

**ISTITUTO SUPERIORE DI SANITÀ**



20<sup>th</sup> Annual meeting

**European Working Group for Legionella Infections**

Istituto Superiore di Sanità  
Rome, May 16-17, 2005

**ABSTRACT BOOK**

Edited by  
Giuseppina Mandarino

*Dipartimento Malattie Infettive, Parassitarie ed Immunomediate*

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The European Working Group for Legionella Infections (EWGLI) was established in 1986. Members are scientists interested in improving knowledge and information on the clinical and environmental aspects of Legionnaires' disease through its diagnosis, management and treatment. In 1987, the Group established a surveillance scheme (EWGLINET) for the detection of the cases in people who travelled and stayed in hotels and resorts. EWGLI aims at rapid identification of the outbreaks and investigations into the source(s) to prevent further cases and increasingly protect European citizens. Collaborators are national or regional representatives from the public health and microbiology institutes, appointed by their Ministry of Health. There are currently 36 countries in EWGLINET (17 EU and 19 non EU). EWGLI activities and updates on the pathogenesis, diagnosis and epidemiology of Legionella infections are presented in an annual workshop hosted every year by one of the 36 countries participating in the scheme.

*Key words:* Legionella infections, Surveillance, Europe

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Lo European Working Group for Legionella Infections (EWGLI), nato nel 1986, è costituito da professionisti europei che operano per il miglioramento delle conoscenze sugli aspetti clinici, epidemiologici, ambientali e diagnostici delle infezioni da Legionella. Dal 1987, lo EWGLI ha istituito una rete di sorveglianza (EWGLINET) dei casi di malattia associati ai viaggi con l'obiettivo di identificare rapidamente eventuali focolai epidemici e mettere in atto tempestivamente adeguate misure di controllo. Lo scopo dei collaboratori dello EWGLI è quindi quello di proteggere i cittadini europei dal rischio di acquisire un'infezione da Legionella, in seguito a un viaggio o ad un soggiorno presso strutture ricettive, sia nel Paese di origine che all'estero. I collaboratori dello EWGLI sono rappresentanti regionali o nazionali di istituti di sanità pubblica nominati direttamente dai Ministri della Salute. Partecipano alla rete 36 Paesi, di cui 17 dell'Unione europea. Lo EWGLI organizza annualmente un convegno scientifico che si svolge ogni volta in un Paese diverso con l'obiettivo di fornire un aggiornamento sui vari aspetti delle infezioni da Legionella.

*Parole chiave:* Infezioni da Legionella, Sorveglianza, Europa

*Scientific Committee:*

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## PROGRAMME

### Monday, May 16, 2005

- 9.00 Opening address  
**A. Cassone, S. Salmaso**
- 9.15 Invited lecture  
*Legionnaires' disease: what's new and useful?*  
**P. Edelstein**

#### Session I

#### LEGIONELLA EPIDEMIOLOGY AND OUTBREAK INVESTIGATIONS

*Chairpersons:* S. Salmaso, J. Etienne

- 10.15 *Epidemiology of Legionnaires' disease in Europe - 2004*  
**C. Joseph**
- 10.35 *European surveillance of travel associated Legionnaires' disease - 2004*  
**K. Ricketts**
- 11.00 Break
- 11.15 *Community outbreak of legionellosis in Sweden*  
**B. de Jong**
- 11.30 *Epidemiology of Legionella infections among children examined in Poland in 1998-2005*  
**H. Stypulkowska-Misiurewicz, K. Pancer**
- 11.45 *Host-related and environmental risk factors associated with sporadic and community-acquired Legionnaires' disease: a matched case-control study*  
**P. Santa-Olalla**
- 12.00 *A water born outbreak of Legionnaires' disease in Soulac sur Mer, France - 2004*  
**M. Charron**

#### Session II

#### PATHOGENESIS

*Chairperson:* Y. Abu Kwaik

- 12.20 Invited lecture  
*Interaction of Legionella with macrophages and amoebae*  
**Y. Abu Kwaik**
- 12.45 *Zinc-dependent cytoadherence of Legionella pneumophila*  
**D. Yaradou**
- 13.00 Lunch

**Session III**  
**SURVEILLANCE, CONTROL AND PREVENTION**

*Chairpersons:* V. Drasar, G. Orefici

14.00 *Portuguese surveillance programme in Legionnaires' disease. One year after, an opportunity for a comment*  
**T. Marques**

14.20 *A four-year experience of a chlorine dioxide treatment for the control of Legionella in a hospital water system*  
**M.L. Ricci**

14.40 *Simultaneous occurrence of Amoebae and Legionellae in cooling towers in South of France*  
**A. Rambaud**

15.00 *Seven cases of travel-associated Legionnaires' disease in Turkey*  
**H. Erdoğan**

15.15 Break and group photograph

16.00 *Is there a professional risk of Legionnaires' disease? Results of a multicentric serological study*  
**P. Borella**

16.20 *Prevalence of Legionella antibodies in two Danish townships*  
**M. Rudbeck**

16.40 *Health Protection Agency spa pool project: an update*  
**S. Surman Lee**

17.00 Poster Session

**Tuesday, May 17, 2005**

**Session IV**

**MOLECULAR EPIDEMIOLOGY AND TYPING SCHEMES**

*Chairpersons:* C.P. Lück, T. Harrison

- 9.00 *Variable genetic element typing: a quick method for epidemiological subtyping of Legionella pneumophila*  
**C.P. Lück**
- 9.20 *Comparison of four methods for molecular subtyping of Legionella pneumophila as implemented during an epidemic cluster of legionellosis in Rome*  
**M. Scaturro**
- 9.40 *Epidemiological genotyping of Legionella pneumophila serogroup 1 by fluorescent - AFLP: an inter-platform comparative study*  
**P. Visca**
- 10.00 *Phenotypic identification and subtyping of Legionella pneumophila strains being insufficient for serogrouping*  
**J.H. Helbig**
- 10.20 *Characterization of clinical strains of L. pneumophila serogroup 1 isolated in Portugal using monoclonal antibodies and amplified length polymorphism and sequence based-typing*  
**M. Chasqueira**
- 10.40 *Update on the consensus sequence-based epidemiological typing scheme for Legionella pneumophila*  
**N. Fry**
- 11.00 Break

## **SATELLITE WORKSHOPS**

### **1. EWGLINET**

- 11.20 Business meeting  
**C. Joseph**

### **2. External Quality Assessment (EQA) Workshop/Update**

- 11.20 Legionella isolation from water sample EQA (J. Lee)  
11.30 Legionella urinary antigen EQA (T. Harrison)

### **3. Typing Workshop**

- 11.40 *Use of the “Dresden Panel Lp A-M” for serotyping of the EWGLI SBT Proficiency Panel n. 2*  
**J.H. Helbig**
- 12.00 *Sequence-based typing of L. pneumophila: results of the second EWGLI multi-centre SBT proficiency panel*  
**B. Afshar**
- 12.20 *The additional use of two gene sequences increases the discriminatory power of the EWGLI sequence-based typing scheme for Legionella pneumophila*  
**P.C. Lück**
- 12.40 *Application of bioinformatic tools to the EWGLI sequence-based typing and identification schemes*  
**W. Bellamy**
- 13.00 Discussion

### **End of the meeting**

- 13.20 Lunch



**Invited lecture**  
**LEGIONNAIRES' DISEASE:**  
**WHAT'S NEW AND USEFUL?**

Edelstein Paul H.

*Department of Pathology and Laboratory Medicine, University of Pennsylvania,  
Philadelphia, USA*

Advances in several areas, including new insights on antimicrobial therapy for Legionnaires' disease (LD), use of ancillary therapies in LD treatment, host genetic factors that predispose to LD, and novel insights into bacterial pathogenesis are discussed. In addition the limitations of current approaches to diagnosis, as well as whether investigation of the home and work environment of sporadic cases is useful or indicated are analysed.



**Session I**  
**Legionella epidemiology and outbreak investigations**



## **EPIDEMIOLOGY OF LEGIONNAIRES' DISEASE IN EUROPE - 2004**

Joseph Carol, Ricketts Kate on behalf of the European Working Group for Legionella Infections  
*Health Protection Agency, Centre for Infections, London, UK*

**Background.** Since 1993, countries participating in the European surveillance scheme for travel associated Legionnaires' disease have contributed their national data to a European data set in order to provide an overview of the epidemiology of Legionnaires' disease in Europe.

**Aims of the study.** This important dataset has been used for studying the effectiveness of surveillance and Legionella control and prevention programmes within Europe.

**Methods.** Data are collected annually through a set of standard tables using agreed case definitions. Epidemiological information includes age and sex distribution, deaths, whether the cases are hospital, community or travel associated, and details of outbreaks by source and type. Microbiological data include method of diagnosis for all cases, and the species, serogroup and subgroup of culture confirmed cases.

**Results.** Improved surveillance in many countries and wider use of the urinary antigen test are reflected in the continuing upward trend of Legionella cases detected in Europe as a whole. Large community outbreaks also continue to occur and have added to this rise. The aggregated annual totals have increased from 1161 in 1994 to 4578 in 2003, an increase of 75% over the ten years, with the majority of the rise taking place since 2000. Aggregated incidence rates per million population have increased from 3.35 in 1994 to 9.8 in 2003. In 2003 34 countries submitted an annual dataset and individual country rates ranged from 0.0 (Latvia, Lithuania) to 28.7 (Spain) per million population. Overall, community outbreaks were associated with more cases than other types of outbreaks detected in 2003.

**Conclusion.** It is expected that this rising trend in reporting has continued in 2004 and that around 5000 cases will be reported from Europe as a whole. An overview of the European data for 2004 will be presented and epidemiological trends in national and European data discussed.

## EUROPEAN SURVEILLANCE OF TRAVEL ASSOCIATED LEGIONNAIRES' DISEASE - 2004

McNaught Bryan, Ricketts Kate, Joseph Carol on behalf of the European Working Group for Legionella Infections

*Communicable Disease Surveillance Centre, Health Protection Agency,  
Centre for Infections, London, UK*

**Background.** The EWGLINET surveillance scheme collects data on travel associated Legionnaires' Disease cases from 37 countries.

**Aim.** To describe and analyse the distribution of travel associated cases in Europe in 2004 and the response by EWGLINET countries to cluster alerts.

**Methods.** Data are reported to the co-ordinating centre in London by national collaborators in EWGLINET. They are entered into a database for detection of clusters, analysis of travel patterns and epidemiological trends.

**Results.** To date, 641 cases have been reported with onset in 2004, a rise in cases from 2003.

The ratio of men to women was 2.88:1 and the most commonly affected age group was 50-59 years. The case fatality rate was 5.1%. Cases peaked in August (n. 106).

22 EWGLINET countries and 1 non-EWGLINET country reported cases. England and Wales reported the highest number of cases (n. 169), followed by France (n. 129) and The Netherlands (n. 120). Altogether, cases had visited a total of 71 countries. France was associated with the highest number of cases (n. 221), closely followed by Italy (n. 204).

82 new clusters were detected in 2004 and 172 cases overall (26.8% of cases) were associated with these and cluster updates. 45% of the newly detected clusters would not have been identified without the scheme's international database. Italy and France were associated with the highest number of newly detected clusters (Italy 17, France 16).

92 Form B reports were returned in 2004, of which 50 reported positive results for detection of Legionella, and four cluster sites were published on the EWGLI website. Some countries investigate the sites of single cases and report the results to EWGLINET. Of those reporting sample results, 48% of sites were positive in 2004.

**Conclusion.** The EWGLINET scheme continues to be successful at detecting and acting upon clusters of cases of travel associated Legionnaires' disease.

## COMMUNITY OUTBREAK OF LEGIONELLOSIS IN SWEDEN

de Jong Birgitta<sup>1</sup>, Svensson Nils<sup>2</sup>, Hageus Ann Christine<sup>3</sup>, Bernander Sverker<sup>4</sup>

<sup>1</sup> *Department of Epidemiology, Swedish Institute for Infectious Disease Control, Solna, Sweden.*

<sup>2</sup> *Regional County Medical Office, Skövde, Sweden*

<sup>3</sup> *Environmental Health Office, Community of Lidköping, Sweden*

<sup>4</sup> *Department of Clinical Microbiology, Uppsala University Hospital, Sweden*

In August 2004 a cluster of cases with legionellosis was simultaneously detected by the surveillance system and local health administration. The initial investigation showed that the cluster consisted of people having connection with the town of Lidköping, by Lake Vänern, either being inhabitants or visitors.

An outbreak investigation team was formed with personnel from the Regional County Medical Office, Environmental Health Office, the County hospital, the local hospital, County Administrative Board, Swedish Legionella referral laboratory and the Swedish Institute for Infectious Disease Control.

Cooling towers and other aerosol producing installations in the town were localised and several installations were sampled. In parallel, all cases and presumptive cases were interviewed using a structured questionnaire including a town map on which they were asked to mark their movements prior to the onset of disease.

Legionellosis was diagnosed in 15 cases including two fatal ones. The majority were diagnosed by the urinary antigen test. Samples from three cases grew serogroup 1 from sputum. Results of environmental sampling showed growth of *L. pneumophila* in very high concentrations ( $3.6 \times 10^8$  CFU/L) from an industrial cooling tower. Lower concentrations were detected from three additional cooling towers. Isolates from two culture positive patients were genotypically indistinguishable by AFLP and PFGE to an isolate obtained from the cooling tower with very high amounts of *L. pneumophila* serogroup 1. This cooling tower is situated less than 1 km from town centre. The isolate from the third patient was different to the other two patients but identical to a second isolate, obtained later, from the same cooling tower.

The local weather service modelled the hypothetical spread and distribution of a bacterial aerosol from the cooling tower. The resulting map showed that the wind at the time blew from the nearby large Lake Vänern, passing by the cooling tower in the industrial area, into the central parts of the town. This southeastward wind direction from the lake was unusual. The probable date of infection for the index cases coincided with a change from a chilly to a rather warm humid weather, which may have also played an important role in the spread of the Legionella bacteria from the cooling tower.

## EPIDEMIOLOGY OF LEGIONELLA INFECTION AMONG CHILDREN EXAMINED IN POLAND IN 1998-2005

Pancer Katarzyna, Stypulkowska-Misiurewicz Hanna  
National Institute of Hygiene, Warsaw, Poland

Identification of the organism responsible for Legionnaires' disease was first associated with an outbreak of the disease among old adults. The population of man over 50 years old is generally more susceptible for pulmonary infections than the younger one and most often suspected as being *Legionella* infected. Little is known about *Legionella* spp. infections among children and the infection is rarely suspected.

The aim of our study was to analyse the results of examination of specimens from children done in our laboratory.

The serum and / or urine specimens collected from 41 children (3-17 yrs old) with symptoms of pulmonary infection were examined with laboratory tests for *Legionella* infection. The diagnosis of *Legionella* infection was confirmed or presumed in 13 cases (31,7%). Two positive and one equivocal urine samples were found. Serological investigations were done using different Methods. ELISA (IgA, IgM, IgG), microagglutination test MAT and IFA. Significant level of *L. pneumophila* serogroup 1 IgM (ELISA) antibody was found in 6 cases, high but insignificant level of IgM was in serum samples collected from 3 children and one 3yrs old girl has significant level of IgG antibody to *L. pneumophila* serogroup 1. In 6 serum samples the high level of antibodies to *L. pneumophila* was found using IFA; in one serum samples titre of antibodies to *L. pneumophila* serogroup 1 determined by MAT was 64, in second-titre to *L. micdadei* was 128.

The patients could be divided into 3 groups: first – from oncology units – 18 children, second – outpatients clinic – 10 and third – from other clinics or wards – 13.

A group of 7 outpatient clinic patients in one small town, children in the age of 4-9 yrs old, with symptoms of bronchial hypersensitivity were examined with MAT, IFA or ELISA tests for persistence of *L. pneumophila* antibodies. Among those patients 5 probably had passed *Legionella* infection. The children were treated with clarithromycin and steroids with good results- recovery.

In the hot-water systems of the buildings where those children lived a high level of contamination with *L. pneumophila* serogroup 2-14 was found. The 3 building water systems were treated with heat and the control examination showed the decrease number of *Legionella* in water.

Conclusion: *Legionella* infection in children might be underdiagnosed as clinical picture of the infection is not characteristic. The early treatment with antibiotics is used without laboratory examination for the etiology of the diseases.



## **HOST-RELATED AND ENVIRONMENTAL RISK FACTORS ASSOCIATED WITH SPORADIC AND COMMUNITY-ACQUIRED LEGIONNAIRES' DISEASE: A MATCHED CASE-CONTROL STUDY**

Santa-Olalla Patricia<sup>1,2</sup>, Campèse Christine<sup>1</sup>, Jacquier Guy<sup>1</sup>, Bitar Dounia<sup>1</sup>, Che Didier<sup>1</sup>

<sup>1</sup>*Institut de Veille Sanitaire, Saint Maurice Cédex, France*

<sup>2</sup>*European Programme for Intervention Epidemiology Training (EPIET)*

**Background.** Legionnaires' disease is a common cause of adult pneumonia, with a high-related mortality. Outbreaks of Legionnaires' disease have been well described, but little is known about sporadic, community-acquired Legionnaires' disease, which accounts for more than 50% of the cases. Knowing the risk factors for acquiring sporadic Legionnaires' disease in the community will help to address the appropriate prevention measures.

**Aim.** To identify the risk factors for acquiring sporadic Legionnaires' disease in the community.

**Methods.** Prospective, matched case-control study. Cases with onset of symptoms from 1<sup>st</sup> September 2002 were included consecutively until the sample size was completed (31<sup>st</sup> September 2004). Six hundred and two cases with sporadic, community-acquired and biologically confirmed Legionnaires' disease, in metropolitan France, were matched with a control subject. Matching variables were age, sex, underlying illness and location of residence within 5 km.

The comparison of cases with their controls was based on an interview regarding host-related factors, potential outdoors and indoor exposures, professional and leisure activities using a standardized questionnaire.

A conditional logistic regression was performed to estimate the risk of acquiring Legionnaires' disease associated with various host-related factors and exposures.

**Results.** The 602 cases included twenty-four (4%) who had died of Legionnaires' disease. Age ranged from 10 to 93 years (median, 56 years). Nine hundred forty were men and 262 were women, corresponding to a 3.6 M/F sex ratio. The diagnosis for 93.9% of the cases was confirmed by detection of soluble *Legionella* antigen in urine. Only 37.7% of cases had an underlying disease known to be a risk factor for Legionnaires' disease.

Bivariate and multivariable analysis is still ongoing and results will be presented further on.

## A WATER BORN OUTBREAK OF LEGIONNAIRES' DISEASE IN SOULAC SUR MER, FRANCE - 2004

Charron Martine<sup>1</sup>, Dejean Gisèle<sup>2</sup>, Jarraud Sophie<sup>3</sup>, Campèse Christine<sup>4</sup>

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<sup>2</sup>*Direction Départementale des Affaires Sanitaires et Sociales de la Gironde*

<sup>3</sup>*Centre National de Référence des légionelles*

<sup>4</sup>*Institut de Veille Sanitaire*

**Background.** During the last week of August 2004, 4 cases of Legionnaires' disease, all living in Soulac sur Mer were notified to the district health authorities. Epidemiological and environmental investigations were conducted to identify the source of the outbreak.

**Method.** Notified cases were living in Soulac sur Mer during the period of ten days before disease onset. Cases were interviewed, using a standard questionnaire. The health authorities asked general practitioners in the area and other districts to notify cases corresponding to case definition. Potential source of environmental contamination in the area were investigated. Human and environmental strains were sent to the National Reference Center of Legionella (NRCL) for identification.

**Results.** Seven cases of Legionnaires' disease (*Legionella pneumophila* séro groupe 1-Lp1) were identified between 11 August and 8 September 2004. One patient died. M to F sex ratio was 2.5, median age was 71 years (range: 54-89 years). In addition, among 3 known cases identified in the area from 2001 to 2003, one strain was available in the NRCL.

The water samples taken from different sections of the water network of Soulac showed concentration levels of Lp1 from 1 000 to 160 000 CFU/l.

Strains isolated from 2 patients, one in 2004 and one in 2003 were identical to the strain isolated from the water network.

The drinking water came from two groundwater sources: one hot spring (33-34 °C) was mixed with cold water (20-21 °C) in order to ensure a water temperature under the limit of 25 °C in the network. The municipal drinking water network was identified as the environmental vector of contamination.

Immediate over-chlorination and purge reduced Lp1 concentration to less than 250 CFU/l three days after the beginning of the treatment. Total decontamination being uncertain, a prospective surveillance of *Legionella* and a technical assessment of water network were set up. Conclusion: This is the first episode of Legionnaires' disease in France in which a drinking water supply using mixed hot and cold water was incriminated. This represents a potential public health problem since this type of water supply is frequently used in tourist cities. At the beginning of the tourist season a surveillance of the water supply should be implemented.

**Session II**  
**Pathogenesis**



**Invited lecture**  
**INTERACTION OF *LEGIONELLA PNEUMOPHILA***  
**WITH MACROPHAGES AND AMOEBAE**

Abu Kwaik Yousef

*Department of Microbiology and Immunology, University of Louisville College of Medicine, Louisville, USA.*

*Legionella pneumophila* is an environmental organism that resides in aquatic environments. In the natural environment, the organism is capable of invading *amoebae* and ciliated protozoa. Once transmitted to humans by aerosols, it causes atypical pneumonia designated Legionnaires' disease. The ability of *Legionella* to invade and replicate within mammalian and protozoan cells is dependent on the type Dot/Icm type IV secretion system, which injects proteins from the bacteria directly into the host cell to modulate host cell biology. One of the functions modulated in both hosts is the evasion of endocytic fusion by the Legionella-containing phagosome. In human macrophages, the bacterium induces caspase-3 activation that does not result in full activation of apoptosis till late stages of the infection. During late stages of intracellular replication, the bacteria escape into the cytoplasm following disruption of the phagosome. Final stages of intracellular replication are completed in the cytoplasm and precede lysis of the host cell and bacterial egress into the cytoplasm.

## ZINC-DEPENDENT CYTOADHERENCE OF *LEGIONELLA PNEUMOPHILA*

Yaradou Diaraf<sup>1</sup>, Raze Dominique<sup>2</sup>, Jarraud Sophie<sup>1</sup>, Vandenesch François<sup>1</sup>,  
Etienne Jerome<sup>1</sup>, Menozzi Franco Dante<sup>2</sup>.

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Microbial adherence to host cells is an early and critical step in the establishment of infection. Following aerosol transmission to susceptible humans, *Legionella pneumophila*, which displays a tropism for monocyte-derived cells, is thought to be rapidly phagocytosed by resident macrophages in which it multiplies. However, since the alveolus is almost entirely delimited by type I and type II pneumocytes, and since *L. pneumophila* can replicate within these epithelial cells, the pneumocytes may also represent a niche for intracellular bacterial multiplication at the beginning of infection. To characterize the *L. pneumophila* interaction with human type II pneumocytes, we conducted *in vitro* investigations using <sup>35</sup>S methionine-labeled bacteria and A-549 pneumocytes. We first demonstrated that the bacterial adherence to the A-549 cells is dramatically increased in the presence of physiological concentrations of zinc ions. This effect was shown to be dose-dependent and was not observed using nickel or copper ions, indicating that *L. pneumophila* has evolved a zinc-dependent adherence mechanism to interact with type II pneumocytes. Using green fluorescent protein-producing *L. pneumophila* and fluorescence microscopy, we showed that this zinc-dependent epithelial adherence mechanism is predominantly expressed when the bacteria are grown at 30 °C compared to 37 °C. In addition, we have shown that i) exponentially-growing *L. pneumophila* adhere more efficiently to A-549 cells than non replicating bacteria, ii) pre-treatment of A-549 cells but not *L. pneumophila* with zinc stimulates the bacterial cytoadherence, and iii) pre-treatment of *L. pneumophila* with trypsin abolishes the zinc-stimulating effect. Together, these observations suggest that the zinc-dependent adherence of *L. pneumophila* relies on the recognition of a zinc-binding pneumocyte receptor by a protein adhesin exposed on the *L. pneumophila* surface. Investigations are currently in progress to isolate this *L. pneumophila* adhesin as well as its pneumocyte receptor.

**Session III**  
**Surveillance, control and prevention**





## **PORTUGUESE SURVEILLANCE PROGRAMME IN LEGIONNAIRES' DISEASE. ONE YEAR AFTER, AN OPPORTUNITY FOR A COMMENT**

Marques Teresa

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Department of Microbiology and Parasitology, Faculdade de Ciências Médicas-  
Universidade Nova de Lisboa, Portugal*

As in many other countries, in Portugal the true annual incidence of Legionnaires' disease is not known and this disease is surely under diagnosed.

Since 1999, two collaborators officially represent our country in the European Working Group for Legionella Infections (EWGLI), for Epidemiology and Microbiology.

Because this is an important problem of public health, efforts have been made by the Microbiology Laboratory of Hospital de Santa Cruz (HSC), one of the collaborators nominated, in order to forward information to different professional groups potentially involved and to improve the laboratory diagnosis.

From 1987 until now, 123 cases have been there diagnosed. In addition, a *Legionella* laboratory equipped with molecular biology techniques was created at the Faculdade de Ciências Médicas (FCM), in Lisbon. Random Amplified Polymorphic DNA (RAPD), Amplified Length Polymorphism (AFLP) and Sequence-based Typing (SBT) give support to characterization of the strains isolated.

On 1999, Direção Geral de Saúde (National Health Authority) established the clinical notification for Legionnaires' disease. Unfortunately, clinical collaboration has not been very strong.

Aiming the reinforce of surveillance, on April 2004, a National Surveillance Programme has been created, coordinated by the National Health Authority (DGS), National Institute of Health (INSA) and HSC/FCM, integrating clinical and laboratorial notifications. EWGLINET case definitions were recommended to standardise information. Laboratories were encouraged to use, at least, urinary antigen detection and isolation by culture.

Evaluating the results of the first year of this "integrated notification system" we can understand that the fight against a heavy and complicated scheme is just beginning.

Our National Surveillance Programme has still a long and difficult journey to achieve a better knowledge of the epidemiology of Legionnaires' disease in Portugal.

## A FOUR-YEAR EXPERIENCE OF A CHLORINE DIOXIDE TREATMENT FOR THE CONTROL OF LEGIONELLA IN A HOSPITAL WATER SYSTEM

Ricci Maria Luisa<sup>1</sup>, Dell'Eva Italo<sup>2</sup>, Scaturro Marisa<sup>1</sup>, Baruchelli Piergiorgio<sup>2</sup>, Fabbri Luca<sup>2</sup>, Ottaviani Massimo<sup>1</sup>, Guizzardi Franco<sup>2</sup>

<sup>1</sup> *Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Roma, Italia*

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Since nineties, several cases of hospital-acquired Legionnaires' disease (LD) occurred in a hospital of 940-bed in Northern Italy. A microbiological investigation revealed a hot water system colonization by *L. pneumophila* serogroups 1, 6, and 8 at a concentration ranging  $10^3$ - $10^5$  CFU/l. Thermal shock and/or hyperchlorination were performed at pre-determined intervals to contain *Legionella* colonization and control measures introduced to ensure circulating hot water temperatures maintained around to 50 °C, the maintenance of all distal sites also carried out regularly. An active cases surveillance was also implemented.

In 2000 a chlorine dioxide generator in continuous was installed at the hospital water system. From 2000 to 2003 according to the fixed limit of the Italian drinking water regulation, an initial concentration of about 0.4-0.5 mg/l of ClO<sub>2</sub> (0.2-0.3 mg/l at the distal sites) was introduced. A computer was used to record all ClO<sub>2</sub> concentration data and microbiological monitoring was performed by collecting hot water samples on a average basis of three/four months. By this procedure no cases of hospital-acquired LD were notified between 2000 and 2001, but six cases occurred between 2002 and 2003. For this reason, at the start of 2004 due to the persistence of variable levels of *Legionella* ( $10^3$ - $10^4$  CFU/l) shock treatments were performed by increasing the ClO<sub>2</sub> concentration to 0.6 mg/l but, surprisingly, six cases still occurred, one in January and five in August.

In conclusion, the establishment of the disinfection procedure did not prevent the occurrence of *Legionella* in the hospital water system nor that of new cases of LD.

Possible causes affecting the efficacy of the disinfecting procedure might be: the complex and old plumbing system with several dead legs; the lack of a continuous flow of water in the plumbing system (e.g. stagnant water in unoccupied rooms or unused showers of occupied rooms); the inconstant water flushing performed by frequently changing staff; the possible existence of a *Legionella* ClO<sub>2</sub> resistance strain in spite of the initial hyperchlorination and continuous treatment with ClO<sub>2</sub>. In fact in years 1990-1994 a single genomic profile was observed in *Legionella pneumophila* serogroup 1 isolates from LD cases and from environmental strains.

These results indicate that the lack of a good control of *Legionella* colonization when using ClO<sub>2</sub> in a complex water system is not only due to human failure but is strongly dependent on several unpredictable events.

## **SIMULTANEOUS OCCURRENCE OF AMOEBAE AND LEGIONELLAE IN COOLING TOWERS IN SOUTH OF FRANCE**

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*Legionellae* survive in environment as intracellular parasites of free living amoebae in which they can multiply. Cooling towers are potential point source contamination due to these organisms and could be responsible of large community outbreaks of Legionnaires' disease. The objectives of this work were determining potential links between *Legionella* and amoebae occurrence and the physical chemical variables that could be explicative of their occurrence. Cooling towers samples were collected during 5 months from May to September 2004 in a region of Southern France where 510 cooling towers are registered. This period, in a Mediterranean climate, is favourable of *Legionella* multiplication. The occurrence of *Legionella* and amoebae on 71 cooling towers was identified by collecting water samples. For some towers, additional information such temperature, conductivity and pH were identified. *Legionella* were analysed according to standard AFNOR T90-431 method for 55 samples or by real time PCR (Polymerase Chain Reaction) for 16 samples. For amoebae, membrane with 0.45 µm porosity was placed on medium NNA covered with *Escherichia coli* after filtration of 1L. For some samples, amoebae were quantified according to MPN method.

Amoebic genera principally prevailing were *Naegleria* sp and *Acanthamoeba* sp; there were also in lowest percentages *Hartmannella* sp, *Willaertia* sp; some small size amoebae could not identified. Amoebae were found in 62 samples (87.3%). *Legionella* were found in 26 samples (36.6 %). *Legionella* sp concentrations range from 0 to 2E+5 CFU/l and from 0 to 8E+6 GU/l and amoebae from 0 to 2300/l. The pH varied between 7.5 and 9.8. Temperatures were between 15 and 36.9 °C and conductivities between 188 and 9900µS/cm. Chi-square test performed for amoebae and *Legionella* occurrence was not significant (p=0.09). Nevertheless, high concentrations of *Legionella* go with high concentrations of amoeba. Temperature higher than 25 °C is significantly linked to *Legionella* occurrence while conductivity values did not discriminate *Legionella*. Cooling towers are generally treated and these disinfectants are active against *Legionella* but generally inactive against amoebae which are more resistant. *Legionellae* were always associated to the presence of amoebae while amoebae presence does not necessary involve the simultaneous presence of *Legionellae*. For one sample, a low contamination with *Legionella* sp (850 GU/l by real time PCR) was not associated to amoebae occurrence. DNA detected could correspond to non cultivable bacteria or to dead cells after disinfection. *Legionella* cultivability could be enhanced by amoebae presence. Amoebae seem to be the predisposing factor to the occurrence of *Legionella* in cooling towers.

## SEVEN CASES OF TRAVEL-ASSOCIATED LEGIONNAIRES' DISEASE IN TURKEY

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We examined clinical, laboratory and radiologic findings in 7 patients (4 men and 3 women; mean age 60.7 ±11 years) with travel-associated Legionnaires' disease in Turkey. All the patients were tourists. Suspected sources of infection for all patients were hotel water systems. Five patients had underlying diseases. The diagnostic methods of Legionnaires' disease were Legionella urinary antigen assay in seven cases, a four fold rise in antibody titer in one cases, and isolation of *Legionella pneumophila* in respiratory samples in one case. The following presenting symptoms and findings that characterize Legionnaires' disease were seen: a temperature of more than 39 degrees C (six patients), disorientation or gait disturbances (four patients), diarrhea (two patients), hyponatremia (four patients), and rhabdomyolysis (one patients). Progression of infiltrates despite appropriate antibiotic therapy occurred in three cases. Regarding severity, the illnesses were moderate in two patients and severe in five patients. Appropriate antibiotic therapy was started all cases in the first eight hours on admission. Only one patient with endotoxic shock on admission died. We conclude that Legionnaires' disease must be kept in mind in patients with severe pneumonia. Early diagnosis and treatment are the important factors predicting prognosis.

## IS THERE A PROFESSIONAL RISK OF LEGIONNAIRES' DISEASE? RESULTS OF A MULTICENTRIC SEROLOGICAL STUDY

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**Aims.** Despite *Legionella* spp. frequently contaminate water distribution systems of private and public buildings, the risk of infection/disease among workers is scarcely known. A multicentric survey was carried out to evaluate the seroprevalence of *Legionella* antibodies in different exposure risk groups accurately studying the related risk factors. Pneumonia events in the last 5 years and flu-like symptoms in the last year were also recorded.

**Methods.** A total of 817 subjects were examined, divided in categories by risk: hospital workers, health personnel of dentistry units, plumbers, HIV positive subjects, subjects affected by COBP, and blood donors as controls. Serum prevalence was evaluated by IFA test, assuming 1:128 as the cut-off titre. All participants filled a standardised questionnaire to collect information about anamnestic data and personal, environmental and work-related risk factors.

**Results.** As a total, 24.5% of subjects were seropositive, and the most frequent titre was 1:256. The *Legionella* antibodies frequency was significantly higher in hospital personnel compared to controls, with a gradient according to job position: nurses (34.1%), physicians (25.5%), and hand workers (17.2%). Geographic differences in *L. pneumophila* serogroups were observed with a gradient from north to south: in Milan, Modena, Torino and Florence (north-centre Italy) more than 80% were *L. pneumophila* sg7-14 antibodies, whereas in south Italy *L. non pneumophila* antibodies prevailed, followed by *L. pneumophila* sg1-6. These trends resembled the environmental contamination of the examined area. HIV-positive subjects showed the lowest prevalence of seropositivity despite these patients were repeatedly recovered in contaminated hospitals. No association was found between seropositivity and both pneumonia and flu-like symptoms. Furthermore, antibodies did not correlate with the main known factors for Legionnaires' disease.

**Conclusions.** Workers exposed to contaminated waters are at major risk for *Legionella* infection in terms of antibodies production. However, the seroprevalence of *Legionella* antibodies is not related to the risk factors for the disease and/or to pneumonia events, suggesting that professional risk for Legionnaires' disease if exists is quite limited.

## PREVALENCE OF LEGIONELLA ANTIBODIES IN TWO DANISH TOWNSHIPS

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**Introduction.** There has been a rather constant high incidence (~20 pr. million) of Legionnaires' disease in Denmark. Denmark differs from most other countries in the fact, that larger outbreaks have never been described. We have previously described a cluster in the township of Randers with an incidence 2.5 higher than in the rest of the county. The present study was intended to describe the seroprevalence in Randers and a similar township (Vejle). During the study period (spring 2004) the incidence in Randers was similar to the overall incidence.

**Materials and Methods.** Blood samples from 312 and 404 blood donors living and in Randers and Vejle, respectively were analysed for antibodies to Legionella by Legionella immunofluorescence test (LAT) with plate grown and heat inactivated *L. pneumophila* serogroup 1 to 6 and *L. micdadei* and *L. bozemanii* as antigens. Donors with a LAT titre of  $\geq 128$  were defined as positive for antibodies to Legionella (any antigen).

Blood donors in Denmark are healthy volunteers, doing it for free.

**Results.** 23% (165) of the blood donors had antibodies, 21% in Randers and 33% in Vejle ( $P=0.157$ ). 6% had  $LAT \geq 256$ . There was a higher seroprevalence of  $LAT \geq 256$  in Vejle ( $P=0.033$ ). No difference in prevalence of antibodies in age groups ( $P=0.801$ ), and sex. 30% were positive for just one serogroup, 62% for 1-3 serogroups, and 38% for more than 3 serogroups. The overall seroprevalence of *L. pneumophila* serogroup 1 was 13% in the study population; 56% of the positive. No difference in serogroups between the towns.

**Discussion.** As there was no difference in distribution of positive LAT in the two towns and as the incidence in Randers is equal to the rest of Denmark in the study period, the seroprevalence in the study can be approximated to the overall seroprevalence in Denmark. The overall seroprevalence for  $LAT \geq 128$  was 23% (13% for *L. pneumophila* serogroup 1) among healthy individuals, despite that no bigger outbreaks have ever been described. The overall seroprevalence in a study population in Sweden was <1% ( $LAT \geq 16$ ) for *L. pneumophila* serogroup 1, which was the only used antigen. The reason for the high seroprevalence of Legionella antibodies in Denmark is unknown. It needs further studies to clarify if the cause is generally continuously exposure for Legionella, or there are other causes.

## **BROMINE TREATED SPA POOLS ARE MORE LIKELY THAN CHLORINE TREATED POOLS TO HAVE POOR MICROBIOLOGICAL QUALITY. AN UPDATE OF THE HEALTH PROTECTION AGENCY SPA POOL PROJECT**

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Typically the water in commercial spa pools is treated with an oxidising biocide, such as chlorine or bromine, and filtered through a sand or diatomaceous earth filter. The temperature of the water is usually 30-40°C with a high bather load in comparison to swimming pools. The potential for nutrient accumulation can make spa pools an ideal environment for infectious organisms to multiply. Problems frequently arise when those operating the pool do not have sufficient understanding of the risks posed by infectious organisms and how they can be controlled. Spa pools have been linked to outbreaks of infectious disease and are the third most common cause of Legionnaires' disease.

During 2002 and 2003 a survey of the microbiological quality of spa pools in the Greater London area was conducted by the Health Protection Agency (HPA) London Food Water and Environmental Microbiology Laboratory (LFWE) together with Local Authority Environmental Health Departments. Water samples were collected by local authority officers and were tested for routine microbiological parameters and for the presence of *Legionella* spp.

A significant finding was that samples taken from bromine-treated pools were more likely to fail both routine parameters and have *Legionella* present than those from pools treated with chlorine. The microbiological quality of water samples taken from sites such as sample taps and drainage points was more likely to be poor than those taken directly from the pool. There is also evidence to suggest an association between test failure and the type of filter used, with samples from pools with a cartridge filter having a higher failure rate than those with a sand filter.





**Session IV**  
**Molecular epidemiology and typing schemes**



## **VARIABLE GENETIC ELEMENT TYPING: A QUICK METHOD FOR EPIDEMIOLOGICAL SUBTYPING OF *LEGIONELLA PNEUMOPHILA***

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Recent analysis of the three sequence genomes of *Legionella pneumophila* suggest that different genes and genetic island are present/ absent in different strains. Based on published sequences and on own sequence data we developed several PCR assays for the detection of 11 different genes that are present/ absent in different *L. pneumophila* strains. The typing system proved to be reproducible and reliable. Data could be obtained within one day and recorded as binary codes. The index of discrimination among unrelated strains was lower than for sequence based typing of pulsed-field gel electrophoresis but could be substantially improved when used in conjunction with serotyping.

In five examples strains isolated from patients with community or nosocomially acquired pneumonia and from environmental sources were shown to be indistinguishable.

## **COMPARISON OF FOUR METHODS FOR MOLECULAR SUBTYPING OF *LEGIONELLA PNEUMOPHILA* AS IMPLEMENTED DURING AN EPIDEMIC CLUSTER OF LEGIONELLOSIS IN ROME**

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The widespread presence of *L. pneumophila* in natural and man-made aquatic environments creates a need to establish epidemiological links between clinical and environmental isolates so as to be able to assess infections and their sources. To this aim, several molecular typing protocols have been proposed based on macrorestriction and/or amplification of genomic DNA. DNA fingerprinting techniques have become the method of choice to assign epidemiological relationships of *L. pneumophila* strains.

In this study, we have used monoclonal antibodies, pulse-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) and sequence-based typing (SBT) methods to establish the clonal relation within an epidemic cluster that caused an outbreak of fifteen cases of Legionnaires' disease and one death, in Rome. In particular, we have compared the international standard AFLP to other two molecular methods, PFGE and SBT, to test their comparative usefulness in establishing the genomic correlation between the clinical and environmental epidemic cluster isolates. The results obtained by PFGE compared well with those of AFLP. Both methods produced the same genomic patterns in clinical and environmental isolates of the epidemic cluster, and they were indeed dissimilar from the control strains. Five genes were sequenced for the SBT analysis and 100% identity was found between the amplicons of the epidemic cluster. We could observed that, while all methods were helpful, the SBT technique is very simple to apply and highly reproducible, with easy-to-interpret results, easy comparable with the database for SBT, so that it could be considered as a useful addition to the actual gold standard AFLP. We will present these results with particular relevance to the relationship robustness-costs of the methods.

# EPIDEMIOLOGICAL GENOTYPING OF *LEGIONELLA PNEUMOPHILA* SEROGROUP 1 BY FLUORESCENT-AMPLIFIED FRAGMENT LENGTH POLYMORPHISM: AN INTER-PLATFORM COMPARATIVE STUDY

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This study compared the reproducibility and epidemiological concordance of double-enzyme fluorescent-Amplified Fragment Length Polymorphism (f-AFLP) analysis for genotyping of *Legionella pneumophila* serogroup 1. F-AFLP was performed on three different platforms (one gel- and two capillary-based) in different laboratories from three Countries (DE, UK, IT): (i) an ALF Express (Amersham Pharmacia), (ii) a CEQ 8000 DNA Analysis System (Beckman Coulter), and (iii) an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). A well-characterised set of 50 strains of *L. pneumophila* serogroup 1 was used (Phase II collection), which had previously been analysed by a standardised non-fluorescent single-enzyme AFLP protocol used by members of the European Working Group for Legionella Infections (EWGLI) for the epidemiological typing of *L. pneumophila*. F-AFLP data were imported into BioNumerics (Applied Maths) and analysed using the Pearson correlation similarity coefficient using a range of parameters. Dendrogram outputs were converted to arbitrary types, after selection of a specified percentage similarity threshold, and results were compared to those obtained using the standard non-fluorescent method. The results were broadly concordant with those generated by non-fluorescent AFLP. Using optimised settings for each f-AFLP method to analyse the panel of 50 strains, epidemiological concordance ( $E$ ) values of 1.00 and reproducibility ( $R$ ) values of 1.00 were obtained and the number of types ranged from 9-15, compared to  $E=1.00$  and  $R=1.00$ , with 16 types, for the non-fluorescent protocol. This study demonstrates the potential of f-AFLP to type strains of *L. pneumophila* serogroup 1 on all three platforms, although inter-platform comparison of f-AFLP data was not achieved. We conclude that F-AFLP analysis may have a role in the fingerprinting of multiple isolates in Legionella outbreak investigation. However, further work demonstrating intercentre reproducibility, epidemiological concordance and discriminatory index, using the same platform, is required prior to type designation and the development of identification libraries.

## PHENOTYPIC IDENTIFICATION AND SUBTYPING OF *LEGIONELLA PNEUMOPHILA* STRAINS BEING INSUFFICIENT FOR SEROGROUPING

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Forty three *Legionella* strains that could not be typed into the known serogroups were confirmed to be *L. pneumophila* by DNA sequencing of the *mip* gene. The phenotypic identification on the species level was performed by (i) Mip protein detection using a *L. pneumophila* specific monoclonal antibody, (ii) whole fatty acid analysis (Microbial Identification System, MIDI, software CLIN40, (iii) MONOFLUO anti-*Legionella pneumophila* Staining Reagent (BIO-RAD Laboratories), and (iv) Legionella Latex Test (serogroup 1 and serogroups 2 to 14 ; Oxoid, Basingstoke, UK).

The immunoblot analysis using the *L. pneumophila*-specific anti-Mip antibody of the “Dresden Panel” recognized all strains. The MIDI software Clin40 recognized unambiguously 79 % of all strains as being *L. pneumophila*, 16 % were identified as *L. pneumophila* but being an atypical strain and the identification was failing for two strains.

The identification using MONOFLUO anti-*Legionella pneumophila* staining was successful for all but one strain, which shows only an equivocal fluorescence.

The Legionella Latex Test serogroup 1 was negative for all strains which confirms the high specificity for serogroup 1. The Oxoid kit recognising serogroups 2 to 14 resulted positive for all strains but one which showed only equivocal agglutination. Nevertheless, the agglutination was weak and was delayed for many of the strains which were judged to be positive. Furthermore, similar reactions were found for four strains which could not be confirmed as *L. pneumophila* by *mip* gene DNA sequencing.

In order to obtain additional information on serological level, all strains were tested by ELISA with five serogroup-cross-reactive monoclonal antibodies which recognise lipopolysaccharide epitopes located on different ATCC or NCTC serogroup reference strains. Altogether, six serological pattern were found. These results substantiate, that the established serotypes do not reflect the true antigenic diversity of *L. pneumophila*, but demonstrate also that a serological fingerprint using monoclonal antibodies is useful for identification of seldom occurring *L. pneumophila* strains being insufficient for the established serogrouping.

# CHARACTERIZATION OF CLINICAL STRAINS OF *L. PNEUMOPHILA* SEROGROUP 1 ISOLATED IN PORTUGAL USING MONOCLONAL ANTIBODIES, AMPLIFIED LENGTH POLYMORPHISM AND SEQUENCE-BASED TYPING

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In Portugal, the majority of Legionnaires' disease cases are caused by *Legionella pneumophila* serogroup (sg) 1. The aim of this study is to provide more information about Portuguese clinical isolates, using three different approaches: Monoclonal Antibodies (MAbs), Amplified Length Polymorphism (AFLP) and Sequence-based typing (SBT) using the housekeeping gene *mip* (macrophage infectivity potentiator).

We studied 45 strains of *Legionella pneumophila* serogroup 1, collected in 12 Portuguese hospitals, from 1987 to 2005. Twenty-two of these strains were isolated from nosocomial infections and 13 from community-acquired infections. On 10 strains we were not able to identify their origin. Serotyping was done using Indirect Immunofluorescent Test with serogroup-specific MAbs of the Dresden Panel. With this method is possible to characterize the isolates as having or not the virulence associated epitope recognized by the MAb 3/1. The molecular typing methods (AFLP and SBT) were done according the EWGLI protocol. The AFLP is a discriminatory and reproducible method that allows the fingerprint comparison of the strains. The SBT is a genotyping method that allows the comparison of the *mip* sequences in study with a EWGLI SBT database, without the disadvantage of the AFLP interpretation of gel images. Otherwise the *mip* gene, encoding a proteic virulence factor, has sufficient sequence variability to be a reliable and discriminatory marker for the epidemiological typing of *Legionella pneumophila* sg1. Most clinical isolates belong to MAb subtype Allentown/France (31/45; 68,9%). The other subtypes found were: Philadelphia (6/45; 13,3%), Knoxville (3/45; 6,7%) and Benidorm (1/45; 2,2%). Four of the strains had no MAb type identified with the panel used. Nine types were obtained with the genotyping method AFLP: 013 London (24/45; 53,3%), 028 Rome (6/45; 13,3%), 019 Dresden (6/45; 13,3%), 012 Rome (2/45; 4,4%), 017 Lugano (1/45; 2,2%), 020 Rome (1/45; 2,2%), 030 Stockholm (1/45; 2,2%), 027 Madrid (1/45; 2,2%) and 024 Copenhagen (1/45; 2,2%). From the SBT analysis we found four different allele number. The majority of the strains (27/45; 60,0%) had the allele number 10, followed by the allele number 15 (7/45; 15,6%), allele number 1 (3/45; 6,7%) and allele number 5 (1/45; 2,2%). Some of the strains don't have an allele number defined by this method. From the three different methods used, we didn't observe different patterns between nosocomial and community acquired strains. The majority of strains had the same MAb subtype (Allentown/France), AFLP type (013 London) and SBT allele number (10).

## UPDATE ON THE CONSENSUS SEQUENCE-BASED EPIDEMIOLOGICAL TYPING SCHEME FOR *LEGIONELLA PNEUMOPHILA*

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A six locus sequence-based typing scheme for the epidemiological typing of clinical and environmental isolates of *Legionella pneumophila* has been developed by EWGLI members and made available on the web ([www.ewgli.org](http://www.ewgli.org)). Multi-centre studies have demonstrated the excellent typability, reproducibility and epidemiological concordance of this method. To date 108 of 134 (>80%) of the *L. pneumophila* serogroup 1 strains in the original EUL collection have been fully characterised by this method resulting in 52 distinct sequence types (STs). Following analysis of additional strains including those from other serogroups, over 67 STs have now been identified. However, further analysis of non-serogroup 1 isolates is required to validate this scheme for these strains. Currently, the number of allelic variants in each locus is as follows: *flaA*, 12; *pilE* 14; *asd*, 16; *mip* 22; *mompS* 18, *proA*, 17. The four most common STs are: 1,4,3,1,1,1 which has been found in isolates from 10 European countries, including the Paris strain for which the complete genome has been determined; 4,7,11,3,11,12 found in 5 European countries; 2,3,9,10,2,1 found in 4 European countries; 7,6,17,3,13,11 found in 3 European countries; 3,4,1,1,14,9 found in 2 European countries and the USA, and is the ST found in the type strain of *L. pneumophila* (Philadelphia-1). Ten STs have been found in only 2 countries, and the remaining STs have so far only been found in isolates from one country, including the Lens strain. As the data-set increases significantly, it is intended to review the contribution of each locus and/or combination to the scheme. This scheme has already been successfully implemented to investigate cases of travel-associated legionellosis as well as localised outbreaks in participants' countries.



## **Satellite workshops**



## **USE OF THE “DRESDEN PANEL LP A-M” FOR SEROTYPING OF THE EWGLI SBT PROFICIENCY PANEL N. 2**

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The lipopolysaccharide of *Legionella pneumophila* is characterized by a high degree of heterogeneity that determines fifteen serogroups and many monoclonal subgroups. The efforts for complete serotyping of isolates, for which at least 22 sera are necessary, are justified only in few cases. More than 90 % of all patient isolates in Europe belong to three serogroups representing in total only eight major serotypes. In order to determine these eight serotypes exactly and to categorize the others on broad-spectrum levels, the “Dresden Panel Lp A-M” was established. It consists of 12 reagents (A to M), being able to distinguish between serogroup 1 (including 8 monoclonal subgroups), serogroup 3, serogroup 6, and different clusters of all other serogroups. The Dresden laboratory has found one to three positive reactions for all *L. pneumophila* strains tested. Results are indicated as code of the positive reagents.

The “Dresden Panel Lp A-M” was distributed to 19 European Legionella laboratories for typing of the EWGLI SBT Proficiency Panel (study code n. 12 to 21). The SBT Proficiency Panel contained two pairs of related strains, (i) two strains not belonging to serogroup 1 and (ii) two strains belonging to serogroup 1, monoclonal subgroup Bellingham. Both pairs were recognized on the same serological level by 95 % of the participating laboratories. Immunofluorescence test was the most commonly used method, but the procedures for antigen preparation were varying between the different laboratories. The obtained results substantiate that for serotyping by using monoclonal antibodies, especially also for recognizing non-serogroup 1 epitopes, a standardized method is essential. A simple procedure will be presented.

The “Dresden Panel Lp A-M” is able to differentiate among the major serotypes of virulent strains but enables also the recognition of all other groups of isolates belonging to rare serotypes which are usually less virulent. Therefore, in the context of timely outbreak investigation this panel is an ideal screening tool for examination of large numbers of isolates in order to find out which should be further investigated by molecular typing methods.

## SEQUENCE-BASED TYPING OF *L. PNEUMOPHILA*: RESULTS OF THE SECOND EWGLI MULTI-CENTRE SBT PROFICIENCY PANEL

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**Introduction.** Previous studies have assessed the reproducibility and discriminatory power of Sequence-Based Typing (SBT) for the epidemiological typing of *Legionella pneumophila* isolates and shown this method to have excellent reproducibility and epidemiological concordance. The standard SBT protocol and associated on-line database are accessible via the EWGLI website ([www.ewgli.org](http://www.ewgli.org)). Users can query the database and identify pre-defined alleles and determine complete allelic profiles. The second SBT proficiency panel of 10 coded *L. pneumophila* strains was distributed by the coordinating centre to 19 centres in 13 countries.

**Aim.** To test the ability of participating centres to correctly type the second proficiency panel, using the standard SBT protocol and on-line database.

**Methods.** The panel consisted of 10 coded *L. pneumophila* serogroup (sg) 1 and non-serogroup 1 strains and comprised two sets of epidemiologically related and six epidemiologically unrelated isolates. Following sequence analysis on-line tools were used to submit allelic profiles.

**Results.** Results were received from 15 centres in 11 countries. Overall the SBT performance was excellent; all six targets (*flaA*, *pilE*, *asd*, *mip*, *mompS* and *proA*) were tested by 12 out of 15 centres, of which six centres reported the correct SBT allelic profile for all proficiency panel strains. Eight out of 15 centres were able to correctly type the entire panel using at least four targets (*flaA*, *pilE*, *asd* and *mip*). Where discrepancies were seen, review of the results revealed two main areas of mis-identification; 1) probable transcription errors, and 2) use of sequencing reactions of poor quality. A more detailed summary of results will be presented at the meeting.

**Conclusion.** Data from the second EWGLI multicentre SBT proficiency panel once again illustrates that the standard SBT method and its associated online database are robust, reproducible and widely applicable. However, proficiency panel testing yet highlights technical difficulties and/or sequence quality issues which are now being addressed by a combination of technical advice and automated quality scoring of DNA sequencing chromatogram files.

## **THE ADDITIONAL USE OF TWO GENE SEQUENCES INCREASES THE DISCRIMINATORY POWER OF THE EWGLI SEQUENCE BASED TYPING SCHEME FOR *LEGIONELLA PNEUMOPHILA***

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In the last years the EWGLI group established a typing scheme based on the DNA sequence of six *L. pneumophila* genes: *flaA* (major flagellin subunit), *pilE* (type IV pilus), *mip* (macrophage infectivity potentiator), *asd* (aspartate dehydronase), *ompS* (major outer membrane protein). By using this approach several clones responsible for cases in patients throughout Europe could be defined (Fry *et al.*, this meeting).

In order to increase the discriminatory power of this typing system we used the sequence data of *gspA* (global stress protein) and *neuA* (neuraminic acid synthetase) with the panel of 79 unrelated EWGLI strains. After PCR and sequencing by standard techniques resulting sequences were assembled and multiple sequence alignment was performed by using the BioNumerics software package (Applied Maths, Belgium).

The additional use of the *neuA* gene sequences increased the discriminatory power whereas use of the *gspA* sequences did not. Thus some clonal groups in the existing typing scheme could be differentiated further.

## APPLICATION OF BIOINFORMATIC TOOLS FOR THE EWGLI SEQUENCE-BASED TYPING AND IDENTIFICATION SCHEME

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**Background.** Previous studies have demonstrated the utility of Sequence-Based Typing (SBT) analysis for the epidemiological typing of *Legionella pneumophila* isolates. An on-line database is now accessible via the EWGLI website ([www.ewgli.org](http://www.ewgli.org)) which can be queried allowing users to identify pre-defined alleles.

**Aims.** To develop and improve bioinformatic tools to facilitate:

- (i) database curation, designation of novel allele types and sequence types (STs)
- (ii) automated quality assessment of sequence chromatograms

**Methods.** Web interfaces were constructed in php and Perl-cgi. Quality assessment of DNA sequencing chromatogram files was accomplished by using Phred and Phrap and manipulation of Bioperl 'SequenceWithQuality' objects.

**Results and Conclusions.** Web interfaces provide a logical, convenient method to allow curatorship of the database. It is anticipated that users will be able upload trace files for individual SBT alleles which are then assessed for quality, assembled into a contig and trimmed to the correct length using markers specific to each allele. These sequences are compared to all allele types in the database, resulting in either an exact match or the closest match. The chromatogram files can then be viewed with any mismatch positions highlighted, together with a table which shows quality and allele information. This allows the user to ascertain whether: the type exists in the current database, is a putative new allele, or is not of sufficient quality to be matched. High quality sequence data is essential for outbreak investigation and data analysis from multicentre proficiency SBT analysis has confirmed that mis-identification readily occurs when poor quality trace files are used. This online tool will help to systemically check sequence quality and will assist in the determination of minimum standards for a range of sequence quality parameters ensuring that only high quality data is accepted for allele and ST identification.

**Poster abstracts**





# **1. LEGIONELLOSIS-EPIDEMIOLOGICAL UPDATE OF SITUATION IN GERMANY, 2004**

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Germany*

**Background.** Since 2001 laboratory confirmed *Legionella* infections are notifiable in Germany. Results of the analysis of notified cases, trends and quality of reported data are presented.

**Methods.** Detection of *Legionella* is reported to local health departments, where complementary clinical information is ascertained. Anonymized case-based data are transmitted electronically via state health departments to the central database established at the Robert Koch Institute (RKI).

**Results.** In 2004, 475 cases (29 (6.1%) deaths) were notified with an overall incidence of 0.6 cases/100,000 population (2001: 331 cases; 2002: 414 cases; 2003: 395 cases). In comparison to the previous years an increase in reported cases was observed. A slight seasonal increase in incidence was found in summer and autumn. The age-specific incidence rises with age. The median age of the cases was 60 years. 66.1% of cases were men. As in the years before most cases were due to *Legionella pneumophila* (86.3%) and urinary antigen detection was the most frequently used laboratory method (51.3%). A history of foreign travel was reported in 89 cases (20.4%), the most frequent travel countries being Italy (24 cases), Turkey (17 cases) and Spain (13 cases). Three outbreaks were detected: one outbreak occurred on a cruise ship (7 cases), one was linked to a hospital (5 cases) and one was assumed to have its cause in a private home (3 cases). In 50.5% of all reported cases information on specific risk factors were available (previous year only 26.8%). In 41.4% the source of infection was assumed to be in the private home followed by exposure in hotels (35.7%) and hospitals (17.2%). In 2.6% the disease was contracted in a nursing home. In addition, in 3.7% it was not possible to classify cases according to disease acquisition, because they were reported as stay in hotel/hospital or nursing home.

**Discussion and Conclusions.** In Germany nationwide surveillance of *Legionella* infections has become well established. Although a rising number of cases were detected the incidence is lower than reported from most other European countries. According to hospital based studies, an underreporting must be assumed in Germany because *Legionella* diagnosis is not sought often enough. Due to modifications in the reporting software reporting of exposure risks has become more specific and complete but should still be improved to allow further enhanced and early detection of outbreaks.

## **2. SURVEILLANCE OF LEGIONELLOSIS OUTBREAKS IN SPAIN, 1999-2003**

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**Background.** *Legionella* causes outbreaks with different impact both in number of cases and case fatality ratio and provokes alarm in the population irrespective of its magnitude. Since big outbreaks have occurred in recent years in Spain, procedures for controlling risk installations have been implemented in most of the Autonomous Regions. In 2003 a new law was approved aimed to the prevention and control of this disease at the national level.

**Methods.** The reports of outbreaks and clusters received in the National Centre of Epidemiology (NCE) have been used as sources of information. These reports are sent by EWGLINET and the Spanish Epidemiological Surveillance Network (SESN). The SESN collects the reports of Legionellosis outbreaks investigated by the health authorities of each Spanish Autonomous Region. Each report includes a summary of the epidemiological and environmental investigation carried out.

**Results.** Two hundred and fourteen outbreaks were notified to the Spanish surveillance scheme from 1999 to 2003. The total number of cases involved were 1,973 and 87 people died. One hundred and ten were community outbreaks and 20 were nosocomial. The 84 left were travel related outbreaks or clusters. The total number of notified outbreaks rose from 16 in 1999 to 60 in 2003. The case fatality ratio was higher in nosocomial outbreaks than in those of community or travel origin (28 % versus 2 % and 6% respectively). Two Regions notified 77% of the community or nosocomial outbreaks (100/130). For travel related outbreaks four regions notified 75% (63/84). The causative agent most frequently identified was *L. pneumophila* serogroup 1 Pontiac. Microbiological results in the environmental investigation were unknown or negative in 47% of the outbreaks or clusters. The source of infection was confirmed (epidemiologically and/or microbiologically) in 53% of the outbreaks (51% were the water systems and 20% the cooling towers). Chlorination deficiencies were the most frequent, followed by deficiencies in the water systems.

**Conclusions.** The number of outbreaks of any origin (community, nosocomial and travel) has increased from 1999 to 2003. In Spain travel associated outbreaks or clusters have special relevance. Outbreaks are more frequent in the Autonomous Regions placed besides the Mediterranean coast. The ascertainment of the source of infection is low.

### **3. ENVIRONMENTAL RESULTS FROM THE EWGLINET SCHEME, POST-GUIDELINES (MID-2002 TO 2004)**

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**Background.** On 1<sup>st</sup> July 2002, EWGLINET introduced European guidelines for the control and prevention of travel-associated Legionnaires' disease. This poster presents the results gathered by the surveillance scheme between July 2002 and December 2004.

**Methods.** When a cluster of travel-associated Legionnaires' disease is detected by EWGLINET, the guidelines allow the country of infection two weeks to complete a 'Form A' report, confirming that a risk assessment has been completed and control measures are in progress. The country of infection then has a further four weeks to complete a 'Form B', confirming that control measures and sampling have been carried out, and stating whether Legionella was found.

If investigations are not completed on time, EWGLINET publishes details of the accommodation site in question on its public website ([www.ewgli.org](http://www.ewgli.org)). This publication does not imply that the accommodation is the source of the infection, but that the co-ordinating centre cannot be confident that the site has adequate control measures in place.

**Results.** Between July 2002 and December 2004, 237 new clusters and 70 cluster updates were identified by EWGLINET. Investigations at 140 sites returned positive samples, and the proportion of positive sites reached over 60% in 2004 (of sites that were sampled). Although single sites do not require investigation, 50% of those that have been investigated and reported to EWGLINET, have been positive.

51 sites have been published on the public part of the EWGLI website since the guidelines were introduced. Tour operators are especially interested in these reports.

35 sites have been investigated satisfactorily under the guidelines, but have subsequently been associated with further cases (so-called 're-offenders').

**Conclusions.** The guidelines have been operating successfully for over two years. The results emerging from the scheme provide interesting and useful feedback for participating countries, allowing them to judge their relative success at investigating cluster sites, and allowing EWGLINET to quickly assist any countries that need to improve their standards of investigation.

## **4. AN EVALUATION OF THE SENSITIVITY OF THE ITALIAN NATIONAL LEGIONELLA SURVEILLANCE SYSTEM**

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Introduction: Italy reports 600-700 cases of Legionnaires' disease each year, the third highest in Europe. There is a wide variation in the number of cases reported in each of the 21 regions. In 2002, 244 cases were reported in Lombardia and no cases were reported in Abruzzo, Molise and Sicilia. It is not known whether these variations are a result of actual differences in the distribution of the disease in Italy or they are a result of differences in the notification of Legionnaires' disease between regions. In this study we attempted to evaluate the completeness of the passive surveillance system by comparing it with the hospital records system (Scheda di Dimissione Ospedaliera, SDO).

Methods. All cases of Legionnaires' disease notified to the national surveillance system in 2002 were compared with cases recorded as a clinical diagnosis of pneumonia in the SDO database and that had laboratory confirmation of Legionella during 2002. Comparison was made on a regional basis, as SDO data were provided by each region. Data linkage was carried out using Access. The number of unreported cases and the total numbers of cases in the population were estimated using the capture-recapture method with two independent, individual data sources.

Results. Three regions did not participate in the study, 13 regions sent SDO data and 5 are in the process of sending data. Very preliminary results indicate that for the 13 regions there were 35 cases of Legionnaires' Disease that were not notified, this is equal to 5.5% of the total notifications to National Registry. The 35 cases would bring the total number of cases diagnosed in 2002 from 639 to 674.

Preliminary conclusions. In the 13 regions, where data was available for analysis, under notification was quite low at 5.5% and homogeneous.

## **5. ISOLATION OF *LEGIONELLA PNEUMOPHILA* FROM HOSPITALS IN NORTHERN GREECE**

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The purpose of this study was to estimate the risk of legionnaire's disease in hospitals of Northern and Central Greece by isolating *Legionella pneumophila*.

During the last year we collected 189 water samples from ten hospitals. The samples were filtered and cultured on BCYE agar enriched with L cysteine. Each sample was cultured in triple, untreated and after heat and acid treatment. *Legionella pneumophila* was isolated in 29 samples from six out of ten hospitals. The main sources of the microorganism were the hot water supply system, (5/10 hospitals), the cooling towers (2/10) and the cold water supply system(2/10).

*Legionella pneumophila* serogroup 1 was isolated in 11 samples obtained from five hospitals in concentrations ranging from 1000 to 100000 CFU/ml. *Legionella pneumophila* strains serogroups 2-14 were isolated in 16 samples obtained from 5 hospitals. Most of the serogroups 2-14 strains were isolated from rooms where high risk patients were hospitalized (ICUs, Bone marrow transplantation units, Immunodeficient patients Units). Our results show that the risk of Legionnaires' disease in the examined hospitals is increased even though no clusters of nosocomial pneumonia were observed at the meantime.

## **6. A PRACTICAL CASE OF *LEGIONELLA* SPP. CONTROL IN COOLING TOWERS**

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In Spain, there has been very important legionellosis outbreaks. Among them: 1996, Alcalà de Henares there were 249 cases; 2000, Barcelona: 47 cases; 2001, Murcia: 449 cases and 2002 Mataró: 80 cases. Most of these outbreaks were caused by cooling towers. In order to control these outbreaks the government published a strict guideline in 2003. This normative oblige to the owners of the cooling towers, sanitary water systems, swimming pools etc to maintain the cleaning and disinfection of the water systems and control physic-chemical parameters and micro organisms regularly, to avoid legionellosis. And if *Legionella* spp. exceeds fixed limits, appropriate corrections have to be applied.

In this work we present the monitoring of five cooling towers from two adjacent buildings of Barcelona city. *Legionella* spp., viable counts and physic-chemical parameters were analysed regularly during one year and a half from water samples of the towers and water inputs, according to the guideline. In spite of the regular cleaning and disinfection with a commercial biocide, the first analysis showed the presence of *Legionella pneumophila* in four of the towers, with numbers ranging from  $1,2 \times 10^5$  CFU/l to  $2 \times 10^2$  CFU/l. To eliminate *Legionella* the towers were decontaminated with a chlorination shock and cleaning two times. But, each time, after one month of the decontamination process, even a commercial biocide was added regularly into the water of the towers, *Legionella* was detected again. Finally, the biocide was changed by hypochlorite added in continuous. From then, *Legionella* has not been detected in any of the towers and as well as the numbers of viable counts diminished.

## **7 THE ITALIAN MULTICENTRIC STUDY ON LEGIONNAIRES' DISEASE: FOUR YEARS OF ENVIRONMENTAL AND EPIDEMIOLOGICAL SURVEILLANCE**

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The Italian multicentric project addresses relevant aspects of Legionnaires' disease with various Aims. a) to deepen the knowledge on *Legionella* diffusion in water systems studying the efficacy of different control measures; b) to evaluate the frequency of nosocomial and community-acquired legionellosis among pneumonia recovered in big hospitals; c) to define risk factors associated with the disease, including the basal immune profile and some genetic markers involved in host resistance/progression to infection; d) to draw an epidemiological map of *Legionella* serotypes/genotypes collected throughout the epidemiological survey; e) to establish the degree of correlation between serotypes, genotypes and virulence.

In order to search, isolate and subtype *Legionella* strains from hot water systems, a large survey on public and private buildings (hospitals, hotels, homes) was conducted. The sampling sessions included a detailed investigation on the environmental and structural factors possibly associated with contamination. *Legionella*, predominantly *L. pneumophila*, was detected in 22.4% of domestic hot waters, in 69% of hotels and 100% of hospitals. The main risk factors for contamination were structural such as the building age (hotels) and the centralisation of water distribution systems (homes), but differences according to *Legionella* species and serogroups were observed. An active surveillance lasting four years was carried out to detect cases among recovered pneumonia. It was based on the systematic detection of urinary *L. pneumophila* antigen (EIA), together with the search of the microorganism in respiratory secretions, and seroconversion (IFA) as confirmation test. Cases were included in a case-control study to evaluate environmental, clinical and personal characteristics associated with the disease. As controls, both healthy subjects and patients affected by other pneumonia were recruited.

The study detected 165 cases on 4,340 examined patients with a frequency of 3.8% for community acquired case and about 5.0% for nosocomial cases. A detailed description of the patients' characteristics and the environmental investigation to detect the source of infection are presented, together with the preliminary results of the case-control study.

## 8. HOSPITAL WATER SYSTEM COLONIZATION

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**Aim.** To find out the origin of 5 cases of pneumonia caused by *Legionella pneumophila* diagnosed in nurse staff.

**Methods.** An epidemiological study has been performed to seek out the agent which was suspected to be present inside the water system. Twelve water taps have been chosen, as sample sites, to be representative in accordance with the behaviour of the affected working people and characteristics of the building. The sampling has been realized consistent with the Health Office Guide Line published on 05/05/2000. Samples, concentrated by membrane filtration (porous size 0,22 µm), have been plated on BCYE agar cultural medium: i without any treatment; ii after acid treatment; iii after heat treatment. Cystein-dependent colonies with typical features have been identified by serologic test.

**Results.** The presence of *Legionella pneumophila* has been excluded from water supply duct, while the agent was present inside the water hospital loop, with contamination ranged value among 300 and 15.000 CFU/l. After first hyperchlorination of the water pipes, the contamination value was found unexpectedly increased, especially in those samples which were found previously positive.

Contamination value remained constant after two more disinfections of water supply consisting in both hyperchlorination treatment and thermal shock, while substitution of all the filters of the sampled taps was finally successful with eradication of *Legionella pneumophila*.

**Conclusions.** Control and prevention measures are essential to avoid colonization of water system and the risk of Legionnaires' disease.

This measures are represented by assessment of exposure risk (occupational hazard inclusive), formation and information of the worker employed in management of water system (ability, experience, instruction), therefore the risk is well handled; consequently it is possible to contain pipes disinfection treatments and to achieve better results in the economical cost-benefits analysis.

In this case study preventive measures to reduce the risk of Legionnaires' disease were not carried out so that the elimination of contamination agent by water system colonized has required several disinfection treatments which were very expensive for the establishment and, moreover, they have greatly inconvenienced patients.



## **9. BIOLOGICAL TREATMENT OF INDUSTRIAL WASTEWATER: AN OVERLOOKED SOURCE OF LEGIONELLA INFECTION?**

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The source for *Legionella* infection can often be found in technical constructions, having ideal conditions for the bacteria to multiply, thereby making the source of infection a man-made problem. Well-known sources of *Legionella* infection are cooling towers, spa pools and lukewarm hot water in showers.

Recently a new source of *Legionella* infection was detected during an epidemiological investigation concerning a case of *Legionella* infection in Sweden.

The case was a 59-year old male and heavy smoker, who fell ill at the end of November 2004. The investigation revealed that the case had a problem with the water system of his house and thereby took all his showers at his work place, which was a paper mill. The shower he used was sampled and *Legionella* was found (>300 000 CFU/l before flushing and 1300 CFU/l after flushing for five minutes). However, typing of the bacterial strains from the case and from the shower showed different types and more possible sources of infection were investigated. It was found that on an average day he worked in a 100-meter vicinity from the biological treatment plant of the industrial wastewater. Water from this plant was sampled and found positive for *Legionella pneumophila* serogroup 1, Benidorm, which matched the strain from the case by molecular typing (AFLP). The amount of *Legionella* in the plant was incredibly high, 180 000 000 CFU/l. Repeated samples from the aerated ponds showed even higher amounts of 1 000 000 000 CFU/l. Both the inlet raw water to the factory and a single sample from the inlet wastewater to the plant, were negative for *Legionella*.

Other paper mills in Sweden have now been sampled at their biological treatment step of wastewater. Five of ten plants have been identified with high levels of *Legionella* bacteria in the aeration ponds. These aeration ponds usually have a set water temperature of 37 °C, to stimulate bacterial growth for nitrogen and phosphorus break down, making ideal temperatures for *Legionella* growth.

A potential problem in identifying contamination is that laboratories may not dilute the wastewater samples they process and thereby give false negative results.

More research is needed to identify and minimize the factors for *Legionella* growth and estimate the risk for public health from this kind of water treatment.

## **10. SEROPREVALENCE OF ANTIBODIES TO *LEGIONELLA* AMONG ELDERLY PEOPLE LIVING IN FRENCH NURSING HOMES**

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Elderly persons are at greater risk of legionellosis than younger people. Also, fatalities mostly occur among subjects older than 50 during Legionnaires' disease epidemics. However, while *Legionella* is an important pathogen for the elderly, its prevalence is unknown. The aim of this study is to investigate the prevalence of *Legionella* antibodies among elderly population.

Participants were recruited in 25 nursing homes between October 2003 and December 2004. More than 500 subjects agreed to participate in the present study and provided blood samples at the end of a 4 months follow-up period. Information on age, gender, smoking and disease histories (respiratory and immunizing) was also collected.

The anti-*Legionella* antibody titers measured by indirect immunofluorescence (Pr. J. Etienne Lyon, France) are: anti-*Legionella pneumophila* (serogroups 1 to 10), anti-*L. micdadei*, anti-*L. bozemanii*, anti-*L. dumoffii*, anti-*L. jordanis*, anti-*L. gormanii*, anti-*L. longbeachae* (serogroups 1 and 2) and anti-*L. anisa* antibodies.

No case of legionellosis was detected during the follow-up period. Seroprevalence for all antibody titers of 1/32 or less was 14%, greater than prevalences observed in european studies of blood donors. For antibody titers  $\geq 1/64$ , this elderly population had a higher prevalence for *Legionella sp.* (13%) than for *L. pneumophila* (5%). Mainly anti-*L. jordanis* antibodies (7%), followed by anti-*L. dumoffii* antibodies (5%) and anti-*L. micdadei* antibodies (5%) were identified, for antibody titer levels greater than 1/64. 26 (6%) volunteers had a antibody titer  $\geq 1/128$  (9 for *L. pneumophila*).

Although the health significance of these prevalence rates remains to be discussed, low titers prevalence of *Legionella* antibodies among elderly adults seems greater than among younger healthy populations. These results will be further analyzed according to the *Legionella* contamination of the nursing homes water system.

## **11. EXPOSURE OF ELDERLY PEOPLE TO *LEGIONELLA* ISOLATED IN SHOWER HOT-WATER AEROSOLS IN NURSING HOMES: FIRST RESULTS FROM THE FRENCH LEGION’AIR MULTICENTRIC STUDY**

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*Legionella* is ubiquitous in aquatic environments. Inhalation of aerosols containing bacteria is the main mode of transmission. Outbreaks of legionellosis are often attributed to aerosols from cooling towers and complex hot water systems.

The epidemiological LEGION’AIR project aims at characterizing exposure of elderly people to *Legionella* while showering, and at studying the relationship between concentrations of *Legionella* in water and/or air and health (clinical and biological responses).

From October 2003 to September 2004, we carried out environmental studies about shower hot-water aerosols in 19 nursing homes. Each sampling point was chosen so as to represent shower water quality for one or a group of elderly people based upon thorough diagnostic of the water system in each nursing home. The sampling protocol was performed using a standardised procedure (pressure and temperature of hot water at maximum, aerosol sampling after shower flow while windows and doors were closed).

At each shower site, water and aerosol samples were taken. Investigations of water samples included temperature and pH measurements, and counting of total bacteria, cultivable *Legionella* and hybridised *Legionella* according to FISH. Two biological aerosol sampling methods were used: impaction and impingement.

A total of 412 water samples were collected at 103 showers. 13 out of the 19 (68%) nursing homes were contaminated by cultivable *Legionella*: 131 (32%) out of 412 water samples contained *Legionellae*, ranging from <100 CFU/L (detection limit) to 2,0.107 CFU/L. Mainly *Legionella pneumophila* was isolated (66%). Cultivable *Legionella* represent only 14 % of Legionellaceae that could be detected by FISH. On 102 aerosol samples: 4 (3%) site points were found contaminated by cultivable *Legionella* and 39 (38%) by aerosolised *Legionella* detected by FISH. In this context, no significant statistical linear relationship was found between water and aerosol *Legionella* contamination (analyzed by culture or FISH).

In conclusion, *Legionella* was detected in aerosol samples by FISH while none or few bacteria were cultivable. *Legionella* contamination in water system in nursing homes is found similar to that described in buildings and hospitals. These first results show no simple relation between water and aerosol contamination by *Legionella*.

## **12. RISK FACTORS AND ANTIBODIES TO LEGIONELLA IN SELECTED GROUPS**

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This is an epidemiological observational study to evaluate, on subjects with *Legionella* high exposition risks, the antibody titles.

Some demographic characteristics or life usage are evaluated, particularly those about behaviours or situations that seem facilitating *Legionella* infection.

Aims. Evaluating, within homogeneous groups in terms of demographic characteristics or life usage, the immunization against *Legionella*; identifying, within homogeneous groups in terms of demographic characteristics or life usage, associated or protecting factors for *Legionella* infection; hypothesizing individual behaviours standard or preventive actions relating to structures potentially subjected to *Legionella* contamination; developing a data bank regarding the antibody title versus time, for people clearly affected by *Legionella pneumoniae*.

Population Target: University students less than 35 years old. Frequent users (at least 20 times a years over the last one year or more) of sport yard facilities. Hospital staff with three years service or more. In-patients, over 70 years, with a back ground of at least 5 hospitalizations over the last three years. Other persons clearly affected in the past by *Legionella pneumoniae*.

The subjects will be informed about the project contents by giving them copy of the information text, the consensus to participate to the project must be collected; they will fill up a data sheet collecting demographic information and life usage regarding past infective lung diseases or chronic respiratory diseases and they will be submitted to double blood samples in order to determine the antibody title versus *Legionella*.

Analysis. Screening tests: Premier test Elisa *Legionella* of Meridian Bioscience Inc.

Analysis and sample storing: S.C. di Analisi Chimico-Cliniche e Microbiologia of Ospedale Fatebenefratelli of Milano with serum storage at -80 °C.

Data evaluation. The data collected will be presented in the final report as follows: the demographic characteristics of all groups will be described.

The relative frequencies associated to the confidence limits to 95% will be calculated for the following parameters: immunity status versus *Legionella*; associated factors to infection; protective factors to infection. The combination of presence/absence of antibodies versus different risk factors (using binary codification standard) will be evaluated with statistical test of univariate type; furthermore, by using the signifying variables resulting from the univariate analysis, the single impact of all factors, properly adjusted taking into account the presence of the others, will be calculated by means of a backward stepwise model (SAS-Release 8.02).

### **13. LEGIONELLA INFECTIONS: INFORMATION AND PREVENTION CAMPAIGN IN THE LOCAL HEALTH UNIT ASL ROMA A**

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The aim of the campaign was to evaluate the diffusion of the disease in the territory of the local health unit (Azienda Sanitaria Locale, ASL) ROMA A in years 2001-2003, in order to verify which were the structures mainly involved. The purpose was also to promote an accurate information campaign.

The structures mainly interested have turned to be those receptive such as hotels. A booklet containing relevant information was prepared and made available to a representative sample of hotel managers. Before the distribution of the above booklet, a questionnaire was submitted to the managers in order to evaluate their knowledge regarding *Legionella*.

The result was an insufficient knowledge of the issue, nevertheless it was of remarkable interest to the participants.

A second questionnaire will be shortly distributed in order to verify booklet efficacy.

## **14. COLONIZATION OF LEGIONELLA SPECIES IN HOTEL WATER SYSTEM IN TURKEY**

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**Aim.** The prevalence of *Legionella* species was evaluated in hotel water distribution systems in Alanya; an important tourism centre in Turkey.

**Method.** Water and swab samples were obtained from 29 hotels during August 2003-June 2004 years. Water samples were collected 100 ml in sterile containers and concentrated by membrane filters with a pore size of 0.45µm. Heat treatment was used for the decontamination of other microorganisms. Samples were spread on BCYE, MWY and GVPC agar plates and incubated at 35°C with 2.5 % CO<sub>2</sub> in a humid atmosphere. Cystein dependent colonies were identified by latex agglutination.

**Results.** *Legionella* species were detected in 26 (19.1 %) of 136 water samples and in 24 (21.6%) of 111 swab samples. In ten hotels all samples were negative for *Legionella* species. *L. pneumophila* was the predominant species, and particularly serogroup 6 (50% of all isolates) was frequently isolated. The colony count was lower than 10<sup>2</sup> CFU/100ml in 12 samples (46.2%), and between 10<sup>2</sup> and 10<sup>3</sup> CFU/100ml in 8 samples (30.7%). In 6 samples (23.1%), the count was higher than 10<sup>3</sup> CFU/100 ml.

**Conclusions.** *L. pneumophila* serogroup 6 is the most common isolate detected in hotel water systems in our study. As the urinary Legionella antigen may be negative in persons infected with *L. pneumophila* serogroup 6, it is important to take sputum culture to enhance recognition of Legionnaires' disease.

## **15. RHABDOMYOLYSIS AND ACUTE RENAL FAILURE ASSOCIATED WITH LEGIONNAIRES' DISEASE**

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**Introduction.** Rhabdomyolysis can be a life-threatening disease if not treated immediately. We reported a case to remind clinicians that *Legionella* can cause rhabdomyolysis followed acute renal failure in the absence of other obvious signs of Legionnaires' disease.

**Case report.** A 67 year-old tourist man with diabetes mellitus presented with a one week history of disorientation and gait disturbance. He didn't report fever and cough. He took no medications apart from oral glucose-lowering agents and warfarin. On presentation to the emergency department, his vital signs were as follows; temperature 37.6°C, pulse rate 96 beat per minute, and blood pressure 110/70mmHg, respiratory rate 28 breaths per minute. Physical examination was unremarkable. Laboratory data were as follows: Blood urea nitrogen: 45mg/dl, Creatine: 2.5mg/dl, Na:132meq/L, K: 3.89mEq/L, CPK: 8109IU/L, AST: 696IU/L, ALT: 154 IU/L. Chest X-ray showed patchy infiltrate. The Legionella urinary antigen assay was positive. Treatment with levofloxacin plus clarithromycin was started in the first eight hours on admission. Although he was also treated with IV fluids, mannitol and loop diuretics, creatine continued to increase. He underwent hemodialysis. After one week of hospitalization, on his demand, the patient flew back home to his country by ambulance airplane.

**Conclusion.** In the present of risk factors for Legionnaires' disease; patient with an unknown cause of rhabdomyolysis should alarm clinicians to consider possibility of Legionnaires' disease.

## **16. ENVIRONMENTAL INVESTIGATIONS AND SEARCH FOR *LEGIONELLA PNEUMOPHILA* IN WATER**

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Recently, an increased frequency of cases of pneumonia caused by *Legionella* has been registered, in both Italy and abroad, particularly in hospitals and among travellers. For this reason, the Health Ministry and the Regional Health Authorities have asked the organizations involved in this field to carry out control and prevention activities. Consequently, in collaboration with the Health Agencies of the Provinces of Udine, Trieste, Gorizia, and Pordenone, the Analytical Service of the Provincial Department of ARPA Friuli-Venezia Giulia in Udine (regional reference laboratory for studies on *Legionella*), in 2002, initiated a program to monitor aqueducts and/or well water in structures where these pathogens find a favourable growth environment and which represent an important reservoir for the circulation of the disease.

Regional health administrations, stimulated by the Regional Health Authority, have also placed into action a biannual auto-regulatory plan in the highest risk wards of their hospitals.

In the three years between 2002-2004, 195 public structures including hospitals, hotels, swimming pools and nursing homes were monitored and 2923 water samples were taken from the hot water circuit; *Legionella pneumophila* was found in 7.76% of these samples. In particular, prevalence ranged from 10.78% in hospitals to 5.68% in hotels. It was 7.18% in nursing homes and 6.33% in swimming pools. *Legionella pneumophila* serotypes 2-14 were more common (71,50%) than serotype 1 (28,50%). Other *Legionella* spp. were uncommon. In 2 hospitals there was mixed contamination from type 1 and types 2-14. The presence of *Legionella pneumophila* did not cause any foci of infection in these structures.

Accurate and timely sampling allowed the identification of contamination sources in private homes and hotels in 7 cases out of the 16 that had been flagged, thus avoiding dangerous re-infections.

Contamination disappeared after adequate treatment (superheating of water, shock hyperchlorination or with stabilized silver and hydrogen peroxide), as demonstrated by later controls in all the structures. It should be noted, however, that in hospitals, contamination tends to reappear some time after the treatment (about 12-24 months). In conclusion, since legionellosis is considered an emerging disease and a serious public health problem with important implications, even economic, we underline the importance of accurate prevention and control activities. In addition, the correct management of hot water systems can even eliminate the problem at its source.



## **17. THE EXPERIENCE OF THE REGIONAL LEGIONELLA REFERENCE LABORATORY OF PIEMONTE (ITALY) IN ENVIRONMENTAL INVESTIGATIONS: PARTICULAR CASES**

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Since 1986, the Regional Legionella Reference Laboratory of Piemonte is involved in environmental investigations. From 1986 to 2004, the demands for support increase so much that the last year more than 2000 samples have been processed (water and other matrices). During these years some particular cases of *Legionella* contamination occur and in particular 4 cases have been distinguished for their peculiarity.

1<sup>th</sup> case. In 2000 because of a case of legionellosis we were involved in an environmental investigation in a private house in north of Piedmont; *Legionella* spp. was not detected in the water supply, but it was detected in the stagnant water of a garden irrigation pipe that was mostly used in summer and always leaved out. *Legionella pneumophila* sg1 was detected in biofilm and *Legionella pneumophila* sg1 and sg6 were detected in the pipe stagnant water and the concentration was 830.000 CFU/l.

2<sup>nd</sup> case. In 2001 because of a Legionnaires' disease linked to a centrifugal pumps manufacture we were involved in an environmental investigation. *Legionella pneumophila* sg1 was detected in the water of the test tank of the centrifugal pumps and in the shower of the dressing room of the staff with a concentration respectively of 1000 of CFU/l and 3000 CFU/l.

3<sup>rd</sup> case. In 2003 we supported a Local Health Authority with the hygienic quality evaluation of the dental unit water systems. The percentage of the water samples contaminated by *Legionella* was 31.3%, the percentage of aerosols samples contaminated by *Legionella* was of 17.6% and the percentage of biofilm samples contaminated by *Legionella* was only 3.8%. This is probably due to the difficulties with biofilm sampling because of the very little diameter of the dental unit water systems pipes. The *Legionella* spp. counts reached 81000 CFU/l in the water samples and 1200 CFU/50 millilitre in aerosol samples. The *Legionella* species and serogroups isolated were *Legionella pneumophila* sg3, sg6, sg8, *Legionella bozemanii* and *Legionella* species.

4<sup>th</sup> case. In 2003 because of a case of Legionellosis occurred to a telephony employee (he died) we were involved in an environmental investigation in the phone company manholes. The phone company manholes were wet and there was stagnant water. We took different kind of samples: water, biofilm and soil. We found *Legionella pneumophila* serogroup 1, 8, 14 and *Legionella* spp, and *Legionella bozemanii* and *Legionella gormanii* with concentration up to 940.000 CFU/l.

## **18. ROLE OF INDIRECT BACTERIAL INTERFERENCE AND PARASITISM IN *LEGIONELLA PNEUMOPHILA* WATER ECOLOGY**

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Numerous studies on *Legionella pneumophila* have widely demonstrated that protozoa play a crucial role in the environmental survival of *Legionellae*. Much less known is the possible influence of aquatic bacteria, which may interfere with protozoan host survival and growth. In order to better understand this indirect interference, we investigated the capability of *L. pneumophila* (serogroup 1 and 2-14) and other aquatic germs to enter the amoebae and multiply within them. Co-cultures of the tested bacteria with a strain of *Acanthamoeba polyphaga* were conducted in Synthetic Tap Water (STW), incubated at 30 °C and observed for 24 h. At regular intervals, intracellular bacterial counts were determined in order to verify the entry capability of the germs. At the same time, extracellular bacteria were counted and their concentrations were compared to the control without amoeba, in order to reveal the effect of the protozoa on the germ survival and the capability of intracellular replication.

Both *L. pneumophila* strains were able to enter and find a shelter inside amoeba, but not to intracellularly multiply, maybe because of the short period of co-incubation. *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Aeromonas hydrophila* showed a much higher capability to enter and multiply.

When *Legionella*-amoeba co-cultures were conducted in the presence of the other aquatic bacteria, we found that *B. cepacia* was able to reduce *Legionella* entry inside *A. polyphaga*.

Finally, by studying endocytosis in the same co-cultures through light microscopy, we observed two different mechanisms. The first was characterized by the presence of few bacteria inside protozoan cells and the absence of lysis within 24 h. This kind of endocytosis was observed with *L. pneumophila* and *B. cepacia*. The second type showed the presence of a high number of bacteria inside infected amoebae and a violent lysis of host cells after a few hours of co-incubation. This mechanism was found with *A. hydrophila* and *P. fluorescens*, whereas *P. aeruginosa* presented a similar behaviour, but didn't cause the host lysis or suffering.

This study confirms that bacteria belonging to the natural micro flora of aquatic environments are able to differently interfere with protozoa and therefore they could influence the ecology of *L. pneumophila*.

## **19. MEASURES FOR ELIMINATING *LEGIONELLA PNEUMOPHILA* FROM A SPA WATER AEROSOL PLANT**

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During a multicentric active surveillance study of pneumonia due to *Legionella pneumophila*, carried out in various Italian hospitals, a case was recruited with suspected exposure to spa water aerosol. The patient was a 74-year-old woman with serologically confirmed Legionnaires' disease. The epidemiological investigation revealed that 10 days prior to the appearance of symptoms, the woman had been exposed to the aerosol of sulphurous spa water. The water, with a source temperature of 16-17 °C, was pumped from the wells, heated at 36-38 °C and fed into a line leading to the aerosol equipment, without undergoing any purification treatment.

The water was analysed by taking samples from the well, the feeding line and from the final aerosol devices. *L. pneumophila* was absent from the well, but was found in the water from the lines at a concentration of 400.000 CFU/l and in the nebulizers at levels ranging from 3.300 to 1.800.000 CFU/l. The colonisation consisted of a mixture of *L. pneumophila* serogroup 1 (12%) and serogroup 5 (88%). Various attempts at decontamination were made, firstly by introducing chlorine dioxide into the line (20 ppm for 6 hours at the distal entry points). This resulted in the elimination of Legionella from the water in the lines, but not from the nebulizers (residual concentrations of 75 to 3.300 CFU/l). A second attempt was therefore made, consisting of decrustation with nitric acid and disinfection with peracetic acid (20 ppm for 6 hours). However, this treatment too failed to resolve the contamination (concentrations of 800 to 80.000 CFU/l). The disinfectants were evidently not able to efficiently reach all the points where the *Legionella* had settled and grown. In order to obtain total abatement it was necessary to carry out a radical restructuring of the plant, involving the replacement of the old nebulizer benches with new aerosol equipment that could be subjected to a new system of programmed hygiene control. The protocol adopted, and still in use, consisted of heat shock treatment at 70 °C for 3 hours, for 3 nights a week, followed by a lowering of the water temperature to 36-38 °C for use in the plant. In addition, 3 times a week superheated steam (1 atmosphere) was introduced for 1 hour into the nebulation machines, before opening to the public. Six months after the application of this protocol the monthly checks have revealed no evidence of *Legionella*.

## **20. AMOEBAE: A NEW CHALLENGE FOR DRINKING WATER DISINFECTION**

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Some amoebae (e.g. *Acanthamoeba*, *Hartmannella*) are recognized hosts for a great number of pathogenic bacteria, such as *Legionella*, which are able to multiply within their hosts, and can be later expelled and thus disseminated in new environments. It has been demonstrated, for instance, that a single amoeba can release thousands of *Legionella*. In that case, it has also been demonstrated that multiplication within protozoa activates *Legionella* virulence.

Because of their ability to form cysts, amoebae are extremely resistant to disinfectants, and thus can also protect the ingested bacteria from the action of these disinfectants. Recent studies conducted at the CIRSEE on *Legionella* control in domestic water systems have confirmed the role of amoebae in the resistance of these bacteria to chemical disinfectants, and their ability to quickly re-colonize water systems once the treatment application is stopped.

Study description. The aim of this study was to assess the ability of the treatments currently recommended for *Legionella* control to reduce amoebae in domestic water systems. Disinfection trials were conducted at laboratory scale on planktonic amoebae.

A first series of disinfection trials was conducted on a mixed population of *Hartmannella*, *Acanthamoeba*, and *Vahlkampfia*, at a concentration of approximately 103 cysts/l.

A second series of disinfection trials was conducted specifically on *Acanthamoeba*, with the aim of quantifying more precisely the efficacy of the treatments on this particularly resistant amoeba.

Results and perspectives. In the first series of trials, *Acanthamoeba* was found to be able to survive 2 hours at temperatures up to 60 °C, or chlorine concentrations up to 50 mg/l, whereas *Hartmannella* and *Vahlkampfia* were totally eliminated in these conditions.

In the second series of trials, the necessary Concentration x Time factors (for chlorine, chlorine dioxide, ozone) and time (for temperature) for a >2 log reduction of *Acanthamoeba* were determined. In all cases, a >2 log reduction could be achieved with high disinfectant concentrations or temperatures. Such concentrations or temperatures can be applied only with the purpose of a shock treatment.

## **21. CLINICAL AND ENVIRONMENTAL SURVEILLANCE OF LEGIONNAIRES' DISEASE IN A NORTHERN ITALIAN HOSPITAL**

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**Objectives.** A surveillance programme was activated within university hospital of about 1,000 beds to search for cases of Legionnaires' disease in patients affected by either community and nosocomial pneumonia and to evaluate the environmental diffusion and control of *Legionella* spp. in hot water distribution systems.

**Methods.** After training courses to awaken the medical and technical staff, the surveillance protocol started. It included the search for Legionella urinary antigen (EIA method) in all recovered patients affected by pneumonia and the control of environmental contamination by planned maintenance and periodical sampling of hot water distribution system. Samples from taps and showers of high risk wards were collected throughout a year both before and after hyperchlorination (sodium hypochlorite 70 ppm).

**Results.** From September 2003 to December 2004, a total of 405 pneumonia were followed, 135 of which considered of nosocomial origin, and 14 cases of community-acquired Legionnaires' disease were detected.

*L. pneumophila* was isolated in 76.5% of the hospital water samples, with a range from 40 to 950,000 CFU/l, and 61% of positive points exceeded 10<sup>4</sup> CFU/l, suggesting the opportunity to carry out appropriate measures of decontamination. Among the positive sites, 53.8% were contaminated by serogroups 7-14, 38.5% by serogroups 2-6, and only one sample by serogroup 1. Disinfection substantially reduced the contamination (<1,400 CFU/l), although only one sample became negative. Six months later the contamination returned to pre-treatment levels and in some units increased. Furthermore, the distribution of *L. pneumophila* serogroups changed, as serogroup 1 and serogroup 6 were more frequent.

**Considerations.** The implementation of preventive measures within a hospital with at risk patients independently on the appearance of cases has undoubted advantages such as to awaken the health personnel. Thus the attention to clinical diagnosis allowed us to detect community-acquired cases otherwise unknown and ascertained the absence of nosocomial legionellosis during the study period. We confirm that non continuous chlorine disinfection may be ineffective to eradicate *Legionella* spp. from hospital water distribution systems, and in case may produce adverse and undue effects such as to select serogroups more resistant to environmental stress situations and frequently associated with the disease.

## **22. ROLE OF DIRECT BACTERIAL INTERFERENCE AND BIOFILM IN *LEGIONELLA PNEUMOPHILA* WATER ECOLOGY**

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In *Legionella pneumophila* water ecology a crucial role may be played by the relationship with other microorganisms which constitute the natural flora of these habitats. Besides protozoa, even aquatic bacteria may positively or negatively influence Legionella survival, representing a possible key factor for the pathogen distribution and persistence in the environment. Aim of the present study was to investigate the interactions occurring between *Legionella* and other aquatic bacteria, particularly within the biofilm. The ability of producing antagonistic compounds such as Bacteriocin-Like Substances (BLS) was tested in eight aquatic strains, selected as representative of water micro flora, by the deferred antagonism method. Successively, studies were focalised on the possible effect of the aquatic strains or their products on the *in vitro* development and stability of *L. pneumophila* biofilms. Suspensions of a strain of *L. pneumophila* serogroup 1 together with each of the aquatic strains in Synthetic Tap Water (STW) were inoculated in 12-well microtiter plates. Biofilm formation on the well bottom was monitored by counting sessile bacteria at regular intervals, during 12 days of incubation at 22 °C.

The tested aquatic strains differently influenced *L. pneumophila* biofilm and these results could be partially correlated with BLS production. *Pseudomonas fluorescens* SSD (from our collection) was the best BLS producer and showed the greatest negative effect on both the pathogen survival and biofilm formation, and strongly enhanced the release of Legionella from biofilm. *Pseudomonas fluorescens* ATCC 49838, and *Burkholderia cepacia* ATCC 25416 were good producers too, but less active against *L. pneumophila* in the deferred antagonism assays, as well as in the biofilm experiments. *Pseudomonas aeruginosa* ATCC 27853 was able to inhibit Legionella growth, but didn't influence the biofilm. *Aeromonas hydrophila*, *Stenotrophomonas maltophilia* (from our collection) and *Pseudomonas putida* ATCC 49128 were endowed with a weak antagonistic activity and didn't affect Legionella biofilm. *Acinetobacter lwoffii* ATCC 15309 did not produce any antagonistic compound and was the only one able to strongly enhance *L. pneumophila* biofilm. We suggest that BLS can vary according to the producer, thus explaining the different effects observed on Legionella under our artificial conditions. Moreover, the present study shows that, besides BLS, other factors may play a role in *Legionella* growth and survival in the environment.

## **23. RESULTS OF AN ACTIVE SURVEILLANCE FOR LEGIONELLOSIS IN SOUTHERN ITALY**

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**Background.** In Italy, the cases of legionellosis have increased progressively in recent years (from 90 in 1997 to 617 in 2003), nevertheless the incidence of this disease is underestimated when compared with that in other European countries. In view of this problem, two Italian Research Programmes (project MIUR, MM06172998\_002 and project PRIN, 2002067337\_006) were launched to verify the real epidemiological situation of the disease. As part of the project, the Operative Unit of Bari carried out an active legionellosis surveillance programme.

**Materials and Methods.** Between the years 2001- 2005, 1000 patients affected by pneumonia, who were admitted to three large hospitals in Southern Italy, were tested for Legionella's urinary antigen (EIA-Ag), specific anti-*Legionella pneumophila* (L.pn) antibody (IFA-Ab) and cultural exam on biological materials (sputum, bronchial aspirate, pleural fluid, etc.) to isolate the *Legionella* species.

**Results.** Legionellosis was diagnosed in 58 patients (5.8%): 46 were found to have a community and 12 nosocomial origin, 65.6% of the subjects were over 50 years of age and 70.7% were male. In 23 patients (39.6%) only the IFA-Ab tested positive; in 21 (36.2%) only the EIA-Ag tested positive, while in 14 (24.1%) both the two tests were positive. Cultures of biological materials were positive for L.pn1 in 5 cases and for L. pn5 in 1 case.

**Comments.** The present study, carried out in only three hospitals, enabled the diagnosis of 58 cases of legionellosis over a period of about four years. When compared with the data obtained by the routine surveillance system (only 9 cases were reported in the five-year period 1996-2000), our data gives some idea to the extent of the lack of legionellosis diagnosis. It could be due to the minor importance often attributed to the etiological diagnosis of pneumonia, but it is also true that not all laboratories are equipped to diagnose this disease.

Although this problem is common to other forms of pneumonia, it is particularly serious in the case of legionellosis, because this disease is subject to compulsory notification and to an environmental surveillance in order to institute suitable disinfection measures, avoiding the further spread of the disease. In light of the above data, it is clear that the surveillance of legionellosis needs to be conducted in a more rigorous fashion.

## 24. A MULTICENTER STUDY COMPARING DIFFERENT COMMERCIAL TESTS FOR DETECTION OF LEGIONELLA URINARY ANTIGEN

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In recent years, the observation of increasing legionellosis epidemics has underlined the need to promote epidemiological surveillance programmes as well as to improve the diagnostic techniques. In Italy, the incidence of legionellosis is underestimated both because the diagnosis of pneumonia is often made by the clinical signs and symptoms (the search for the etiological agent is limited only to special cases) and because not all the laboratories are equipped for a correct diagnosis of the disease. The detection of the specific Legionella Urinary Antigen (LUA) is actually the most used method to diagnose legionellosis, but some different tests are commercially available at the moment in Italy. The Italian Working Group on Legionella Infections carried out an investigation to compare the reproducibility of three commercial tests for LUA detection versus the Biotest Legionella Urinary Antigen EIA (B-LUA), which is declared to have the highest sensitivity (SE) and specificity (SP) (99.8% and 100%), as well as the ability to recognize all species of Legionella.

**Materials and Methods.** A total of 84 urine samples (coming from the Operative Units of Bari, Bologna, Milano, Modena, Napoli and Torino) were stratified into two groups: 42 positive and 42 negative for B-LUA. Both the two groups were re-tested, in blind, for the detection of LUA by three other different tests: Bartels Elisa Legionella Urinary Antigen (BE-LUA), Binax Legionella Urinary Antigen EIA (BI-LUA) and Binax Now Legionella Urinary Antigen (BN-LUA). The SE and SP for each method were evaluated.

**Results.** The 42 B-LUA positive samples were confirmed in 41 cases by both the BE-LUA and BI-LUA and in 40 cases by the BN-LUA. The B-LUA negative samples were confirmed by all tests. The SE and SP values respectively were: BE-LUA, 97.6% and 100%; BI-LUA, 97.6% and 100%; BN-LUA, 95.2% and 100%.

**Discussion.** The difference shown in the SE and SP could be attributed to the etiology of the disease: when the infection is caused by *Legionella pneumophila 1 (Lpn1)*, some of the investigated tests are not able to identify the *Lpn1* urinary antigen. Although all the tests showed a high SE and SP, it is always recommendable to use the test with the highest SE and SP. This could be important not only to identify all the cases of the disease but also to know the real spread of *Lpn1* in the etiology of legionellosis.



## **25. CHILDREN EXPOSURE TO LEGIONELLA IN DAY NURSERIES IN THE PROVINCE OF HAINAUT (BELGIUM)**

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From September 2004 to January 2005, the laboratory for the study and the prevention of indoor pollutions (LPI) from the Provincial Institute of Hygiene and Bacteriology (in Mons) has realised systematic investigations for the presence of indoor pollutants susceptible to impair the health of young children in day nurseries. 41 crèches were included in this study. Systematically, water sampling was performed from the water heater. Culture, isolation and quantitation of *Legionellae* were performed according to the norm NF T 90-431:2003. A confirmatory test was applied by a direct immunofluorescent-antibody technique (Monofluo *Legionella pneumophila* IFA test Kit Biorad 32514). Serotype determination of the *Legionella* identified was performed by an agglutination test (Legionella latex kit, Oxoid) in order to separate serogroup 1 and serogroups 2-14.

*Legionella* were found in 12 of the 41 day nurseries (29,3%), with 10 samples containing more than 10.000 CFU/l. We found more than 10.000 CFU/l in 8 samples (5 crèches).

*Legionella pneumophila* serogroup 1 was identified in 12 samples (5 crèches), *Legionella pneumophila* serogroups 2-14 were found in 14 samples (8 crèches); a mixed representation of serogroups 1 and 2-14 was found in 2 samples (1 crèche).

From our results, it seems that, beside hospitals or workplace, water contamination in day nurseries could be an important source of children exposure to and infection by *Legionella* spp. Then, it seems that regular microbiological controls of water heater systems should be mandatory and that water heating should be applied in order to decrease *Legionella* proliferation in day nurseries.

## **26. PROGRAMME TO MINIMIZE THE RISK OF LEGIONELLA INFECTIONS IN OLOMOUC UNIVERSITY HOSPITAL**

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The University Hospital in Olomouc is a tertiary care teaching institution with 1450 beds – 166 of these are in intensive care units. In 2004, 49,000 in-patients were admitted and 20,000 operational procedures were performed. The teaching hospital comprises 50 clinics and departments with 3100 employees, including 470 physicians and 1700 nurses. Immunocompromised patients are hospitalised especially in oncological, haemato-oncological, anaesthesiological and intensive care departments and pulmonary disease clinics.

The hospital development plan to build independent operating and examination centres for 4 clinics was changed due to financial cuts and a less expensive solution had to be found. Finally, it was decided to construct a single new building with operating theatres and to connect it with existing clinics by corridors. Before the new centre was opened, samples from cold and hot tap waters were taken. Since a large number of *Legionella* were found, the start of the operating centre was postponed. The authors comment on the main reasons responsible for colonization of the water distribution system (e.g. thermoisinfection causing temperature increase in the cold tap water, blind ends of the piping system etc.) and they describe the repressive measures that had to be implemented. In order to prevent *Legionella* colonization and infections in the future, an effective programme was introduced that includes the frequent monitoring of the water distribution system, chemical disinfection by means of a chlorine dioxide generator and early screening of patients with respiratory symptoms for legionellosis. Thanks to the preventive measures, only 9 cases of legionellosis were diagnosed in our teaching hospital between 2002 and 2004 – most of them community-acquired. The preventive programme focused on *Legionella* infection prevention that is currently in place in our teaching hospital ranks among the best in Czech Republic.

## **27. ACTIVE LEGIONELLA SURVEILLANCE IN CAMPANIA REGION (ITALY): REPORT OF ACTIVITY OF LEGIONELLA REGIONAL REFERENCE CENTRE (CRL) IN 2004**

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**Background.** In 2001 in Campania Region was identified Legionella Regional Reference Centre (CRL). Since then many efforts have been done to improve the prevention of legionellosis in Campania.

**Methods.** CRL performs environmental investigation either after notification of cases to identify the source of infections, either for surveillance plans in hospitals as recommended by Italian Guidelines for Prevention and Control of Legionnaires' disease.

Water and air samples were collected from several sites (tap, shower, swimming-pools, thermal systems, cooling tower, climatization system and other).

**Results.** In 2004 CRL analyzed a total of 1040 samples.

25 structures (hotels, public offices, private houses, barracks, ships) were investigated after case notification; 18 were found to be contaminated by *Legionella*, 315 samples were collected and 58% of positive samples (32%) showed *Legionella* concentration > 10<sup>3</sup> CFU/l. Mainly *Legionella pneumophila* serogroup 1 was isolated (36%).

31 hospitals were examined and 441 samples collected with 51% containing *Legionella*. 62% of positive samples showed >10<sup>3</sup> CFU/l. *Legionella pneumophila* serogroup 1 (35%) was isolated, followed by *Legionella pneumophila* serogroup 6 (6%) and *Legionella pneumophila* serogroup 3 (5%).

284 samples were collected after disinfection methods and 21% samples was found still positive.

**Conclusion.** Regional Health Board has applying in Campania a straight surveillance network for Legionnaires' disease. As consequence hospitals have started up prevention plans. The recent Italian Guidelines for receptive structure will extend active surveillance even to touristic complexes so largely diffused in Campania.

## **28. MOLECULAR POPULATION GENETICS ANALYSIS OF *LEGIONELLA PNEUMOPHILA* IN THE COMUNIDAD VALENCIANA, SPAIN**

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*Legionella pneumophila* is associated to recurrent outbreaks in several Comunidad Valenciana (Spain) localities, especially in Alcoi (Alicante province), where social and climatic conditions seem to provide an excellent broth for bacteria growth. We have obtained the nucleotide sequence of three protein coding genes, *fliC*, *mompS*, and *proA*, previously used in MLST analysis, and 13 intergenic loci from 31 environmental isolates from Alicante province localities, including Alcoi. The analysis of these isolates has revealed a substantial level of genetic variation, with consistent patterns of variability across loci, and comparable to that found in a large, Europe-wide distributed sampling. We detected 5, 6, and 8 allele types in the three coding loci (with one new allele in *proA* and 5 new alleles in *mompS*), yielding a total of 9 different profiles. The number of alleles in non-coding loci ranged between 4 and 9, and the combined analysis of all loci resulted in 15 allelic profiles.

Among the three coding genes studied, *fliC* showed the highest level of nucleotide diversity (0.0208). Values of this parameter for non-coding loci ranged from 0.0036 to 0.0648. The partitioning of genetic variability within and between groups defined by geographical location revealed that the within population component is the largest one, as it holds about 80% of the total variation detected. The analysis of isolates sampled in different years revealed a clear temporal differentiation, with samples from 2001 being significantly distinct from those obtained in the other years. Furthermore, although linkage disequilibrium measures indicate a clonal nature for population structure in this relatively reduced sampling, the presence of some recombination events cannot be ruled out.

## **29. SEQUENCE-BASED TYPING OF *LEGIONELLA PNEUMOPHILA* AS AN AID IN THE INVESTIGATION OF HOSPITAL-ACQUIRED LEGIONNAIRES' DISEASE**

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Sequence-based typing (SBT) was used to determine the allelic profiles of three sporadic clinical isolates as well as seven environmental isolates of *Legionella pneumophila* serogroup 6, isolated at the bacteriology laboratory unit of the Karolinska University Hospital during 2004. The clinical isolates were cultured from patients with suspected nosocomial Legionnaires' disease (LD), while the environmental isolates were cultured from potable water sources of the hospital wards in the near vicinity of the three patients being investigated. The genes sequenced included *flaA*, *pilE*, *asd*, *mip*, *mompS* and *proA*, and the (SBT) protocol for the epidemiologic typing of *Legionella pneumophila* was the one proposed by the European Working Group for Legionella Infections (EWGLI). The allelic profile of all the 10 isolates in the above pre-determined order of genes was the same for all the isolates (3, 13, 1, 28, 14, 9). We conclude that the environmental strain isolated from our hospital's drinking water is identical genotypically to the 3 clinical isolates of *Legionella*. SBT may prove to be an effective aid for the epidemiological investigation of nosocomial (LD).

## **30. REAL-TIME AND NESTED PCR ASSAYS FOR THE SPECIES-SPECIFIC DIAGNOSIS OF *LEGIONELLA PNEUMOPHILA* IN WATER SAMPLES**

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*Legionella pneumophila* is a contaminant of potable waters and the most common causative agent of legionellosis, a respiratory infection which may give rise to restricted outbreaks. To detect *L. pneumophila* in water samples we developed a species-specific nested PCR and TaqMan real-time PCR for the molecular diagnosis of *L. pneumophila*. We targeted the *mip* gene, which is highly conserved among all serogroups of *L. pneumophila* species, but is divergent in other members of the genus *Legionella*. *mip* gene codes for a protein of the FK506 binding protein class and is a critical component for the intracellular life cycle of *Legionellae*. A multiple alignment of the sequences of the *mip* gene from a number of *L. pneumophila* species representative of different serogroups was performed by means of the ClustalW program in order to identify the regions of the gene with highest sequence homology, and all oligonucleotides were designed to anneal in these in regions.

Purified genomic DNA extracted from *L. pneumophila*, subs. *pneumophila*, obtained from ATCC (ATCC<sup>®</sup> 33152D) was employed as target sequence for the amplification of a 114 bp fragment from *mip* gene in the TaqMan real-time PCR reaction. The detection limits of the assay were assessed to be 10 fg. The nested PCR assay had a sensitivity of 10 pg of purified genomic DNA (ATCC<sup>®</sup> 33152D), in the first step PCR, which amplified a 338 bp amplicon, while had a sensitivity of 10 fg of purified genomic DNA (ATCC<sup>®</sup> 33152D) in the second step PCR, which amplified a 124 bp fragment. In order to validate the specificity of both the assays, the DNA extracted from *Legionellae* and non-*Legionellae* bacteria was subjected to amplification by both assays, none of the DNA belonging to bacteria other than *L. pneumophila* yielded any amplicon.

Both protocols were validated by the repetitive analysis of 124 of water samples, collected in private habitations and hospitals of Bologna district, simultaneously analysed by cultural isolation and molecular diagnosis, and the two assays exhibited overlapping sensitivities. Remarkably, both PCR assays exhibited a somewhat higher (about 10%) sensitivity than cultural isolation.

Cumulatively, the results indicate that both assays described are sensitive and specific tools for the molecular diagnosis of *L. pneumophila*. In contrast to the real-time PCR assay, the nested PCR protocol applied here was relatively simple, and did not require a specific instrumentation and competences.

## **31. PRELIMINARY RESULTS OF LEGIONELLA DETECTION FROM WATER SAMPLES WITH RT-PCR**

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**Introduction.** Culture is the gold standard for the diagnosis of *Legionella* infection and for the detection from environmental samples. However, several days are required to obtain a positive result, with most *Legionella* colonies being detected within 3-5 days. DNA techniques have shown promise for the rapid detection. In this study direct detection of *Legionella* from hospital water samples was performed by real time (RT)-PCR samples. Preliminary results are presented.

**Materials and Methods.** 33 water samples belonging to 5 hospital were analysed. Culture was performed following a standard protocol. Water samples (5l) were concentrated by filtration through cellulose acetate membrane filters (0.2  $\mu$  pore size) and resuspended in 10 ml of the same water. Aliquots (0.1 ml) of the untreated, heat-treated (50 °C for 30 minutes) and acid-washed suspensions were plated on BCYE, BMPA and MWY. The plates were incubated at 37 °C for 15 days. The strains of *Legionella* isolated were serologically typed by slide agglutination and by immunofluorescence assay. RT-PCR was performed on all water samples (1 l) after two extraction Methods. A- direct extraction on filter after water filtration; B- extraction of resuspended water used for culture. Qualitative and quantitative detection of *L. pneumophila* and *L.spp.* were then performed on each DNA extracted samples using iCycler and BIO-RAD reagents, following producer instructions.

**Results.** Culture was positive (*L. pneumophila*  $\geq$  20 CFU/l) in 14 (42%) samples. Strains were identified as *L. pneumophila* serogroup 1, 3, 6, 2-14. Results of qualitative RT-PCR were as follows: *Legionella* spp. was positive (133 G.U./L) in 32 (96.9%) samples with method A and in 33 (100%) samples with method B. *L. pneumophila* was positive (133 G.U./L) in 25 (75.7%) and in 24 (72.7%) samples respectively. Concordances between culture (detection limit 20 CFU/l) and RT-PCR (detection limit 133 G.U./l) were: 39.4% for *L. spp.* and 51.5% for *L. pneumophila* with method A, 42.4% and 69.7% respectively with method B. Discordant results of qualitative detection showed that RT-PCR was more sensitive than culture for *L. pneumophila* and *Legionella* spp. with both methods. Quantitative results for *L. pneumophila* and *Legionella* spp. were determined by comparison to standard DNAs. Precise quantitative results (CV lower than 25%) were possible on the quantification range.

**Conclusion.** Culture is gold standard for *Legionella* detection from water. RT-PCR offers rapid results, as total time required was about 5 hours. *Legionella* detection by molecular methods seems to be promising. At the moment correlation between the two methods, results interpretations and public health significance need further evaluations.

## **32. USEFULNESS OF PFGE COMBINED TO MONOCLONAL ANTIBODY TYPING IN A NOSOCOMIAL *LEGIONELLA* PNEUMONIA**

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**Introduction.** Nosocomial *Legionella* infection must be confirmed by strains typing. The aim of the study was to demonstrate the suspected nosocomial origin of a case of *L. pneumophila* serogroup 1 pneumonia by using monoclonal antibody (MAb) typing and molecular techniques.

**Materials and Methods.** Pneumonia occurred in a 53-year old immunosuppressed man, hospitalized for nine days. *L. pneumophila* serogroup 1 was isolated from sputum. Urinary antigen and serology were positive. The patient, treated with levofloxacin, recovered completely. As a nosocomial infection was suspected, epidemiological and microbiological investigations for a hospital source of *Legionella* were initiated. The strains isolated from patient and from water were typed by monoclonal antibodies (Dresden panel) and by molecular techniques. PFGE (Sfi I restriction enzyme) was performed on 1% agarose gel (CHEF DRIII). RAPD-PCR was done with Ready-To-Go RAPD analysis beads; RAPD binding pattern was visualized on 2% agarose gel electrophoresis and on polyacrylamide gel (CleanGel and Silver Staining, GenePhor).

**Results.** *L. pneumophila* serogroup 1 strains were isolated from hospital water supply and particularly from water in two rooms where the patient stayed. Tap water (103 cfu/L), shower heads water, swabs from the same sites of these rooms were all colonized by *Legionella*. A total of 31 related isolates were typed. *L. pneumophila* serogroup 1 strains isolated from the patient and hospital water showed the same monoclonal subtype (Philadelphia). Water isolates showed four PFGE profiles. *L. pneumophila* serogroup 1 strains from patient's respiratory sample shared the same PFGE pattern of some strains isolated from tap and shower water, collected from one of the two rooms where the patients stayed. PFGE binding pattern of these isolates was different from the unrelated strains. After laboratory confirmation of the suspected nosocomial case, environmental cultures for *Legionella* were immediately performed and hospital water was disinfected by thermal shock. No other case occurred.

**Conclusion.** In this study *L. pneumophila* serogroup 1 isolates belong to the same monoclonal subtype, but only some strains isolated from one room were genetically indistinguishable from patient's strains. MAb subtyping and molecular techniques are both useful to confirm the nosocomial origin of the infection. PFGE, however, is more discriminative in order to recognize the source of infection.



### **33. IQ-CHECK™ KITS, NEW REAL-TIME PCR SYSTEM FOR THE DETECTION AND QUANTIFICATION OF LEGIONELLA SPP. AND LEGIONELLA PNEUMOPHILA**

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**Introduction.** Water safety is an important part of Bio-Rad's R&D investment and commitment for industrial control.

Regular testing for the presence of *Legionella* in water supply systems is the first necessary action for preventing the disease.

**Background.** Conventional culture-based methods for the detection of *Legionella* spp. and *Legionella pneumophila* is long, time consuming and not sensitive enough; therefore they are not satisfactory for routine laboratory applications. More rapid, simple and sensitive techniques are required. PCR methods provide an accurate measure of the bacterial DNA genomic units (GU) originally present in the sample. Combined with the specificity of fluorescent detection probes, real-time PCR assays are simple, fast and sensitive.

**Project Description.** Bio-Rad has developed four kits for the detection and quantification of *Legionella* spp. and *L. pneumophila* bacteria in water samples based on real-time PCR. The genus *Legionella* is identified by amplifying DNA sequences with specific primers complementary to conserved regions in the 5S ribosomal RNA gene. The amplicons are detected in real time using a 5S rRNA specific fluorescent probe (Molecular Beacon). The identification of *Legionella pneumophila* is performed using the mip (macrophage infectivity potentiator) gene as a target. Using the iCycler iQ thermal cycler (Bio-Rad), the fluorescence generated by the binding of the fluorescent probes to the amplified DNA sequences is monitored during the PCR annealing step.

We present here the performances of the method: specificity, limit of detection, limit of quantification and linearity. In collaboration with LVD of Niort (France), the method was tested on water samples from different origins (hot sanitary water and cooling water). 108 water samples were tested for the presence of *Legionella pneumophila* using iQ-Check™ Quanti *L. pneumophila* kits. 84 samples were tested for the presence of *Legionella* spp. using iQ-Check™ Quanti *Legionella* spp. kit. Results were compared to culture reference method AFNOR T90 431.

**Conclusions.** This evaluation shows that real-time PCR is a very rapid, accurate and sensitive method allowing a better risk assessment of *Legionella* proliferation in water. Less than 2% of PCR inhibitors on sample tests has been found after using Bio-Rad's extraction protocols.

iQ-Check™ *Legionella* kits allow fast monitoring of *Legionella* spp. and *L. pneumophila* and a real-time accurate quantitative analysis that is more adapted than CFU counts for follow-up of installations or to prevent any contamination in hospitals.

## **34. DETECTION OF *LEGIONELLA* spp. BY A MULTIPLEX REAL TIME PCR BASED ON THE *RNPB* GENE**

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**Objective.** To develop a multiplex real time PCR for detection of the most important species in the *Legionella* genus.

**Methods.** The *rnpB* gene, coding for the RNase P RNA, was determined for 40 species specific reference strains of *Legionella* and all 15 serogroups of *L. pneumophila*. A set of three primers and a TaqMan probe for specific analysis of *L. pneumophila* was designed. In addition seven primers and a group specific probe were selected for detection of *L. anisa*, *L. bozemanii*, *L. dumoffii*, *L. feeleii*, *L. gormanii*, *L. longbeachae* and *L. micdadei*. Species identification of *non-pneumophila* species was performed by terminator labeled cycle sequencing of the amplification product.

**Results.** The *rnpB* gene had species specific sequences for 38 species, while identical sequences were found for *L. micdadei* and *L. maceachernii*. For *L. pneumophila* 8 sequence variants were found.

In the real time PCR the detection limit for *L. pneumophila* was 10 genome copies per reaction. For other *Legionella* spp. the sensitivity was typically between 10 and 60 copies when using a mix of primers. In addition to the above mentioned species, nine other *Legionella* spp. of less medical importance were detected at varying copy numbers. A sensitivity comparison between our method and the ProbeTec assay for detection of *L. pneumophila* (BectonDickinson) showed that our method could detect a 200 fold higher dilution of a DNA preparation from a reference strain.

To analyse the detection capacity of the real time PCR five clinical isolates/reference strains of the above mentioned *Legionella* species and 20 strains of *L. pneumophila* were tested. All strains were detected, except one strain of *L. feeleii*. This strain had no mismatching sequence in the primer/probe regions that could explain this failure.

Our method was further assessed by examination of a quality assurance panel from the European Working Group of *Legionella* Infections and all 10 specimens of *L. pneumophila* were detected.

A minor evaluation of the method for detection of *Legionella* spp. was performed on ten clinical respiratory tract samples, previously positive in a mip PCR for *L. pneumophila* or culture positive for *L. bozemanii*. The *rnpB* PCR detected all except one case. Evaluation of the new assay on environmental water samples is ongoing.

**Conclusion.** We have developed a sensitive and specific multiplex real time PCR for detection of the most important *Legionella* species in clinical diagnostics.

## **35. AUTOMATED RIBOTYPING OF LEGIONELLA ISOLATES FROM HUMAN AND ENVIRONMENTAL SAMPLES FOR IDENTIFICATION AND CHARACTERIZATION**

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**Background.** The environmental variability of strains and species of the genus *Legionella* is very high, even more than it is in samples taken from humans. Serotyping is routinely performed, but the interpretation is sometimes difficult and it is not unusual to isolate presumptive *Legionella* spp. strains from e.g. water samples which do not agglutinate with the commercially available antisera. The aim of this study was to test the usefulness of the automated ribotyping for the identification of wild *Legionella* species and to support their characterization for epidemiology purposes.

**Methods.** We collected 136 strains of *Legionella*, mainly isolated from water samples, and screened 124 of them by serotyping using the *Legionella* latex test (Oxoid Ltd, UK). All strains, included those which did not agglutinate with the test, or which were not serotyped but showed typical growth characteristics, were ribotyped using RiboPrinter (DuPont Qualicon, Wilmington, USA). The obtained Ribotype patterns were then transferred to BioNumerics (Version 4.0, Applied Maths, Belgium) for cluster analysis.

**Results.** Among the 124 strains screened with the *Legionella* latex test, 22 were assigned to serogroup 1, 77 to serogroup 2-14, 2 to *Legionella* ssp. while 23 did not agglutinate.

The RiboPrinter System, with its 31 ribopatterns of 25 different *Legionella* species present in its resident database was able to identify 111 strains, *L. pneumophila* 15), *L. pneumophila* ss. *pneumophila* (57), *L. rubrilucens* (10), *Legionella species* (25), *L. nautarum* (1), *L. anisa* (1), *L. jordanis* (1), Two strains were assigned to the species *Arthrobacter cumminsii*. The system was able to differentiate 8 different RiboGroups by the *L. pneumophila* strains, 2 RiboGroups by *Legionella* species, whereas the *L. rubrilucens* strains were all assigned to the same RiboGroup. The strains not identified by the system were assigned to 8 different RiboGroups.

The cluster analysis performed using the UPGMA coefficient after Pearson correlation, distinguished clearly the different identified species, and divided the remaining strains in 8 main clusters.

**Conclusions.** The RiboPrinter system is actually able to identify most of the *Legionella* strains isolated from environmental samples. Extending the pattern library could even lead to an improved identification performance. The ability of the system to differentiate several *L. pneumophila* strains could be relevant for epidemiological purposes.

## **36. MICROAGGLUTINATION TEST IN SEROLOGICAL INVESTIGATIONS OF LEGIONELLA INFECTION IN POLAND**

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According to EWGLI data 10% of cases are caused by *Legionella* spp. other than *L. pneumophila* serogroup 1, but in Central European or Scandinavian countries infections due to *L. pneumophila* serogroup 2-14 or other *Legionella* spp. are more frequently reported.

The serum samples are specimens the most frequently examined for diagnosis of legionellosis in Poland. In our laboratory the microagglutination test (MAT) with prepared in-house antigens of *L. pneumophila* serogroup 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, *L. micdadei*, *L. bozemanii*, *L. longbeachae*, *L. jordanis* and *L. anisa* was evaluated. The significant titre of antibodies for each of the antigen was determined on the base of statistical analysis of results obtained in examination of blood-donors serum samples. The value of significant titres varied in range 64 -256, depending on antigen.

Next, the results of microagglutination tests of the panel of antigens with latest examined serum samples (56) collected from patients were analysed. Totally 1214 assays were done. The significant titre of antibodies only for one antigen was found in 15 of tested sera (in 4 sera – for *L. micdadei*, 4 – *L. pneumophila* serogroup 12, 3 – *L. pneumophila* serogroup 7, 2 – *L. pneumophila* serogroup 5 and one – *L. pneumophila* serogroup 3 and one - *L. bozemanii*). In the 13 serum samples the significant titre for more than one antigen was determined, among them more often for *L. pneumophila* serogroup 12 (11) and *L. pneumophila* serogroup 7 (5).

The interpretation of some of MAT results causes a problem, especially when the significant titre for more than one antigen were determined. The structure of antigens of *L. pneumophila* is compound, and it is possible that each infection due to *L. pneumophila* rise the antibody level. From that point of view the high prevalence of significant titre of antibodies for *L. pneumophila* serogroup 12 might indicate the past *Legionella* infection and/or the high prevalence of those bacteria in the water environment.

Serological investigations are the most required laboratory examinations for *Legionella* spp. infections in Poland. The assay of choice is commercial ELISA detecting *L. pneumophila* serogroup 1 infections, but for searching the enlarged spectrum of *Legionella* spp. the MAT is irremissible for completing examination.

## **37. COMPARISON OF SEROLOGICAL AND SEQUENCING BASED METHODS FOR *LEGIONELLA* IDENTIFICATION**

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Background. *Legionella* genus includes 48 species and 70 serogroups. Species and serogroup distribution of *Legionella* Spanish isolates, by IF using policlonal rabbit antisera, were as follows: 83,8% of clinical isolates and 50% of environmental ones were identified as *L. pneumophila* serogroup 1, 12,7% of clinical and 42,7% of environmental ones were *L. pneumophila* serogroup 2-14, and 3,4% of clinical and 7,2% of environmental ones were *Legionella* spp. Several serological methods are used for *Legionella* identification, as well as sequencing based methods.

Aim of the study. The aim of the study was to compare several serological and sequencing methods used for *Legionella* identification, in order to know their agreement, and to establish a strategy to use them.

Methods. 70 *Legionella* isolates were identified, including 27 *L. pneumophila* (9 clinical and 18 environmental ones) and 43 *Legionella* spp. strains (21 clinical and 22 environmental ones).

Serological methods were used for *Legionella* identification: 1) IF using policlonal rabbit antisera home-made (*L. pneumophila* serogroups 1 to 14 and other 8 *Legionella* species) and 2 *Legionella* identification kits: IFD (Bio-Rad) and latex agglutination (OXOID). 2) Sequencing based methods were performed using *mip* and 16S rRNA genes.

Results. Comparing serological methods, 95,72% of the strains presented agreement results when the three methods were used. Comparing sequencing methods, 90% of strains presented agreement results by sequencing with the two genes, and discrepancies were only detected with environmental strains.

96,6% of clinical strains were assigned to the same species by all methods (serological and sequencing), the only discrepant result was between *L. jordanis*/*L. bozemanii* by serology and *L. parisiensis* with both genes. However, the agreement was lower with environmental strains, 61,53% of the strains presented identical species identification using serology and *mip* gene and 79,48% using serology and 6S rRNA gene.

Conclusions. Sequencing methods allow to identify any strain, detecting all species described in *Legionella* genus. To achieve this complete identification with serological methods is necessary to have a large number of antibodies.

A complete agreement was detected with both genes when sequencing were used to identify *Legionella* clinical strains, so either of the two genes could be used. Discrepancies were detected with both genes, when environmental strains were sequenced, 16S rRNA gene showed more agreement with serology than *mip* gene.

## **38. MOLECULAR TYPING OF CLINICAL AND ENVIRONMENTAL *LEGIONELLA PNEUMOPHILA* SEROGROUP 1 ISOLATES IN PALERMO**

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*Legionella* spp. infection is an important cause of nosocomial and community-acquired human diseases that can range from a mild respiratory illness to an acute pneumonia. Among the 42 described *Legionella* species all environmental microorganisms, a great majority of cases are caused by *Legionella pneumophila* strains, particularly belonging to serogroup 1.

Consequently, several phenotypic and genotypic typing methods have been developed in the last years in epidemiological studies to characterize clinical and epidemiologically linked environmental isolates of this common serogroup in order to locate the source of an outbreak, to evaluate the extent of infection, or even to perform epidemiological surveillance.

One of the molecular typing techniques, the amplified fragment length polymorphism (AFLP) analysis, is fast, efficient and reproducible for typing strains of *Legionella pneumophila* serogroup 1 isolated both from humans and the environment. The AFLP methodology has been accepted as an international standard protocol for epidemiological typing of *Legionella pneumophila* serogroup 1 and is today widely used by the members of the European Working Group for Legionella Infections (EWGLI). Nevertheless, difficulties have been reported from some users in terms of the assignment of types when AFLP banding patterns had to be compared of isolates too similar to be consistently discriminated one from the other.

Recently, a sequence-based typing (SBT) method has been proposed as the new “gold standard” for epidemiological typing of *Legionella pneumophila* serogroup 1. This method, based essentially on sequencing of three genes under selective pressure (*flaA*, *proA*, and *mompS*), has been suggested as being epidemiologically concordant, highly discriminatory and suitable to any laboratory involved in the investigation of outbreak of legionellosis caused by *Legionella pneumophila* serogroup 1.

For this reason we used AFLP analysis and the sequence-based typing method to investigate the source of a culture confirmed Legionnaires’ disease in a young patient admitted to the Palermo city hospital because of thoracic trauma. Both techniques allowed us to exclude the nosocomial origin of the infection.

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