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Cytopathogenicity and molecular subtyping of *Legionella pneumophila* environmental isolates from 17 hospitals

M. GARCIA-NUÑEZ[1,2]*, M. L. PEDRO-BOTET[1,2], S. RAGULL[1], N. SOPENA[1], J. MORERA[3], C. REY-JOLY[1] AND M. SABRIA[1,2]

[1] *Infectious Diseases Section, Fundació Institut d'Investigació Germans Trias i Pujol, Badalona, Autonomous University of Barcelona*
[2] *CIBER de Enfermedades Respiratorias, Spain*
[3] *Pneumology Department, Hospital Germans Trias i Pujol, Badalona, Autonomous University of Barcelona*

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SUMMARY

The cytopathogenicity of 22 *Legionella pneumophila* isolates from 17 hospitals was determined by assessing the dose of bacteria necessary to produce 50% cytopathic effect ($CPED_{50}$) in U937 human-derived macrophages. All isolates were able to infect and grow in macrophage-like cells (range $\log_{10}$ $CPED_{50}$: 2·67–6·73 c.f.u./ml). Five groups were established and related to the serogroup, the number of PFGE patterns coexisting in the same hospital water distribution system, and the possible reporting of hospital-acquired Legionnaires' disease cases. *L. pneumophila* serogroup 1 isolates had the highest cytopathogenicity ($P=0{\cdot}003$). Moreover, a trend to more cytopathogenic groups (groups 1–3) in hospitals with more than one PFGE pattern of *L. pneumophila* in the water distribution system (60% *vs.* 17%) and in hospitals reporting cases of hospital-acquired Legionnaires' disease (36·3% *vs.* 16·6%) was observed. We conclude that the cytopathogenicty of environmental *L. pneumophila* should be taken into account in evaluating the risk of a contaminated water reservoir in a hospital and hospital acquisition of Legionnaires' disease.

INTRODUCTION

Hospital-acquired Legionnaires' disease (LD) has been reported in many hospitals since the first outbreak in 1976 [1]. Although cooling towers were linked to many of the cases of LD in the years after its discovery, potable water has been the environmental source for almost all reported hospital outbreaks [2–4]. *Legionella* spp. are common commensals of large building water-supply systems. In a previous study we demonstrated that *L. pneumophila* was present in 17 out of 20 hospital water systems [5] and prospective surveillance showed that cases of hospital-acquired LD appeared in 11 of these hospitals [6]. The remainder did not report any cases of LD, although appropriate techniques were available for the diagnosis of *Legionella* spp. and most (70%) reported >30% positive peripheral sampling points.

There are some clinical data indicating that the virulence of *Legionella* spp. may vary, although it unclear whether this is a consequence of differences in bacterial virulence or in host immunity. Examples are: the survival of patients with LD despite not receiving adequate antibiotic treatment [7], the variability in the mortality observed in different

* Author for correspondence: M. Garcia-Nuñez, M.Sc., Infectious Diseases Unit, Fundació Institut Investigació en Ciencies de la Salut Germans Trias i Pujol, C/Can Ruti.Camí escoles s/n 08916 Badalona, Barcelona, Spain.
(Email: mgarcia.igtp.germanstrias@gencat.net)

community outbreaks [8, 9], or the fact that most cases are caused by *L. pneumophila* serogroup (sg) 1 [10]. In experimental studies, isolates from amoebas exhibit a variety of phenotypes that appear to increase the incidence and complications of LD in humans [11–13]. Furthermore, some authors have demonstrated that *Legionella* strains causing pulmonary disease express more virulence traits than those that do not cause human disease [14]. Virulence traits show differences between species [14, 15] and strains of *L. pneumophila* [16–19].

As the demographic characteristics of the patients in the 17 hospitals reported earlier [5, 6] were similar, we questioned why hospitals with *L. pneumophila* in their water distribution systems did not experience cases of hospital-acquired LD and hypothesized that the virulence of the *L. pneumophila* strains may have influenced the appearance of clinical cases. We therefore assessed the cytopathogenicity of *L. pneumophila* environmental strains isolated from each hospital and determined their relationship with sg1 and the number of different strain genotypes coexisting in the same water distribution system to gain insight into the contribution of cytopathogenicity and potential to cause disease in each hospital.

Table 1. *DNA patterns,* Legionella pneumophila *serogroup, cytopathogenicity and number of cases of hospital-acquired Legionnaires' disease reported in each hospital during the surveillance period*

Strain/ DNA pattern	Serogroup	log $CPED_{50}$ (mean ± S.D.)	Cytopathogenic group	Hospital
Lp01	Non-sg1	5·60 ± 0·20	4	H 1
Lp02	Non-sg1	4·48 ± 0·28	3	H 2*
Lp03	1	6·64 ± 0·54	5	H 6*
Lp04	Non-sg1	6·73 ± 0·11	5	H 7*
Lp05	1	6·55 ± 0·24	5	H 8
Lp06	Non-sg1	6·3 ± 0·19	4	H 9
Lp07	1	5·57 ± 0·13	4	H 10*
Lp08	1	3·45 ± 0·15	2	H 10 *
Lp09	1	4·52 ± 0·18	3	H 11*
Lp10	Non-sg1	6·08 ± 0·19	4	H 11*
Lp11	1	2·67 ± 0·32	1	H 12*
Lp12	1	4·73 ± 0·16	3	H 12*
Lp14	Non-sg1	5·67 ± 0·11	4	H 13*
Lp15	Non-sg1	5·50 ± 0·10	4	H 14*
Lp16	Non-sg1	6·03 ± 0·14	4	H 15
Lp17	Non-sg1	5·84 ± 0·14	4	H 16*
Lp19	1	4·10 ± 0·07	3	H 17
Lp20	Non-sg1	6·63 ± 0·59	5	H 18
Lp21	Non-sg1	5·59 ± 0·2	4	H 18
Lp22	1	5·40 ± 0·30	4	H 19*
Lp23	1	5·94 ± 0·13	4	H 19*
Lp24	Non-sg1	5·90 ± 0·16	4	H 20*

$CPED_{50}$, The $CPED_{50}$ was defined as the minimum number of bacteria necessary to produce a cytopathic effect in 50% of the U937 cell infected monolayers after 72 h incubation; Non-sg1, *L. pneumophila* non-serogroup 1.
* Hospital that reported hospital-acquired Legionnaires' disease (1996–2001).

MATERIAL AND METHODS

Bacteria and culture

A total of 22 environmental *L. pneumophila* strains from 17 hospital water-supply systems previously characterized [5] by serogroup, and chromosomal DNA subtype by pulsed-field gel electrophoresis (PFGE) were included in the present study (Table 1). The bacteria had a $\leqslant 2$ passage history and were stored in brain heart infusion broth (Oxoid, Wesel, Germany) supplemented with 10% glycerol at −80 °C. All strains were susceptible to 100 mM NaCl [20], a marker of laboratory attenuation (data not shown).

For cytopathogenicity and intracellular growth assays, strains were cultured on BCYE-α agar for 72 h and resuspended in antibiotic-free tissue culture medium containing 5% fetal calf serum (FCS).

Cytopathogenicity assay

The human monocyte cell line U937 was cultured in RPMI 1640, 2 mM L-glutamine and 10% FCS. Prior to infection, the cells were differentiated for 72 h with 10^{-8} M phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co., St Louis, MO, USA) as described previously [21]. The relative cytopathogenicity of *L. pneumophila* strains was determined by the 50% cytopathic effect (CPE_{50}). $CPED_{50}$ was defined as the minimum number of bacteria necessary to produce a cytopathic effect in 50% of the U937 cell infected monolayers after 72 h incubation. Briefly, eight replicate monolayers containing 2×10^5 macrophage-like cells in 96-well microtitre plates were infected with serial dilutions of each strain (range of inocula, 10^1–10^9 bacteria per monolayer) for 90 min. The monolayers were washed and treated with 80 μg/ml of gentamicin and then incubated with RPMI containing 10% FCS at 37 °C for 72 h. The monolayers were stained with tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide (MTT; Sigma)

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and the viability of the cells was determined measuring the optical density (OD) and expressed as percentage of cell death compared with uninfected cells using the formula

$$1-\frac{\text{mean OD of infected cells}}{\text{mean OD uninfected cells}}\times 100.$$

The number of bacteria that reduced the OD by 50% compared with the OD of uninfected cells was defined as the $CPED_{50}$ value. Each experiment was performed in triplicate.

Intracellular growth

U937 macrophage-like cells plated at a density of 1×10^6 cells per well in 24 tissue culture dishes were infected at a multiplicity of infection (MOI) of 0·2. Thereafter, extracellular bacteria were removed by washing the monolayers with RPMI followed by incubation with gentamicin in RPMI for 30 min. Intracellular growth was measured in duplicate at 0, 24 and 48 h and expressed as c.f.u./monolayer. Variations between experiments were corrected with the MOI value (dividing the result c.f.u./well by MOI). The relative increase in c.f.u. was calculated by subtracting the c.f.u. at each time-point from the first time-point.

Statistical analysis

Groups of cytopathogenicity were determined with the *K* means cluster analysis using SPSS software program version 12.0 (SPSS Inc., Chicago IL, USA). The Mann–Whitney *U* and Kruskal–Wallis tests were used for statistical analysis of quantitative variables and Fisher's exact test was used for qualitative variables. *P* values $<0·05$ were considered significant.

RESULTS

All strains were able to infect and grow in macrophage-like cells and produce a significant cytopathic effect. Values ranged from log $CPED_{50}$ 2·67 c.f.u./ml to log $CPED_{50}$ 6·73 c.f.u./ml and five groups were determined, ranging from 1 (most) to 5 (least) cytopathogenic strains (Fig. 1). Cytopathogenic groups (1 and 2) had a higher intracellular infection efficiency ($T=0$ h, $P=0·031$). At 24 h all isolates showed increased intracellular growth to $2·05\pm0·74$ log units (range 1·04–3·07) and at 48 h to $3·29\pm0·74$ log units (range 1·75–4·93) (Fig. 2).

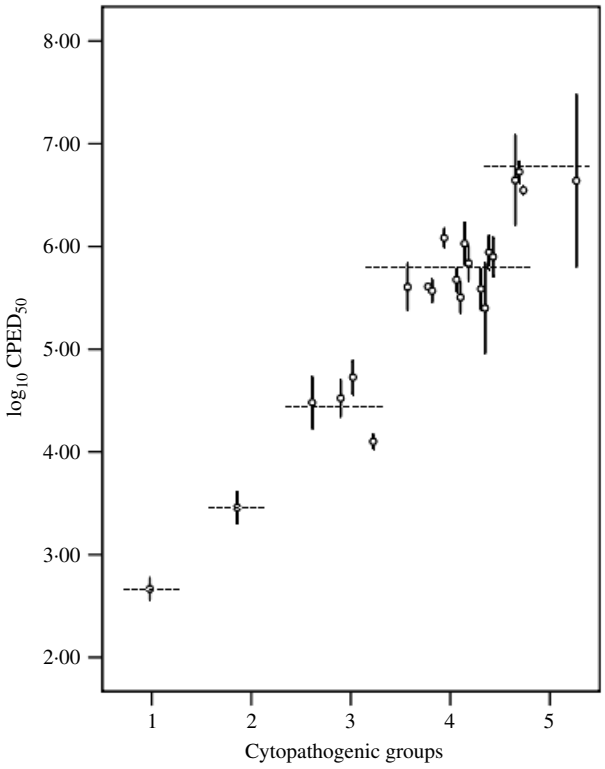

Fig. 1. Cytopathogenic groups of the different environmental isolates. Data of each isolate are represented by a point and error bars represents standard deviations. Mean data of each cytopathogenic group is represented by a horizontal bar. Groups were established with the *K* means cluster analysis (SPSS). The $CPED_{50}$ groups were statistically different ($P<0·001$, Kruskal–Wallis test).

L. pneumophila sg1 strains had a significantly higher mean cytopathogenicity (log $CPED_{50}$ $4·92\pm1·28$ *vs.* $5·75\pm0·68$, $P=0·003$) and were distributed in the top three groups more frequently than other strains [50% (5/10) *vs.* 8·3% (1/12), $P=0·06$] (Table 1). Strains from hospital water systems harbouring more than one DNA type (PFGE) more often fell into the top three cytopathogenic groups than strains from systems that yielded a single genotype (3/5 *vs.* 2/12 hospitals respectively, $P>0·05$). Similarly, cytopathogenicity was greatest among strains from hospitals reporting hospital-acquired LD cases than those without clinical cases (4/11 *vs.* 1/6 hospitals respectively, $P>0·05$).

DISCUSSION

It is not clear why some hospitals with environmental colonization by *L. pneumophila* have nosocomial LD and others do not. This is often attributed to

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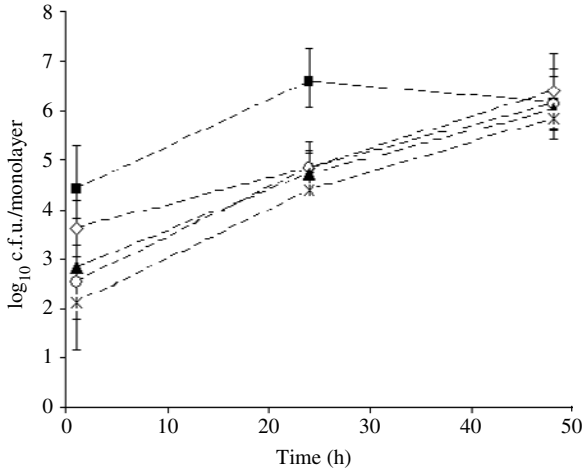

Fig. 2. Intracellular growth of cytopathogenic groups of *L. pneumophila* isolates in co-cultures with U937 macrophage-like cell monolayers. ◇, Cytopathogenic group 1; ■, group 2; ○, group 3; ▲, group 4; ×, group 5. The values are the mean of groups and error bars represents standard deviations.

differences in engineering design and maintenance, bacterial concentration at peripheral points (taps and shower faucets) and host susceptibility. The virulence of the bacteria is also thought to play a major role.

We found that all environmental isolates of *L. pneumophila* tested were able to infect and grow in U937 macrophage-like cells resulting in a significant cytopathic effect. Almost three-quarters of the strains fell into weakly cytopathogenic groups and were invariably non-sg1; half of the 10 strains of sg1 were grouped as strongly cytopathogenic. Moreover, strains from mixed *Legionella* populations in water systems were more often cytopathogenic than those recovered in single strain culture and importantly, this association held for environmental strains from hospitals with cases of LD compared with those free of cases.

It is well established that *Legionella* spp. differ in virulence determined by cytopathogenicity and other assays and can be grouped accordingly [14, 19, 22–25]. However, there are few studies evaluating virulence traits of environmental isolates of *L. pneumophila* [10, 18, 24, 26]. Most cases of disease are caused by sg1 strains and, consequently, it has been suggested that this serogroup is more virulent than other serogroups of *L. pneumophila*, but this has not been demonstrated experimentally [14, 26, 27].

The higher cytopathogenicity of *L. pneumophila* from mixed strain populations in water systems has not been previously described but our data are consistent with the finding of the coexistence of different *L. pneumophila* genotypes in cooling towers where the limitation of nutrients in the non-potable water induces an overgrowth of the most virulent strain and displacement of other strains leading to an outbreak of disease [28]. Indeed, it has been demonstrated by *in vitro* studies that depleted nutrients enhance the expression of virulence traits [20]. The coexistence of more than one strain population in the hospital water distribution system may provide the microbial complexity to facilitate biofilm formation and intracellular growth in protozoa. *L. pneumophila* growing within amoeba changes phenotypically and exhibits increased invasion ability [29] compared with cells grown in conventional media. The similarities in the model of intracellular infection between protozoa and alveolar macrophages [30] suggest that the virulence of *L. pneumophila* for alveolar macrophages is a consequence of its evolution as a parasite of amoeba [31]. Thus, considering that protozoa are important determinants in the ecology of *L. pneumophila* in aquatic environments, studies to determine the different populations of amoeba in water supplies of hospitals and the ability of different strains to grow in them and alveolar macropages would be informative of the ecology and pathogenicity of the species.

Hospitals harbouring cytopathogenic strains of *L. pneumophila* strains in water supplies tended to report hospital-acquired LD cases more frequently. Unfortunately, owing to a lack of active surveillance clinical isolates were not available for comparison with the environmental isolates. However, several authors have noted the importance of strain virulence in the appearance of hospital-acquired LD since in hospitals colonized by more than one strain genotype, the clinical strain was often identical to the most virulent environmental strain [16, 17].

Some authors have pointed out that the presence of *Legionella* spp. in a hospital water distribution system, the use of specific techniques for the diagnosis of *Legionella* pneumonia and in-patient host factors such as immunosuppression, are the most important prognostic factors for the appearance of hospital-acquired cases [1]. Nevertheless, since the complete eradication of *Legionella* spp. from a potable water distribution system is impossible without highly cost-effective disinfection methods, knowledge of the cytopathogenicity of an environmental *Legionella* spp. isolate may be useful in evaluating the risk of the emergence of hospital-acquired LD from a contaminated water source.

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DECLARATION OF INTEREST

None.

REFERENCES

1. **Sabria M, Yu VL.** Hospital-acquired legionellosis: solutions for a presentable infection. *Lancet Infectious Diseases* 2002; **2**: 368–373.
2. **Best M, *et al.*** *Legionellaceae* in the hospital water supply-epidemiological link with disease and evaluation of a method of control of nosocomial Legionnaires' disease and Pittsburgh pneumonia. *Lancet* 1983; **2**: 307–310.
3. **Johnson JT, *et al.*** Nosocomial legionellosis uncovered in surgical patients with head and neck cancer: implications for epidemiologic reservoir and mode of transmission. *Lancet* 1985; **2**: 298–300.
4. **Stout J, *et al.*** Ubiquitousness of *Legionella pneumophila* in the water supply of a hospital with endemic Legionnaires' disease. *New England Journal of Medicine* 1982; **36**: 466–468.
5. **Sabria M, *et al.*** Presence and chromosomal subtyping of *Legionella* species in potable water systems in 20 hospitals of Catalonia, Spain. *Infection Control and Hospital Epidemiology* 2001; **22**: 673–676.
6. **Sabria M, *et al.*** Environmental cultures and hospital-acquired Legionnaires' disease. A five-year prospective study in twenty hospitals in Catalonia. Spain. *Infection Control and Hospital Epidemiology* 2004; **25**: 1072–1076.
7. **Macfarlane JT, *et al.*** Erythromycin compared with a combination of ampicillin and amoxycillin as initial therapy for adults with pneumonia including Legionnaires' disease. *Journal of Infection* 1983; **7**: 111–117.
8. **Lettinga KD, *et al.*** Legionnaires' disease at a Dutch flower show: prognostic factors and impact of therapy. *Emerging Infectious Diseases* 2002; **8**: 1448–1454.
9. **Garcia-Fulgueiras A, *et al.*** Legionnaires' disease outbreak in Murcia, Spain. *Emerging Infectious Diseases* 2003; **9**: 915–921.
10. **Marston BJ, Lipman HB, Breiman RF.** Surveillance for Legionnaires' disease. Risk factors for morbidity and mortality. *Archives of Internal Medicine* 1994; **154**: 2417–2422.
11. **Barker J, *et al.*** Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga*: physiological status and susceptibility to chemical inactivation. *Applied Environmental Microbiology* 1992; **58**: 2420–2425.
12. **Cirillo JD, *et al.*** Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. *Infection and Immunity* 1999; **67**: 4427–4434.
13. **Rowbotham TJ.** Current views on the relationships between amoebae, *Legionellae* and man. *Israel Journal of Medical Sciences* 1986; **22**: 678–689.
14. **Alli OA, *et al.*** Comparative assessment of virulence traits in *Legionella* spp. *Microbiology* 2003; **149**: 631–641.
15. **Joshi A, Swanson MS.** Comparative analysis of *Legionella pneumophila* and *Legionella micdadei* virulence traits. *Infection and Immunity* 1999; **67**: 4134–4142.
16. **Bezanson G, *et al.*** Virulence of patient and water isolates of *Legionella pneumophila* in guinea pigs and mouse L929 cells varies with bacterial genotype. *Canadian Journal of Microbiology* 1994; **40**: 426–431.
17. **Bollin GE, *et al.*** Difference in virulence of environmental isolates of *Legionella pneumophila*. *Journal of Clinical Microbiology* 1985; **21**: 674–677.
18. **Molmeret M, *et al.*** Different growth rates in amoeba of genotypically related environmental and clinical *Legionella pneumophila* strains isolated from a thermal spa. *Epidemiology and Infection* 2001; **126**: 231–239.
19. **Samrakandi MM, *et al.*** Genetic and phenotypic differences between *Legionella pneumophila* strains. *Journal of Clinical Microbiology* 2002; **40**: 1352–1362.
20. **Byrne B, Swanson MS.** Expression of *Legionella pneumophila* virulence traits in response to growth conditions. *Infection and Immunity* 1998; **66**: 3029–3034.
21. **Pearlman E, *et al.*** Growth of *Legionella pneumophila* in a human macrophage-like (U937) cell line. *Microbial Pathogenesis* 1988; **5**: 87–95.
22. **Fields BS, *et al.*** Comparison of guinea pig and protozoan models for determining virulence of *Legionella* species. *Infection and Immunity* 1986; **53**: 553–559.
23. **Izu K, *et al.*** Grouping of 20 reference strains of *Legionella* species by the growth ability within mouse and guinea pig macrophages. *FEMS Immunology and Medical Microbiology* 1999; **26**: 61–68.
24. **Neumeister B, *et al.*** Multiplication of different *Legionella* species in Mon Mac 6 cells and *Acanthamoeba castellanii*. *Applied and Environmental Microbiology* 1999; **63**: 1219–1224.
25. **O'Connell WA, Dhand L, Cianciotto NP.** Infection of Macrophage-Like cells by *Legionella* species that have not been associated with disease. *Infection and Immunity* 1996; **64**: 4381–4384.
26. **Luck PC, *et al.*** Analysis of *Legionella pneumophila* strains associated with nosocomial pneumonia in a neonatal intensive care unit. *European Journal of Clinical Microbiology and Infectious Diseases* 1994; **13**: 565–571.
27. **Neumeister B.** Intracellular multiplication of *Legionella* species and the influence of amoebae on their intracellular growth in human monocytes: mono mac 6 cells and *Acanthamoeba castellanii* as suitable in vitro models. *Methods in Molecular Biology* 2004; **268**: 141–151.

28. **Ragull S, *et al.*** Diversity of *Legionella* subtypes in cooling towers. Why only one environmental strain causes de clinical cases. In: *Program and Abstracts of the 6th International Conference on Legionella*. Chicago: American Society for Microbiology; 2005, pp. 64
29. **Cirillo JD, Falkow S, Tompkins LS.** Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infection and Immunity* 1994; **62**: 3254–3261.
30. **Gao LY, Harb O, Abu Kwaik Y.** Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant hosts, mammalian and protozoan cells. *Infection and Immunity* 1997; **65**: 4738–4746.
31. **Swanson MS, Hammer BK.** *Legionella pneumophila* pathogenesis: a fateful journey from amoebae to macrophages. *Annual Review of Microbiology* 2000; **54**: 567–613.

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