Olle Järnmark, 1980.

The mode of transmission has, however, been a matter of controversy and aspiration has been suggested by some authors to be more important than inhalation (91, 92, 93). This is likely in a hospital environment with immunocompromised patients who have poor respiratory tract reflexes. In such cases, drinking water contaminated with *Legionella* is a potential risk.

In the original outbreak in Philadelphia in 1976, drinking water at the hotel was the only statistically significant relationship that could be associated with disease in a case-control study (1). Inhalation of aerosols is, however, probably an important factor (87).

Cooling towers, evaporative condensers, mist machines, whirlpool spas and showers are among the causes of transmissions. Air conditioning devices in general, however, are not a source of infection, as is sometimes assumed by public media. An air conditioning system is only hazardous if a cooling tower or evaporative condenser is positioned in such a way that the generated aerosol can pass into the air intake of a building or be transmitted directly to a passer-by or bystander (85, 87) (Fig. 2 and 4).

Inhalation or microaspiration of amoebae could be a potential risk, since one single amoeba might harbour >1000 legionella cells (25). Furthermore, intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances the virulence of *L. pneumophila* (3, 5, 94). Thus, it is conceivable that infection in humans may require the presence of both *Legionellae* and an amoebal host (5). This could explain why the attack rate in LD outbreaks is low, despite the presence of *Legionellae* in the plumbing system. In one study occurrence of LD cases was related to the presence of both protozoa and *L. pneumophila* (89).

Epidemiology. *Legionella* cause approximately 4% - 6% of hospitalised cases of pneumonia in Sweden (95, 96) although geographical variations probably occur. Between 74 and 86 cases of confirmed or presumptive LD were reported by the Swedish Institute of Infectious Disease Control (SMI) to the European Working Group on Legionella Infections (EWGLI) in 1999 - 2002 (B. De Jong, personal communication). The case fatality rate was 3.6% (2002) - 16.2% (1999). The reported yearly incidence of LD was thus 8,3 – 9.7 cases per million in the Swedish population. In 1999 the rate per million in Europe as a whole was 5.4 cases (97). However, some countries reported as many as 16 – 20 cases per million in the same year. It is generally believed that the true incidence of LD in communities is seriously underestimated. In a prospective study in 1991 of community-acquired pneumonia in Ohio, USA, Marston et al. found an LD incidence of 7 cases per 100000 (70 per million) people, the equivalent of 3% of the total number of hospitalised cases (98). If this incidence is projected on the total US population, it would mean between 8,000 and 18,000 cases of legionella

pneumonia each year. In fact, only 300 – 400 cases per year were reported to Centers for Disease Control and Prevention (CDC) (51). In a German prospective study LD was diagnosed in 5% of community–acquired pneumonia and in 6.8% of hospital-acquired pneumonia (99). In a similar study on hospital-acquired pneumonia in USA, 14.3% of the total number of cases were LD (100).

Since there is an inherent bias for *L. pneumophila* sg 1 infections in surveillance studies, it is difficult to assess the true incidence of other legionella infections (51). In the Swedish reports to EWGLI (1999 – 2002) mentioned above, between 31% and 66% of patients with legionellosis had been diagnosed by urinary antigen. In 2002, however, 80.6% of the total number of European cases reported to EWGLI were diagnosed by the urinary antigen test. The corresponding figure in the US in 1998 was 70% (51). In a study of 33 cases in Sweden during the latter part of the 1980s, 58% were caused by *L. pneumophila* sg 1, 27% by *L. pneumophila* non-sg 1 and 15% by other *Legionella* species (95). Looking at clinical isolates from culture-proven cases, Helbig et al. found *L. pneumophila* sg 1 in 83.7% and *L. pneumophila* non-sg 1 in 16.3% of community-acquired cases (52). However, in Scandinavia the corresponding figures were 65.5% and 34.5%. *L. pneumophila* non-sg 1 constituted 71.2% of nosocomial patient isolates in Scandinavia compared to 35.9% in Europe as a whole. Thus, more non-sg 1 strains were isolated in the northern parts of Europe than in the south. Whether this reflects a true epidemiological situation or is an artefact, is open to question. Only 17% of diagnosed cases reported to EWGLI in 1999 were based on culture of the causative microorganism. It is clear, however, that *L. pneumophila* non-sg 1strains are frequently found to cause LD, if looked for (52, 97).

Table 2. Major outbreaks of Legionnnaires´ disease reported in Sweden. The three earliest outbreaks are found in references no. 102, 103, and 104 respectively. The number in brackets is the sum of cases from the outbreak and sporadic cases caused by the same strain.

Year	Place	No. of cases	Category
1979	Västerås	68	Community
1983	Huddinge	5	Nosocomial
1990	Värnamo	31	Nosocomial
1993	Uppsala	8 (12)	Nosocomial
1996	Uppsala	18	Nosocomial

Legionellosis may occur sporadically or epidemically (3,97). Sometimes infections are said to occur endemically. These distinctions are not clear-cut since both sporadic and epidemic infections might be caused by endemic legionella strains (101). Furthermore, it is conceivable that sporadic infections are sometimes part of an unrecognised outbreak. Epidemic clusters of LD may be either community-acquired or nosocomial (hospital-acquired) (3, 97). Three outbreaks of LD, one community-associated and two nosocomial (102, 103, 104), and one of Pontiac fever (50), have been reported in Sweden. Two other nosocomial clusters are reported in this thesis (Table 2). Community-acquired clusters are sometimes related to hotels, giving rise to travel-associated pneumonia. The European Working Group on Legionella Infections (EWGLI) is conducting a European surveillance scheme on legionellosis (www.ewgli.org). In 1999 20% of 2,136 LD cases reported to EWGLI were travel-associated and 9.1% hospital-associated (97). In Sweden roughly 50% of reported cases are travel-associated (B. De Jong, SMI, personal communication).

Environmental investigations. *Legionellae* are ubiquitous in aquatic habitats and in water distribution systems (Fig. 2) (16, 17, 18, 19, 20). The infecting legionella bacteria are thus mostly acquired from water, notably potable water, either by inhalation of aerosols or by microaspiration (87, 92). Thermal baths have also been found to be a source of infection with *L. pneumophila* (105) and potting mixes have been shown to be an important source for *L. longbeachae* infection (23). It is therefore important to relate patient strains to environmental isolates in epidemiological investigations, whether it be an outbreak or a sporadic infection.

Legionellae are cultured from water, especially hot water systems in buildings, and from the biofilm of plumbing fixtures, e.g. taps, showerheads etc. (18, 19, 20, 21, 29). Currently, concentration of the sample by filtration of water through a black filter (pore size 0.45 μm) is the method of choice (106, 107). The filter is placed on the growth medium and can then be screened in a low power microscope for colonies with typical cut glass appearance. The culture of *Legionellae* from water distribution systems is performed much the same way as has been described above for clinical samples (10, 107). Acid treatment of samples and culture on selective media is of prime importance.

Epidemiological typing and molecular epidemiology. Several different methods have been used for typing *L. pneumophila*, particularly *L. pneumophila* sg 1, which is the most common serogroup causing legionella infections. Subgrouping (subtyping) with monoclonal antibodies (MAbs) directed against lipopolysaccharide epitopes on the surface of legionella cells has been used for several years as a rapid procedure and, in recent years, as an adjunct to select legionella strains for genotyping (7, 108, 109). The MAb panels, notably the international panel and the Dresden panel (Fig.5), have been useful for subgrouping *L. pneumophila* sg 1 strains and also for detecting strains expressing the virulence-associated epitope recognised by the MAb 3/1 in the Dresden panel (MAb 2 in the International panel) (110, 111). MAb 3/1-positive strains are more hydrophobic than MAb 3/1-negative strains (111). MAbs may also be used for subgrouping non-*L. pneumophila* sg 1 strains (7). MAb typing is a rapid technique that produces stable and reproducible typing patterns. Discrimination is however unsatisfactory, since only about 10 phenons of *L. pneumophila* serogroup 1 strains are readily distinguished.

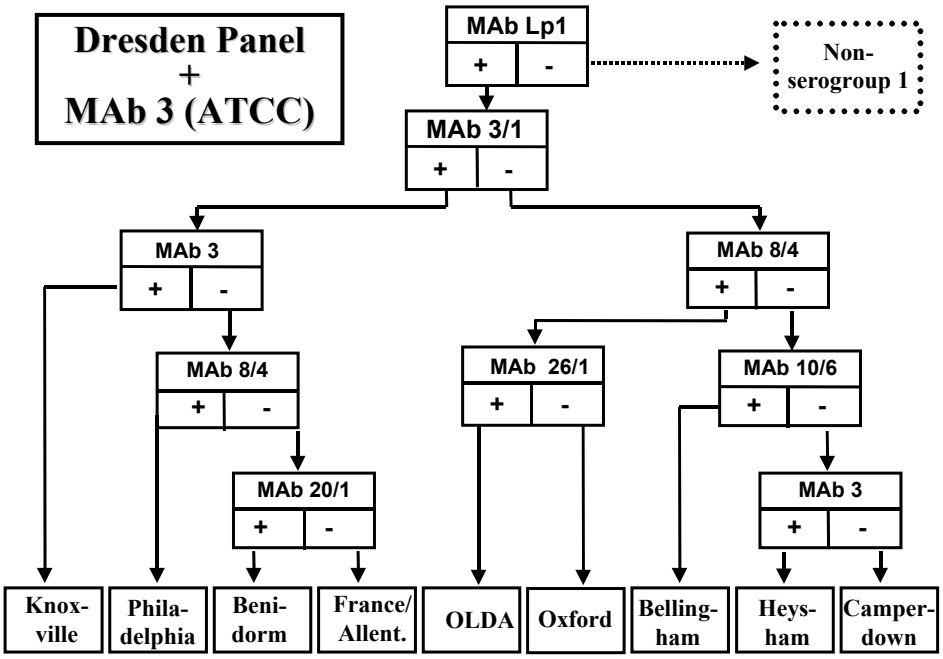


Fig. 5. Flowchart for monoclonal subgrouping (subtyping) of *L. pneumophila* sg 1 using the Dresden panel plus MAb 3 from ATCC.

Genotypic methods have been developed for use in epidemiological investigations of which some are reviewed in this thesis. Currently, the main methods used are macro-restriction with subsequent pulsed-field gel electrophoresis (PFGE) (39, 112, 113, 114, 115, 116), amplified fragment length polymorphism analysis (AFLP) (116, 117), and PCR methods using arbitrary primers (39, 112, 118, 119). All three methods have been effective tools in *L. pneumophila* sg 1 typing. Guidelines for the appropriate use and interpretation of genotyping methods have been reviewed in recent years (120, 121, 122).

The choice of genotyping method depends on the specific infection control scenario and on available resources (122). In macro-restriction, on the one hand, >90% of the chromosome is scanned but restriction enzyme cutting is infrequent and the electrophoresed fragments are large. Thus minor genetic changes may go undetected. PCR methods, on the other hand, generally cover <10% of the genome. Fragments are usually much smaller making it possible to detect small genetic changes affecting their size. This explains the discrepency that is sometimes observed between the two types of methods (122).

A new, recent approach to genotyping is known as sequence-based typing (SBT), which is similar to multilocus sequence typing (MLST). MLST uses housekeeping genes that are not under selective pressure, whereas SBT is based on sequencing any set of suitable genes showing nucleotide variability. Advantages include portability between laboratories and a consequent ease of establishing databases. Gaia et al found SBT of *L. pneumophila* serogroup 1 using only three gene loci to be epidemiologicallly concordant and highly discriminatory, thus having a potential of becoming a future “gold standard” for epidemiological typing (123).

AIMS OF THE THESIS (AIMS OF STUDY)

- To construct and evaluate a DNA-based amplification method for diagnosis of legionella pneumonia.

- To evaluate molecular methods for use in epidemiologic typing and to establish a database containing molecularly defined *Legionella pneumophila* serogroup 1 genotypes in a multicentre study.

- To describe the molecular epidemiology of three *Legionella* outbreaks in Sweden.

Diagnosis of Legionnaires´ disease by polymerase chain reaction (PCR). **Paper I:** A nested polymerase chain reaction for detection of *Legionella pneumophila* in clinical specimens.

Introduction

Methods based on amplification of specific target genes provide a possibility of detecting minute amounts of DNA in clinical specimens. The sensitivity and relative rapidity of these methods give laboratories the opportunity of an ideal test. Currently there are few evaluated commercial systems available for legionella detection (54). A summary of different target genes used is given in Table 1. The gene loci are essentially 5S rDNA, 16S rDNA, and *mip.* Earlier studies used the *mip* together with the 5S r RNA gene, since it was found that the *mip* primers did not react equally well with both *L. pneumophila* and other *Legionellae* (124, 125, 126, 127). The commercial EnviroAmp kit, which was designed to detect *Legionellae* in water and which contained both *mip* and 5S rDNA primers, was used for clinical specimens in two studies (125, 126). This kit is not available now. In an early study, in 1992, Jaulhac et al. used the *mip* gene alone and found that their method detected all serogroups of *L. pneumophila,* but only two other *Legionella* species (128). In that study *L. pneumophila* could be detected at a level of 10-20 cfu/mL. Lindsay et al. later used the *mip* for detecting legionella DNA in serum (72). Subsequently, primers specific for the 16S rRNA gene, which seemed to detect a broader range of *Legionella* spp, were also used (129, 130). Approximately 100 base-pair 5S rRNA gene sequences have been used for detection of *Legionella* DNA in urine (70, 71, 73). The results from these studies need, however, to be evaluated further.

The above-mentioned PCR methods required a final hybridisation step with an internal probe in order to achieve adequate sensitivity and specificity. Another format for PCR performs the reaction in two steps with two sets of primers in a nested configuration. Such a procedure decreases the risk of amplifying non-specific sequences and the final hybridisation step may, therefore, be omitted. In the present study a nested PCR method was developed for detecting *L. pneumophila* in clinical samples. Preliminary data on the principle of the method and its application to clinical specimens were given at the 7th meeting of the European Working Group on Legionella Infections (EWGLI) in 1992 and at the 9th meeting of EWGLI in 1994.

Material and methods

Strains: Seventeen *Legionella* strains from ATCC were tested in pure culture, namely *L. pneumophila* sg 1 – 10, *L. micdadei*, *L. bozemanii* sg 1 and 2, *L. longbeachae* sg 1 and 2, *L.dumoffii*, and *L. gormannii*. In addition 12 clinical isolates consisting of *L. pneumophila* sg 1, sg 3, sg 5, and sg 6 and one *L. bozmanii* sg 2 were tested. Seventeen non-*Legionella* bacterial species, including *Bacillus* spp., *Escherichia coli, Haemophilus influenzae, H. parainfluenzae, Klebsiella pneumoniae, Moraxella (Branhamella) catarrhalis, Mycobacterium tuberculosis, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae,* and *S. pyogenes* were tested as controls.

Patients: PCR was performed on 25 specimens from the respiratory tract of 23 patients who proved positive in routine culture and/or urinary antigen test. The interval between initiation of adequate macrolide therapy and sampling of the patient was recorded. The control group consisted of 23 samples from consecutively tested patients, who suffered from proven clinical pneumonia of other causes. A further 10 samples were obtained from bronchoalveolar lavage fluid or bronchial aspirates of 9 consecutively tested patients suffering from pneumonia that had not responded to antimicrobial therapy. These patients were all serologically tested by indirect immunofluorescence antibody test (IFAT) in addition to other legionella tests.

Culture and urinary antigen detection: Culture of respiratory tract specimens was performed on non-selective and selective BCYEα medium on both undiluted and acid-treated diluted specimens (10, 11). Urinary antigen was detected using Equate Legionella Urinary Antigen RIA kit (Binax, Portland, Me, USA). Suspected *Legionellae* were identified by cysteine requirement and serogrouping using polyclonal sera.

Preparation of DNA for PCR: Suspensions of pure bacterial cultures were heat lysed. DNA in clinical samples was prepared using two methods:

1. A conventional method using extraction with phenol/chloroform/isoamyl alcohol subsequent to treatment with a lysis buffer containing proteinase K.

2. A commercially available kit, the QIAamp Tissue kit (QIAGEN, GmbH, Hilden, Germany). This method is based on the principle of adsorbing nucleic acids onto a silica gel membrane in the presence of chaotropic salts.

Primers: The external primers used were identical to those used by Mahubani et al., reported to be specific for *L. pneumophila* all serogroups, amplifying a 649-bp product (131). The sequence was: 5′-GCT ACA GAC AAG GAT AAG TTG-3′ (920-940) and 5′-GTT TTG TAT GAC TTT AAT TCA-3′ (1568-1548). The internal primers were specially chosen for the study, giving rise to a 403-bp product. The sequence was: 5′-CAT GCA AGA CGC TAT GAG TG-3′ (1024-1040) and 5′-CAA GTT GAT CCA GCT GGC AT-3′ (1423-1392).

PCR amplification: Briefly, following preparation of a PCR mixture, the first step of the nested PCR, with the prepared DNA and external primers added, was performed with 30 cycles in a DNA Thermal Cycler (Perkin Elmer Cetus). Each cycle consisted of denaturation at 95°C for 30 s (5 min in the first cycle), annealing at 55°C for 30 s, and extension at 72°C for 1 min (5 min in the last cycle). The second step with the internal primers was run under the same conditions as in the first step except that 5μL of amplified DNA from the first step diluted 1:10 was used as a template. Ten μL of the amplified product from the second step was then run on a 3% agarose gel, stained with ethidium bromide and photographed under UV light. Lanes with a distinct band at the exact level of the positive control band (403 bp) were regarded as positive. Two amplificates, one from a *L. pneumophila* sg 1 strain (ATCC 33152) and one from a clinical isolate of *L. pneumophila* sg 6, were sequenced to check specificity.

Results

Lysates of *L. pneumophila* produced distinct bands (Fig 6). Other *Legionella* species proved negative except for the clinical *L. bozemanii* sg 2 strain that showed a weak band. The sensitivity was in the range of 10 – 50 CFU of *Legionella* in the sample volume from the original material.

PCR using the *mip* gene

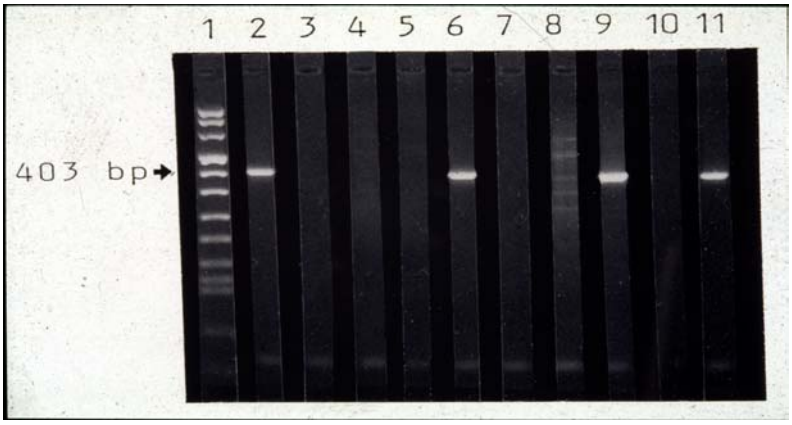


Fig 6. Agarose gel electrophoresis of nested PCR products. Lane 1: DNA molecular weight marker VIII (Boehringer Mannheim GmbH, Mannheim, Germany). Lanes 2, 4 and 6: three aliquots from the same DNA preparation of a sputum sample from a patient with legionella infection. Lanes 3, 5, 7 and 10 are negative controls. Lane 8. A negative sputum. Lane 9: The same sample as in lane 8, but with 50 Cfu of *L. pneumophila* sg 1 (ATCC 33152) added. Lane 11: positive control of heat-lysed *L. pneumophila* sg 1 (ATCC 33152).

The results of testing 25 samples from 23 patients with confirmed legionella infection are given in Table 3. Ten of 13 culture and PCR-positive specimens were collected <2 days after initiation of therapy. However, 7 of 9 culture-negative but urinary antigen positive specimens, that were obtained 3 – 7 days subsequent to initiation of therapy, were PCR-positive. The only culture-positive specimen that was PCR-negative grew one colony on each of two plates. Five clinical specimens proved PCR positive when DNA was prepared by the QIAamp method instead of by the conventional phenol/choloroform extraction. Also, if three aliquots of the same DNA preparation were tested from specimens of patients that were culture negative but urinary antigen positive, one or two of these could prove to be PCR positive (Fig. 6). Thus, in some cases there was an uneven distribution of DNA template in samples with few bacteria. The 33 specimens from two

control groups were negative in PCR. Sequencing 250 bp of an amplificate showed full agreement with previously published sequences of the *mip* gene.

Table 3. Results of PCR testing on patients with legionella pneumonia in relation to previous antibiotic treatment: 25 samples from 23 cases.

	No. of samples		
	eatment		
Test results	**≤ 2 days**	**3 – 7 days**	**Total**
Culture+ / PCR +	10	2	13[a]
Culture +/ PCR -	1	0	1
Culture -/ PCR +	2	7	9[b]
Culture -/ PCR -	1	1	2[c]
Total	14	10	25[a]

[a] No information about therapy for one patient.
[b] Includes one bronchial aspirate overgrown by *Serratia* sp. and one pleural fluid. Both patients proved culture positive in an earlier sample. Seven specimens are from patients with positive urinary antigen test.
[c] Both specimens from patients with a positive urinary antigen test, of whom one was positive in serology (IFAT) for *L. pneumophila* sg 4.

Discussion

Methods used for diagnosis of Legionnaires´ disease lack sufficient sensitivity and sometimes also specificity. (3, 33, 54, 55). Culture is specific but sensitivity varies and at least 3 – 5 days are needed to obtain growth of the *L. pneumophila.* Serology is not of value in an acute phase of the disease and serologic methods have not been satisfactorily validated in the diagnosis of *L. pneumophila* non-sg 1 infections. Urinary antigen is primarily specific for *L. pneumophila* sg 1.

PCR methods have the potential of being very sensitive and specific. However, many laboratories refrain from using nested formats because of an increased risk of obtaining false-positive results due to cross-contamination. No such problems were observed in this study, since the different steps were strictly separated physically. By using a nested format there was no need of a final hybridisation step with an internal probe. Further, use of the QIAamp method increased sensitivity and rapidity. The method allowed for detection of 10 – 50 CFU in original samples, which should be adequate unless hampered by the presence of inhibitors in the clinical specimens. It is prudent to perform PCR on more than one aliquot of each sample preparation since legionella bacteria are randomly distributed in specimens; a clinical specimen containing only few bacteria may thus turn out negative.

The target sequence used in this study was relatively long, 649 bp, and, in general, shorter sequences are preferable. Studies, detecting DNA in urine, have used sequences of about 100 bp from the 5S rRNA gene (70, 71, 72). More recently, the nested PCR format from this study was tested on urine specimens, which were strongly positive in the legionella urinary antigen test. None became positive (unpublished results). Possibly, only short sequences are excreted in urine and are thus detectable. However, DNA has been detected in serum from a few anecdotal cases (unpublished results).

Because legionellosis is rare it was not possible to conduct a prospective study on consecutive cases. Specificity was probably adequate since there were no positive results in the control groups. However only *L. pneumophila* was detected and only serogroups 1 – 10 were tested. Experience of the method in routine clinical use has shown that it also detects other serogroups of *L. pneumophila* (unpublished data). The narrow specificity might be a disadvantage but still covers approximately 90% of legionella infections. Notably, seven of nine culture-negative patients who had been treated with macrolides for 3 – 7 days were PCR-positive, which is an important advantage with the PCR technique. All culture-positive samples were positive in PCR except for one, the result of which was probably incidental to a random distribution of a low number of bacteria in the sample.

Summary: A nested PCR was designed for the detection of *L. pneumophila* which was as sensitive and specific as other previously described PCR methods. A final step, using a hybridisation probe, was not needed. A new commercial kit for sample preparation, adsorbing DNA to a silicate gel, was shown to be superior to a conventional technique.

Evaluation and harmonisation of molecular methods for epidemiological typing of *Legionella pneumophila* serogroup 1 and designation of genotypes for an international database: A multicentre study. Paper II, III, and IV.

Introduction

The increase in leisure travel, particularly within the European Union (EU), together with a vigilant surveillance scheme has led to a heightened recognition of travel-associated legionellosis (97). In 2002 675 cases of travel-associated LD representing 94 clusters were reported to the EWGLI co-ordination centre (B. de Jong, SMI, personal communication). When a case of LD is recognised, others may have become infected from the same environmental source. Thus, the source of the outbreak must be established by characterisation of the clinical and environmental strains if action is to be taken to reduce the risk (Fig 7).

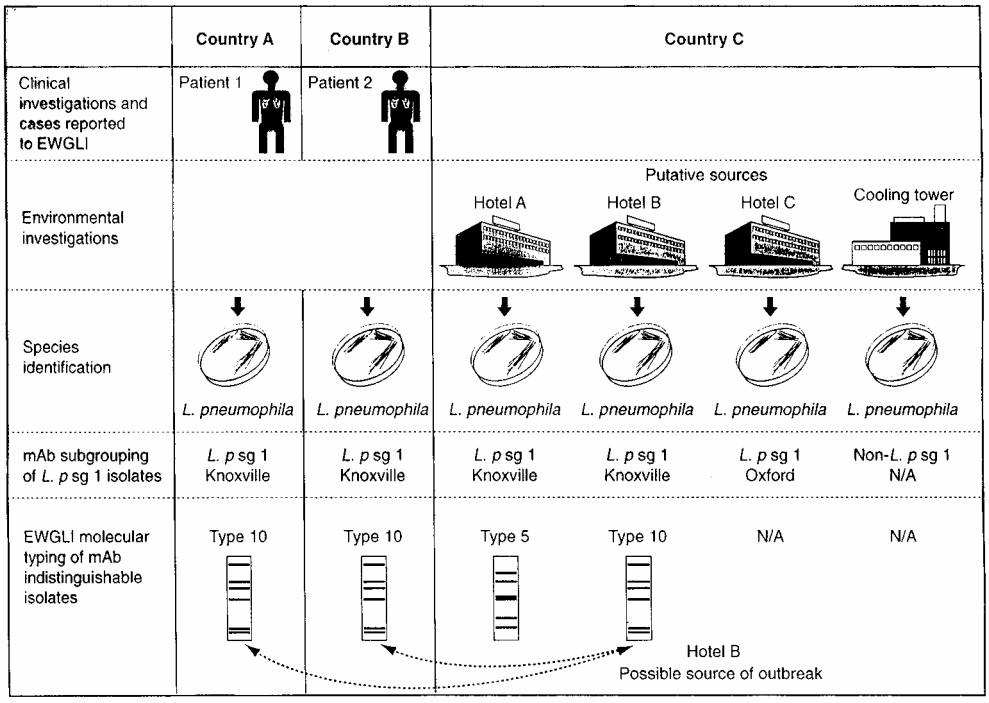


Fig.7. Strategy adopted by the European Working Group on Legionella Infections (EWGLI) for the epidemiologic typing of strains examined in the investigation of travel-associated legionellosis. NA= not applicable.

Many methods have been described for the genotypic and phenotypic typing of *L. pneumophila* serogroup 1 (paper II), which causes the majority of legionella infections (52, 97). These methods have however usually been used in a single laboratory for examination of a few strains. In such circumstances, it is only necessary to compare fingerprints of isolates at the time of analysis. In a context of travel-associated legionellosis, the situation arises, where a patient isolate is obtained in one laboratory (e.g. when the patient returns home) whilst the associated environmental isolate is obtained in the country where the infection was acquired (Fig 8). This necessitates sending strains between laboratories for comparison, an undertaking which has become increasingly complex and expensive.

These problems might be overcome if agreed standardised methods could be adopted that allowed laboratories to allocate an isolate to a type and then transmit this information rather than the isolate. Consequently, a strategy to develop a standardised approach of typing *L. pneumophila* sg 1 within Europe was proposed and agreed upon by members of EWGLI.

The overall aims of the study was to (1) create a reference collection of representative European clinical isolates, (2) to identify valid and electronically portable DNA-based typing methods among those currently used, and (3) to designate defined genotypes that may be kept in a readily accessible European database. Thus, In phase 1 (paper II) 14 different DNA-based methods or variants thereof were used to analyse 114 related and non-related strains. In phase 2 (paper III) reproducibility and epidemiological concordance were assessed using amplified fragment-length polymorphism analysis (AFLP) on 50 isolates of well-characterised strains. In phase 3 (paper IV) 31 AFLP types were designated among 130 strains in the EWGLI culture collection. Furthermore, 17 centres using a standardised protocol tested a proficiency panel consisting of 12 isolates.

Phase 1

Paper II: A multicentre evaluation of genotypic methods for the epidemiologic typing of *Legionella pneumophila* serogroup 1: results of a pan-European study.

Materials and methods: Participants from 12 institutions, representing 11 European countries took part in the study. Ten participating laboratories contributed with their 10 most recent (i.e. consecutive) endemic clinical isolates that conformed with the following criteria: (i) that they were

obtained from patients resident within that country and had not travelled to another country within two weeks before onset of symptoms; (ii) that they were not epidemiologically related (temporally or geographically) to any other isolate included in the panel. Seventy-nine isolates were obtained, since all participating centres were not able to provide 10 strains. These 79 isolates constituted an "unrelated" panel 1. A second panel 2 consisting of 35 "related" isolates, clinical and environmental, was also collected. Each of these was epidemiologically related to one of the isolates in the "unrelated" panel. Altogether, in both panels, there were 114 isolates, which were given a unique EU *Legionella* (EUL) culture collection number, by the coordinating laboratory (Respiratory and Systemic Infection Laboratory, PHLS Central Public Health Laboratory, London).

Each centre examined coded isolates by using their current genotypic methods. Thus, one or more of the following methods were used in each laboratory: restriction endonuclease analysis (REA), restriction fragment length polymorphism analysis (RFLP), two methods, ribotyping, two methods, macro-restriction endonuclease (*Sfi*I or *Not*I) analysis of genomic DNA resolved by pulsed-field gel electrophoresis (PFGE), six methods, PCR using arbitrary/repeat sequence primers (AP- and AP/rep-PCR), two methods, and amplified fragment length polymorphism analysis (AFLP). Furthermore, two laboratories performed subgrouping (subtyping) using monoclonal antibody (MAb) panels. AP/rep-PCR and PFGE were used at the Department of Clinical Microbiology at Karolinska Hospital, Stockholm.

The results were submitted to the coordinating centre by dendrograms, photographs or tagged image file format (TIFF) files. Typability, epidemiological concordance and index of discrimination were calculated for each method.

Results and discussion: It became apparent during the study that some of the epidemiologically related isolates could be distinguished by MAb subgrouping and by the majority of genotyping methods. Therefore, these "related" sets were re-examined and subsequently subdivided into three groups. Subdivision I contained 4 sets with compelling evidence of relatedness. Subdivision II contained 13 sets for which the evidence was consistent with the supposition of relatedness, supported by MAb grouping data and a substantial number of genotypic methods. Subdivision III contained sets either lacking epidemiologic data or lacking an established

epidemiologic link between patient and environmental isolates, and was excluded from further analysis.

The discriminatory index (D) of genotypic methods ranged between 0.840 (ribotyping) and 0.990 (one PFGE method). In general, the highly discriminatory PFGE and AP/rep-PCR methods showed a low value of epidemiological concordance (E, whilst the least discriminatory methods showed high values of E. This was also true of MAb subtyping, which therefore is more suitable as an initial screening tool. AFLP showed a D value of 0.891 and an E value of 1.0.

EWGLI decided to continue with AFLP and PFGE using *Sfi*I (Fig. 8 & 9). Of the two restriction enzymes used in PFGE *Sfi*I was the most discriminatory. Data obtained by PFGE were, however, difficult to interpret by computer analysis, which was reflected in the low E values. Possibly a careful standardisation of methodology and data analysis would obtain a better inter-laboratory reproducibility. The AFLP method was first tested by Valsangiacomo et al in 1995 and consists of a simple restriction-ligation reaction with a subsequent PCR amplification (117). In a single step reaction genomic DNA is digested (*Pst*I) and restriction fragments ligated to specially constructed adapters. PCR amplification of the tagged restriction fragments is then performed with primers complementary to the adapters allowing detection of RFLPs upon resolution on agarose gels. AFLP combines speed with adequate discrimination and a high value of epidemiological concordance.

Phase 2

Paper III: Assessment of intercentre reproducibility and epidemiological concordance of *Legionella pneumophila* serogroup 1 genotyping by amplified fragment length polymorphism analysis.

Introduction: Because of difficulties in standardisation of PFGE, also shown by de Zoysa et al (114), EWGLI decided, subsequent to the phase 1 study, to continue work with AFLP only. The aim of this work was (i) to assess intercentre reproducibility (R value) and epidemiological concordance (E value), and (ii) to assess the suitability of the method for standardisation and implementation by members of EWGLI.

Material and methods: Fifty coded isolates were sent to 13 centres in 12 European countries. The isolates were composed of two panels, a reproducibility panel (n 20) and an epidemiologically related panel (n 30). The reproducibility panel comprised 10 duplicate isolates, which had been characterised in the phase 1 study, whilst the epidemiologically related panel comprised nine related sets and an additional set with variants of the same strain.

A standard protocol was implemented (paper 2, www.ewgli.org). Despite this, minor variations occurred, i.e. in the use of DNA preparation methods, agarose, electrophoresis parameters, image capture, and analysis software. Participants analysed their own data. However, all data were submitted as photographs, TIFF files, or outputs from image capture devices, to the coordinating centre, PHLS, in London and subjected to visual analysis. Only bands between 100 bp and 3500 bp were included in analyses. The coordinating centre used a 90% similarity threshold to define the output in types using GelCompar anlysis software.

Results and discussion: The R value using data analysis ranged from 0.2 – 1.0 and the E value from 0.11 – 1.00 . One centre compared 4 different similarity thresholds demonstrating that an increase from 74% to 100% decreased R and E values and increased the number of types. When the coordinating centre made a visual analysis of submitted gel images, R and E values increased, ranging between 0.78 and 1.00 (R value) and 0.67 to 1.00 (E value). Thus, visual analysis clearly demonstrated that the AFLP method could be both highly reproducible and epidemiologically concordant: 9 of 13 centres had R and E values of >0.9.

However, not all laboratories achieved high values. Modifications that appeared to influence results significantly included use of agarose with different electroendosmosis values, different concentrations of agarose, and different electrophoresis parameters. The quality of images submitted varied considerably. Using high-contrast data sets in software analysis greatly increased both reproducibility and epidemiological concordance. In summary the AFLP method, as described in this study, was found suitable for standardisation and implementation within Europe by EWGLI.

Phase 3

Paper IV: Designation of the European Working Group on *Legionella* infection (EWGLI) amplified fragment length polymorphism types of *Legionella pneumophila* serogroup 1 and results of intercentre proficiency testing using a standard protocol.

Introduction: The utility of AFLP for the epidemiological typing of *L. pneumophila* sg 1 was demonstrated in the previous study (phase 2). In this study (phase 3), recommendations for designation of EWGLI AFLP types using a standard protocol were presented and AFLP types were designated from the 130 strains in the EWGLI culture collection.

Materials and methods: Twenty epidemiologically unrelated isolates from the culture collection were analysed by the coordinating centre (PHLS, London) in order to establish a proficiency-testing panel. At least three separate restriction-ligation and PCR amplification reactions were carried out for each strain. Only bands between 300 and 3500 bp were included in the description patterns. This yielded 16 major AFLP types following normalisation with a molecular size marker. Each type was assigned a unique EWGLI AFLP type number using the following format: *Legionella pneumophila* serogroup 1 standard EWGLI AFLP type (number) (city of the submitting laboratory). The remaining 110 strains in the culture collection were subsequently analysed.

Twelve isolates representing 9 of the 16 standard AFLP types, including three sets of two belonging to the same type, were selected for the proficiency panel. Each participating laboratory received these isolates coded and in addition a TIFF file containing the 16 standard AFLP type strains. Analysis of the coded isolates was then undertaken by using the standard protocol. Results were scored with reference to the previously designated standard EWGLI AFLP types, either visually or using locally available gel analysis software.

Results and discussion: The coordinating centre identified thirty-one AFLP types among the 130 strains in the EWGLI culture collection. Details may be obtained from the website: www.ewgli.org. Of the 17 participating laboratories 7 achieved maximum score in the analysis of the proficiency panel and another 7 scored 11 of 12 types correctly. The results were considered to be encouraging. There are, however, a number of recommendations that might improve future results:

(1) strict adherence to the standard protocol is required;

(2) the quality of image captures is of paramount importance;

(3) visual confirmation of computer-generated analysis by careful inspection of original images is always recommended.

Summary (paper II – IV)

A European culture collection of *L. pneumophila* sg 1 isolates was created. Fourteen different genotypic methods or variants thereof were analysed for typing these isolates in a first phase of the study. Amplified fragment length polymorphism (AFLP) was favoured because of high values of discriminatory index, reproducibility and epidemiological concordance. This was assessed in the second phase of the study. Standard EWGLI AFLP types of *L. pneumophila* serogroup 1 were designated in the third phase and tested internationally by the distribution of a proficiency panel. For the first time an international database was established containing defined *L. pneumophila* genotypes.

Molecular epidemiology of three *Legionella pneumophila* outbreaks in Sweden. Paper V, VI, and VII.

Introduction

Three major hospital-associated outbreaks of LD occurred in Sweden during the 1990s. In December 1990 to February 1991 an outbreak comprising 31 patients occurred at the General Hospital in Värnamo (104). The causative bacterium was *L. pneumophila* sg 1. In 1993 another outbreak with 8 patients occurred at the University Hospital in Uppsala caused by *L. pneumophila* strains not belonging to sg 1. Again, a new outbreak began at the same hospital in 1996, comprising 18 patients, this time caused by *L. pneumophila* sg 1. Following these outbreaks surveillance programmes were implemented and the hot water distribution systems were intermittently superheated. Water from the plumbing systems was also cultured for *Legionella* spp. at regular intervals subsequent to acid treatment. Isolates were then stored at - 70°.

Experience with the EWGLI harmonisation study, described earlier, prompted an interest to look closer at those strains that had been isolated in connection with these three nosocomial outbreaks. In the case of Värnamo, there was a possibility of studying the distribution of the causative strain geographically and temporally in the environment over a 12-year period. A similar scenario also existed in Uppsala, but in that case there had been two consecutive clusters appearing at different times corresponding to the presence of two different strains of *L. pneumophila* in the environment. Again, in Uppsala one strain, a *L. pneumophila* sg 1, had not been isolated from either patients or environment before 1996, when the new outbreak started.

The aim of the three presented studies was thus to characterise patient and environmental strains in each outbreak by using amplified fragment length polymorphism analysis (AFLP) and to some extent macro-restriction endonuclease analysis resolved by pulsed-field gel electrophoresis (PFGE), and if applicable MAb subgrouping. Furthermore, there was the possibility of comparing the outbreak strains with other strains, which had been isolated in Sweden previously and during this period.

The second outbreak in Uppsala, starting 1996, was the first one studied. It soon became apparent that the causative genotype of *L. pneumophila* sg 1 could vary between two MAb subgroups e.g. Knoxville and Oxford. For this reason a cooperative study was established with JH Helbig and PC Lück at Institut Medizinische Mikrobiologie und Hygiene, Medizinische Fakultät TU Dresden, Dresden, Germany, the aim being to study the possible insertion or deletion of the virulence associated *lag-1* gene. The Dresden group also contributed with the MAb panel used.

Definition of nosocomial disease

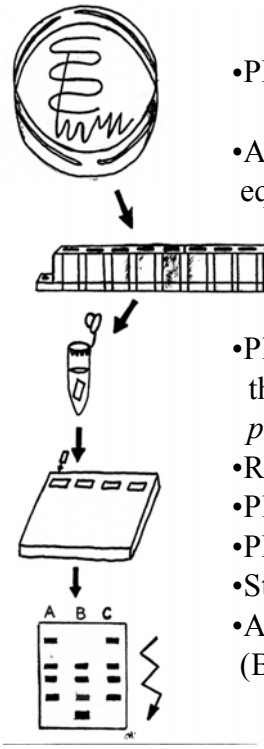
A case of LD is considered to be nosocomial, (i) if the patient has been admitted to the hospital >9 days before onset of symptoms, (ii) if the patient has spent 1 – 9 days in hospital prior to onset of symptoms, and either (a) became ill at a hospital where other patients have suffered from LD, or (b) yielded an isolate that is indistinguishable from an associated environmental strain as determined by MAb subgrouping or one genotypic method (52, www.ewgli.org).

A case is defined as travel-associated if a person suffering from LD has spent at least one night from home during a 10-day period prior to onset of symptoms.

Genotypic methods

PFGE: This method involves endonuclease macro-restriction using *Sfi*I resolved by pulsed field gel electrophoresis. The principle is shown in Fig. 8.

Restriction enzyme cutting is rare and large DNA fragments are produced. A more detailed description is given in paper V. In the present studies both lysozyme and proteinase K were used in the process of releasing DNA from legionella cells. Preparing DNA plugs without the use of lysozyme, reported by others (114), was found to be inadequate in preliminary experiments. Fewer and inconsistent bands were produced in absence of lysozyme. PFGE was also used for comparison in paper VII.



•PFGE total running time 30 h

•A suspension of 10^9 cfu is mixed with an equal volume of 2% agarose.

•Plugs are incubated in *lysozyme* solution 1h, then incubated again in SDS with *proteinase K* (1h), and washed
•Resticton ensyme *Sfi*I gives fragments of 50-700 Kbp.
•Plugs are loaded onto 1% agarose gel.
•PFGE 6V/cm, pulses 6.75-63.8 s at 14°C in 22h.
•Staining with ethidium bromide.
•Analysis with the aid of Molecular Analyst Software (BioRad).

Fig. 8. Flowchart showing the procedure of macro-restriction followed by pulsed-field gel electrophoresis (PFGE) used in this study. Drawing by Susanne Lenander.

AFLP: The principle of AFLP, originally described by Valsangiacomo et al. (117), is shown in Fig. 9. A more detailed description of the protocol is given in papers II – V and at the EWGLI website (http://www.ewgli.org). The extracted DNA is subject to an initial one-step restriction-ligation reaction. The *Pst*I enzyme cuts the bacterial chromosome at a certain site and restriction fragments are ligated at the restriction site to a specially constructed adapter oligonucleotide, which subsequently is used as a template in a PCR reaction. The primers that are complementary to the adapters on the tagged fragments contain an extra G. Because of this the number of amplified restriction fragments are limited and fewer bands are generated in the gels subsequent to electrophoresis. Analysis would, otherwise become difficult because of too many bands. The amplified restriction fragments are then electrophoresed 4 h at 100V. Two methods of DNA extraction were used in the present studies. The QIAamp tissue kit was used in the investigation of the Uppsala outbreaks (paper V and VII), whereas lysis of legionella bacteria by lysozyme and proteinase K digestion

was used in the Värnamo investigation (paper VI). The EWGLI collaboration group found that the different DNA extraction methods used did not significantly influence the results of AFLP genotyping. However, in the Värnamo study the second method was shown to be more effective in extracting adequate amounts of DNA.

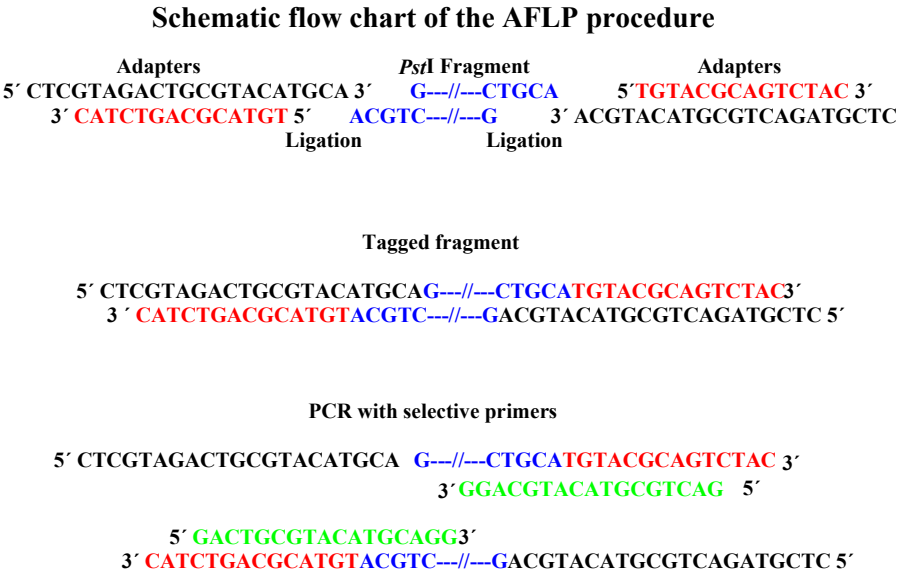


Fig. 9. AFLP: The *Pst*I fragment is tagged with an adapter at the restriction site and then amplified by primers complementary to the adapter. Restriction/ligation is a one-step procedure.

Gel analysis: Subsequent to electrophoresis and ethidium bromide staining the gels were scanned in a Geldoc instrument (Bio-Rad) and the band profiles converted into a TIFF file. All gels were analysed visually and in a gel analysis software programme (Molecular Analyst Software, Bio-Rad). Group analysis of bands was performed with the Dice coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering. Isolates were considered to belong to the same genotype in PFGE if the difference between strains was < 3 bands (Tenover et al., 119). Similarly in AFLP, either a similarity index of $\geq$ 95% or a difference by $\leq$ 1 band would indicate a common genotype (115).

Paper V: A hospital-associated outbreak of Legionnaires´ disease caused by *Legionella pneumophila* serogroup 1 is characterized by stable genetic fingerprinting but variable monoclonal antibody patterns.

Isolates: An outbreak of 18 pneumonia cases caused by *L. pneumophila* sg 1 occurred at the Uppsala University Hospital between 1996 and 1999. The causative bacterium was isolated in 8 cases. These clinical isolates were compared to 20 environmental isolates from the hospital and to 21 epidemiologically unrelated isolates in Sweden, mostly patient isolates by using PFGE, AFLP and monoclonal antibody (MAb) using the Dresden panel (7). Selected isolates were tested for the presence of the *lag-1* gene (132).

Results and discussion: All patient isolates and most environmental *L. pneumophila* serogroup 1 isolates from the outbreak hospital in Uppsala belonged to the same cluster in both PFGE (cluster A) and in AFLP (cluster 1) (Fig.10 A and B). This strain differed distinctly from other patient isolates and from another cluster in Sweden (cluster B/2), which mainly originated from Göteborg. Altogether 10 PFGE types and 13 AFLP types were recognised. There were some minor discrepancies between the two methods. Interestingly one isolate (no. 51) belonging to the A/1 cluster originated from a patient with community-acquired pneumonia who lived in a another town about 70 kilometres to the east and who had not visited Uppsala at the time of illness (the Danderyd/Norrtälje isolate in Fig. 14). The B/2 cluster included two isolates from patients with community-acquired pneumonia who lived in other parts of the country.

The MAb subtype (also termed subgroup) of the Uppsala outbreak strain was Knoxville. Most environmental isolates belonging to the A/1 cluster were also Knoxville. However, three isolates were MAb subtype Oxford and were indistinguishable genotypically from the Knoxville strain (Fig. 10 A and B). A similar variation was observed in the other B/2 cluster, in this case between MAb subtypes Philadelphia and OLDA. Some of the tested Knoxville and Philadelphia strains were quite unrelated genotypically from the two main clusters (A/1 and B/2). Altogether 6 phenons based on MAb typing were recognised.

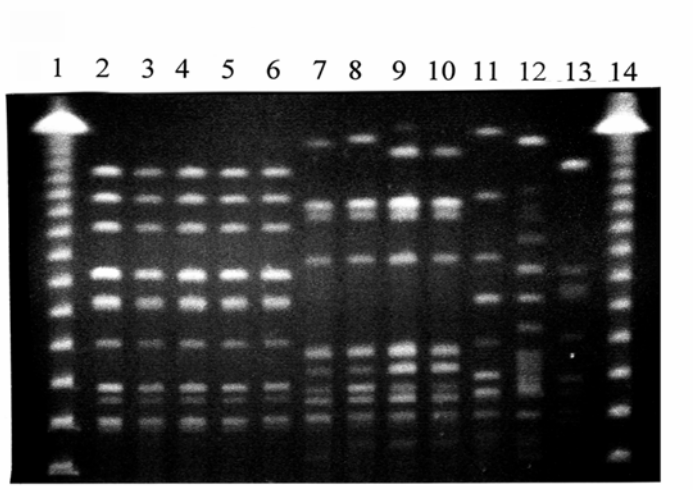


Fig. 10 A. PFGE. Lanes 2 to 6 represent strains from cluster A (hospita I, Uppsala), two patient (isolates 3 on lane 2 and 138 on lane 4, both Knoxville strains) and three environmental (isolates 10 on lane 3, and 139 on lane 5, both Oxford strains, and isolate 83 on lane 6, a Knoxville strain). The patient isolate in lanes 7 (Philadelphia, isolate 59) and 8 (OLDA, isolate 60) show a genotypic similarity with other strains from cluster B (hospital II, Göteborg) in lanes 9 (Philadelphia, isolate 21) and 10 (OLDA, isolate 22) but are epidemiologically unrelated. The strains in lanes 11 to 13 (Benidorm, isolates 28 and 56, and Philadelphia isolate 54) have been isolated from travellers pneumonia and are completely unrelated epidemiologically.

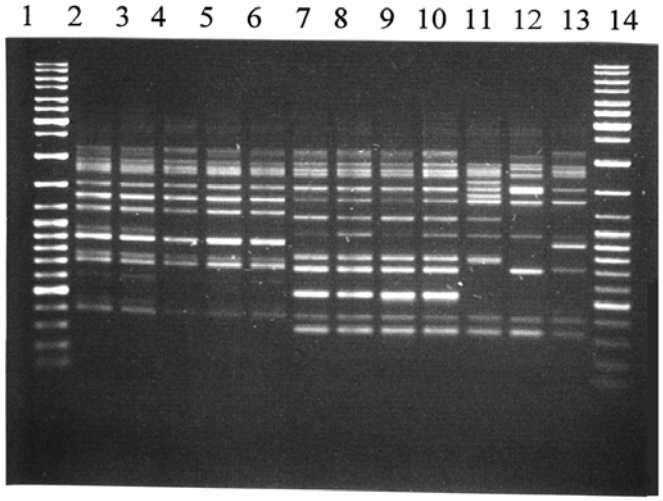


Fig. 10 B. AFLP. The same series of strains as in Fig. 10 A is shown. Note the similarities between isolates in lanes 2 to 6 (cluster 1) and between the isolates in lanes 7 to 10 (cluster 2).

The serological MAb subtypes Benidorm, France/Allentown, Knoxville, and Philadelphia belong to the so called Pontiac group, which expresses the virulence associated epitope recognised by MAb 3/1 (MAb 2 in the international panel). This epitope is only found in strains that possess the *lag-1* gene, which codes for an *O*-acetyltransferase of the lipopolysaccharide O-chain (111, 132). *L. pneumophila* sg 1 strains that cause large outbreaks are often MAb 3/1-positive and thus possess the *lag-1* gene (110). Therefore, it became important to show if the variation between MAb subtypes mentioned above was due to a phenotypic expression or a genetic event since they belonged to the same clusters. This question could be answered for the first time in the present study. The Oxford and OLDA isolates that belonged to the A/1 and B/2 clusters respectively did not possess the *lag-1* gene, whereas the Knoxville and Philadelphia isolates did. There were 797-bp direct repeats on both sides of the fragment containing the *lag-1* gene. It was therefore conceivable that a 2,857-bp fragment had been either deleted from the MAb 3/1-positive isolates or inserted in MAb 3/1-negative (Fig. 11).

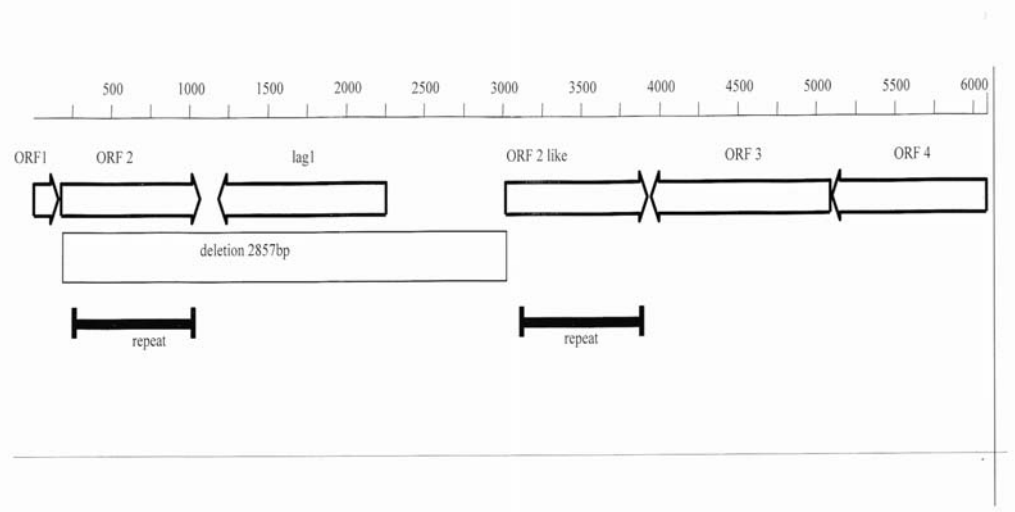


Fig. 11. Schematic representation of the gene organisation in the *lag*-1 region in the MAb 3/1-positive strain 3. Open reading frames are represented as open boxes, with arrowheads indicating the orientation. The design of the open reading frame was based on published sequences. The closed box indicates the 2,857-bp fragment that was lacking in the MAb 3/1-negative strain 10, which has the same genotype as the strain 3. Two repeats of 797 bp that showed an identity of 99.6% are also shown.

Interestingly, the Knoxville strain causing the outbreak starting in 1996 in Uppsala had not been isolated from patients or environment before 1996. A

previous outbreak, caused by another *L. pneumophila* strain, had, however, occurred in 1993 (paper VII).

Paper VI: The presence of a specific genotype of *Legionella pneumophila* serogroup 1 in a hospital and municipal water distribution system over a 12-year period.

Isolates: Only one clinical isolate was obtained from the outbreak at the Värnamo General Hospital in 1990. One isolate was also obtained from a patient with community-acquired pneumonia. Diagnoses were generally made by serology and urinary antigen detection. Water samples were obtained from selected fixtures at the hospital and from buildings in the surrounding community from 1991 to 2002. Altogether 367 water samples were collected during this period, yielding growth of *Legionellae* in 130 instances. Fifty-seven samples from the hospital and 28 samples from the community grew *L. pneumophila* sg 1, of which 33 and 19 isolates respectively survived storage. The Värnamo isolates were compared to isolates from 6 other regions in Sweden and to16 EWGLI standard AFLP types. The aim of the study was to define the molecular epidemiology of the outbreak strain geographically and over time. AFLP was used for genotyping and the Dresden panel for MAb subtyping.

Results and discussion:
The water distribution system of all hospital buildings was periodically colonised with *L. pneumophila* sg 1 during a 12-year surveillance. The patient isolate from the 1990 outbreak and all environmental isolates belonged to a specific genotype (AFLP type A). The majority of isolates shared a common AFLP pattern, subtype $A_{1.}$ Subtype $A_{2,}$ which included the clinical isolate, was only detected in 1991 prior to raising the circulating hot water temperature to >55°C. Of the 34 hospital isolates, 91% including the patient isolate belonged to MAb subtype Benidorm, the others to Bellingham.

Eight of 9 residential buildings in the community were colonised by *L. pneumophila* sg 1 belonging to genotype A. However, in 14 of 20 tested isolates the MAb subtype was Bellingham rather than Benidorm. The community-acquired case of pneumonia was caused by a Benidorm strain belonging genotype B.

The dominating A_1 genotype in Värnamo was also found in Jönköping, 70 km to the north, and was identical to the EWGLI standard AFLP type 8 Stockholm (EUL 101), also subtype Benidorm. This latter isolate was originally cultured from a patient in Falkenberg, a town situated about 100 km west of Värnamo. It was not known if this patient had been staying elsewhere prior to the onset of illness. However, it can be conceived that the A_1 clone is distributed over a wide geographical area. The dendrogram in Fig. 12 shows the relationship between strains from different geographical regions in Sweden and 4 EWGLI standard AFLP types. Of the 16 EWGLI AFLP types tested these were the ones showing relatedness to isolates in the present study.

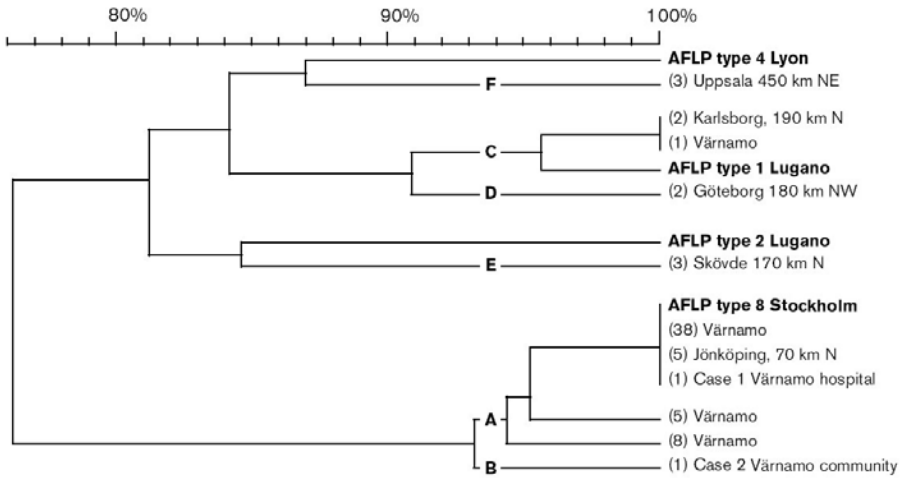


Fig. 12. Genetic relatedness using AFLP typing on Legionella pneumophila serogroup 1 isolated from 7 communities in Sweden. AFLP types defined by the European Working Group on Legionella Infections (EWGLI) are written in bold type. The dendrogram is based on a cluster analysis of 15 representative Swedish isolates and 4 EWGLI types (of 16 that were tested) with a similarity index of >80% in relation to the Swedish strains. AFLP types identified in the visual inspection of gel images are indicated with capital letters (A-E). Figures in parenthesis indicate the total number of isolates. Geographical distances are given in relation to Värnamo.

Also the genotypic cluster in Värnamo (genotype A) showed a variation of MAb subtype between Benidorm and Bellingham in the water distribution system. Since Benidorm is MAb 3/1-positive, whereas Bellingham is MAb 3/1-negative, it is conceivable that there existed a variation in this environment between *lag-1* gene positive and negative strains, as was seen in Uppsala and Göteborg (paper V).

Paper VII: A hospital-associated outbreak of Legionnaires´ disease caused by *Legionella pneumophila* serogroups 4 and 10 with a common genetic fingerprinting pattern.

Materials and methods: A nosocomial outbreak of LD was recognised at the University Hospital in Uppsala in 1993. The causative organism was eventually identified as *L. pneumophila*, though not belonging to serogroup 1. Preliminary serological data using polyclonal reagents showed cross-reactions with several serogroups. Five sporadic cases caused by similar strains were diagnosed 1988, 1995, 1997, and 1998. Thus, during a 10-year period altogether 13 cases were registered. However, only 12 isolates were available. Due to this outbreak and the following outbreak in 1996 caused by *L. pneumophila* sg 1 a surveillance program was implemented. This included culturing water quantitatively for *Legionella* spp from the hospital hot water distribution system at selected sites and regular intervals. Thus, 45 environmental *L. pneumophila* non-sg 1 isolates were obtained during the following period until 1999. Other *L. pneumophila* isolates originating from community-acquired pneumonia cases and from other outbreaks including the 1996 outbreak at the same hospital were included for comparison.

All isolates were serogrouped or subtyped using MAbs belonging to the Dresden Panel (7). AFLP was used for genotyping all isolates, and PFGE was used in selected cases to check results of AFLP.

Results and discussion: Of the 12 isolates that originated from nosocomial *L. pneumophila* non-sg 1 infections four isolates belonged to sg 4, seven to sg 10, and one to sg 6. The 1993 cases were all epidemiologically connected with a building containing a renal transplant unit. Three of these cases were caused by sg 4 and four cases by sg 10. Of the 45 non-sg 1 environmental isolates from the hospital 8 were sg 4 and 37 sg 10.

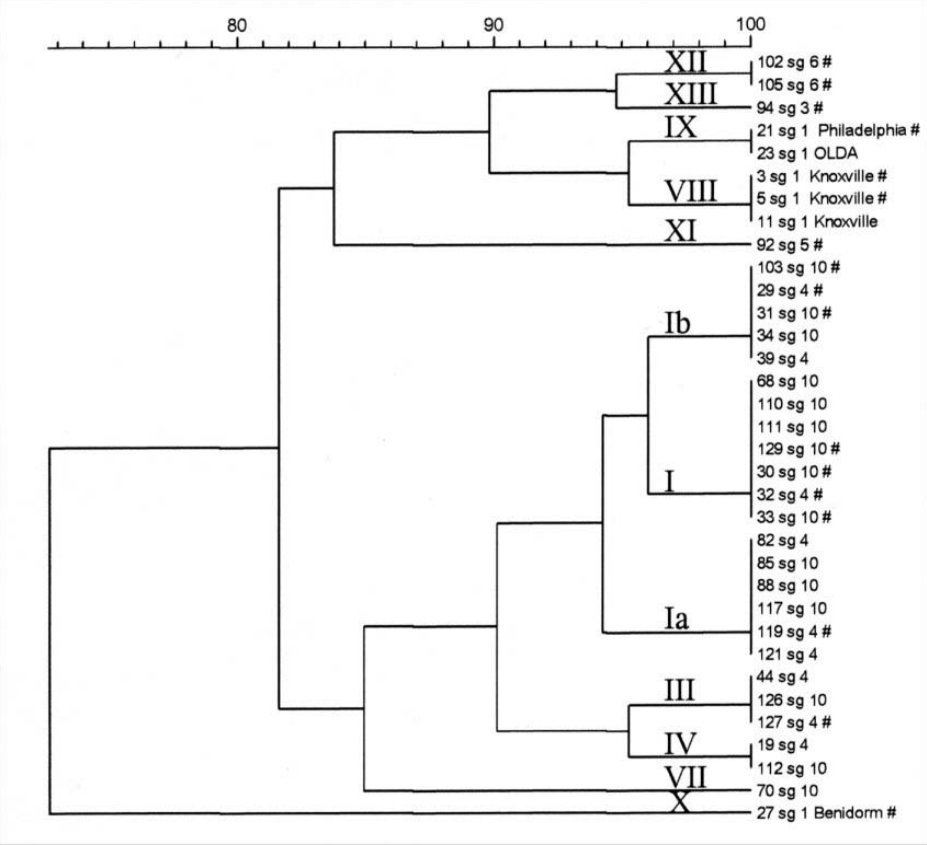


Fig. 13: Dendrogram of AFLP, with a Dice coefficient with 5% tolerance and UPGMA clustering of 34 selected *L. pneumophila* isolates. Patient isolates are marked by "#" symbols. The isolate number is shown, (though not in numerical order), and the serogroup (sg) or MAb subtype (subgroup) in case of sg 1 isolates. AFLP genotypes are given in roman numerals, and subtypes in lower case letters. The difference between genotype I and its subtypes is one band (approx. 95% relatedness). Type II – V differ from Type I by two bands. Type Ic , II, and V not shown. Note the occurrence of sg 4 and 10 in each AFLP subtype.

Nine of 12 non-sg 1 patient isolates clustered in AFLP with genotype I or with its subtybes Ia, Ib, and Ic, which differed from genotype I by one band. Two isolates were related to genotype I but differed by 2 bands. The sg 6 patient isolate was not related to genotype I at all but clustered with an isolate from a case of community-acquired pneumonia. Thus, this supposed nosocomial case might not have been hospital-acquired at all. Thirty-seven environmental non-sg 1 isolates belonged to genotype I or to its subtypes and were found in several hospital buildings, including those buildings that were associated with LD cases. Fig. 13 shows a dendrogram based on AFLP patterns of selected isolates. The AFLP genotype I and related genotypes

were quite different from other tested strains including the strain that caused the 1996 outbreak at the same hospital.

In Fig.13 it can also be seen that *L. pneumophila* sg 4 and sg 10 patient and environmental isolates cluster together, thus belonging to the same genotype. This was also clearly demonstrated when checking selected isolates with PFGE.

Thus, one genotype of *L. pneumophila*, defined by AFLP and PFGE, contained two serogroups, which caused the outbreak at the University Hospital in Uppsala in 1993. It is therefore conceivable that the sg 4 and sg 10 strains were closely related. This raises the question if the variation in phenotype was a result of a variable phenotypic expression or of a genetic event. Both explanations are possible. In practice genetic fingerprinting should be used in conjunction with serogrouping in epidemiological investigations. MAbs in the Dresden Panel react with serogroup-specific polysaccharide epitopes on the bacterial surface (7). Their use is therefore helpful in epidemiological investigations. Some other epitopes, recognised by MAbs, are found in several *L. pneumophila* serogroups. This probably explains why the initial work on serogrouping isolates from the outbreak yielded cross-reactions and inconsistent results.

Discussion (paper V – VII)

The present study of the molecular epidemiology in three hospital outbreaks is the first one in Sweden using modern typing methods. Furthermore, genotypic methods were used in conjunction with MAb panels. The AFLP method was chosen because studies by the EWGLI collaboration group had shown the method to be adequately discriminatory and highly epidemiologically concordant. In addition, it had been used to designate standard *L. pneumophila* sg 1 types in the EWGLI culture collection, from which the genotype patterns are electronically available. PFGE subsequent to macro-restriction with *Sfi*I was used as second method in two studies.

A variation in phenotype, defined by the MAb panel, was recognised among isolates belonging to a specific genotypic cluster in all three outbreaks. This was also true of a fourth cluster included in paper V. Thus MAb subtype (subgroup) Knoxville varied with Oxford, Philadelphia with OLDA, Benidorm with Bellingham, and *L. pneumophila* sg 4 with sg 10. In the case of the Oxford and OLDA strains it could be shown for the first time that

these phenotypes lacked the *lag-1* gene. Thus, despite complete similarity in fingerprinting patterns a genetic event had taken place, either a deletion of the gene from MAb 3/1-positive strains or an insertion in MAb 3/1-negative strains. Thus it seems as if Knoxville, Philadelphia, and Benidorm strains possess a *lag-1* gene-negative counterpart in Oxford, OLDA, and Bellingham strains respectively, though the universal applicability of such a theory is not proven at this time. There is presently no explanation as to the serogroup variability among the patient and environmental strains that occurred in the 1993 outbreak caused by *L. pneumophila* non-sg 1 strains.

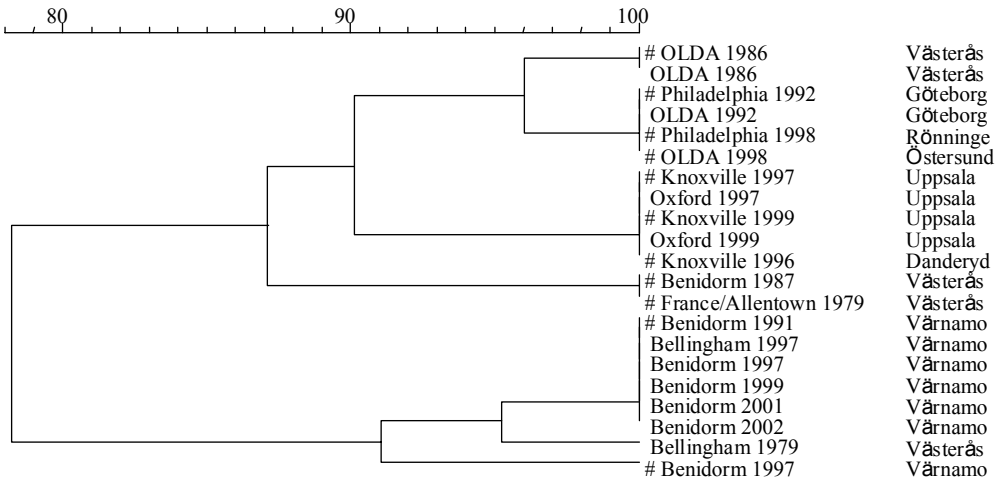


Fig. 14. Dendrogram based on AFLP patterns representing different clusters of *L. pneumophila* sg 1 that have caused Legionnaires´ disease in Sweden. The place of isolation of the strains is given. Patient isolates are marked by the "#" symbol. There is a variation inside genotypic clusters between Philadelphia and OLDA, Knoxville and Oxford, and Benidorm and Bellingham. The Knoxville strain from Danderyd originated from a patient living in Norrtälje. The patient strain from the Västerås outbreak in 1979 is identical to another patient strain from the same city isolated in 1987 but quite different from the strain isolated from the suspected source, a cooling tower on the roof of the department store (Fig. 4). Furthermore, a nosocomial strain, patient and environmental, from 1986 in Västerås is related to the Göteborg strain (cluster B/2 in paper V), but not to the 1979 outbreak strain

Fig. 14 shows a dendrogram of AFLP results summarising the relationship between *L. pneumophila* sg 1 strains studied in the present work with the addition of isolates from clusters inVästerås. Unexpectedly, the 1979 patient isolate, representing the Västerås outbreak strain, does not cluster with the environmental strain obtrained from the cooling tower. The clusters in the figure are adequately discriminated by the AFLP method. However, the Värnamo genotype was found in a large geographical area. The Göteborg strain was also found with patients from other parts of Sweden. Such

widespread occurrence of a single genotype has been reported earlier (101, 133, 134). The Uppsala and Värnamo studies also demonstrate that a certain genotype may remain in the water distribution system for a long period of time despite the implementation of disinfecting procedures.

Using genotyping methods and MAb reagents together increases the discrimination between strains of *L. pneumophila*. However, typing methods should always be used in conjunction with clinical and epidemiological data.

Summary (papers V – VII)

The following conclusions may be drawn from three studies of the molecular epidemiology of hospital-associated legionella outbreaks in Sweden:

1. The strains of *L. pneumophila* belonging to the different clusters were unrelated, each outbreak being caused by a different strain.
2. Phenotypic variations were found inside specific genotypic clusters. For the first time such a variation in *L. pneumophila* sg 1 was proven to be caused by a genetic event.
3. In one outbreak there was a phenotypic variation between two different serogroups, sg 4 and sg 10, in a genotypic cluster, which has not been demonstrated earlier.
4. A specific genotype can occur endemically in a large geographical area and for a long period of time in a water distribution system.
5. The PFGE and AFLP methods were in agreement with few exceptions.
6. The use of MAbs is a valuable adjunct to genotyping.
7. Typing methods should be used in conjunction with clinical and epidemiological data in investigations of Legionnaires´ disease.

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