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Combined monoclonal antibody typing, multilocus enzyme electrophoresis, soluble protein profiles and plasmid analysis of clinical and environmental *Legionella pneumophila* serogroup 1 isolated in a Portuguese hospital

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Summary: Sixteen strains of *Legionella pneumophila* serogroup 1 isolated from patients and the environment at Santa Cruz Hospital were examined using four typing methods. Monoclonal antibodies showed that all the isolates belonged to subgroup Pontiac and mainly to subtype Allentown. With multilocus enzyme electrophoresis nine subtypes (ETs) were revealed; each strain had the same profile with electrophoresis of soluble proteins, and plasmid analysis revealed that only two of the strains studied contained plasmids. Among these methods, multilocus enzyme electrophoresis was the most discriminative as a single epidemiological marker. Problems concerning the microbiological examination of environmental specimens and correlation with clinical strains confirmed the difficulty of investigating an outbreak source of legionellosis and the need for careful evaluation of the results.

Keywords: *Legionella pneumophila*; nosocomial infection; bacterial typing methods; clinical and environmental specimens.

Introduction

Since 1977, when *Legionella pneumophila* was first recognized as a causative agent of pneumonia,[1] over 30 species of this new genus and different serogroups have been described. These microorganisms have been shown to be an important cause of community-acquired and nosocomial pneumonia particularly in immunocompromised patients.[2–4] Most clinical isolates of *L. pneumophila* belong to serogroup 1 (sg 1). In Portugal the incidence of

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hospital- and community-acquired pneumonia is not known but most clinical isolates of *L. pneumophila* also belong to sg 1.[5]

Since 1987, 19 strains of *Legionella* spp. have been isolated in Santa Cruz Hospital and 16 of them were *L. pneumophila* sg 1. To characterize these 16 isolates, investigating a possible correlation between clinical and environmental strains and contributing to a better understanding of *Legionella* strains isolated in Portugal, four epidemiological markers have been used: monoclonal antibodies; multilocus enzyme electrophoresis; soluble protein profiles; and plasmid analysis.

Materials and methods

Cultural methods

Isolates were obtained from specimens which were cultured using buffered charcoal yeast extract agar containing alpha-ketoglutarate (BCYE-α), also BCYE agar containing cefamandole, polymyxin B and anisomycin (BMPA-α) (Oxoid, Unipath Ltd, Hampshire, UK). Colonies were characterized by conventional procedures and identification confirmed by immunofluorescence using specific *L. pneumophila* sg 1 fluorescein-labelled rabbit antisera.

For the environmental study, small water samples (250 mL) and tap and shower swabs were used. Each water sample was concentrated by centrifugation and 0·1 mL of the concentrate was acid washed by adding 0·1 mL of HCl-KCl solution (pH 2·2) to the tube. The mixture was vortexed and allowed to stand at room temperature for 15 min and then neutralized by adding 0·01 N KOH. Then 0·1 mL aliquots of non-treated and acid-treated samples were plated on BCYE-α and selective Wadowsky-Yee Medium supplemented with polymyxin B, anisomycin and vancomycin (MWY).

Isolates of Legionella *spp.*

Sixteen strains of *Legionella pneumophila* sg 1 were studied (Table I). Twelve were clinical isolates at Santa Cruz Hospital: 11 caused nosocomial infections in immunosuppressed patients—heart transplant (HT): HSC_1, HSC_6, HSC_7, HSC_8, HSC_{10}, HSC_{11}; renal transplant (RT): HSC_2, HSC_9; and other immunosuppressed patients of the Nephrology Department (OIS): HSC_3, HSC_4, HSC_5. Strain HSC_{13} was an agent of a community-acquired pneumonia (NIS). The other four were environmental strains and were isolated from water supplies in the hospital: Nephrology Department (IS): A_6; Cardiac Surgical Department (HT): A_8, A_{10} and A_{11}.

Strains HSC_3, HSC_4, and HSC_5 were isolated in 1989, from three patients treated with immunosuppressive drugs during a small outbreak of nosocomial legionellosis in the Nephrology Department. A_6 was an environmental strain isolated from the shower in the room of the patient

Table I. *Origin of the 16* Legionella pneumophila *serogroups 1 strains studied*

Clinical strains
Nosocomial infections (HSC) HT: HSC_1, HSC_6, HSC_7, HSC_8, HSC_{10}, HSC_{11}. RI: HSC_2, HSC_9. OIS: HSC_3, HSC_4, HSC_5.
Community-acquired infection NIS: HSC_{13}.
Environmental strains (water supplies—HSC)
ISR: (Nephrology Department) A_6. HTR: (Cardiac Surgery Department) A_8, A_{10}, A_{11}.

HSC, Hospital Santa Cruz; HT, heart transplant; RT, renal transplant; OIS, other immunosuppressed patients; NIS, non-immunosuppressed patient; ISR, immunosuppressed patient's room; HTR, heart transplant patient's room.

from whom HSC_5 was isolated. Strains A_8, A_{10} and A_{11} were recovered from patients' rooms in the Cardiac Surgical Department some months later at the time of a second outbreak of legionellosis, which had occurred despite the implementation of a programme of environmental legionella control measures.

Immunofluorescence profiles

A panel of 12 monoclonal antibodies was used to produce patterns of immunofluorescent staining (IFA). Tobin: 114·2; 112·4; 415·23; 324; 221·2; 268; Joly: 33 G_2; 144 C_2; 32 A_1; and McKinney: MAb 1; MAb 2; MAb 3. Fluorescence was recorded on a scale from 0–3 (6, 7).

Multilocus enzyme electrophoresis (MEE)

Relative electrophoretic mobilities were studied using starch gel electrophoresis as described by Selander.[8] Cell extracts were prepared by sonication of cells grown on BCYE-α agar and suspended in buffer: 10 mM Tris, 1 mM EDTA, 0·5 mM NADP (pH = 6·8). They were stored at −70°C.

Gels were stained selectively for 16 metabolic enzymes: esterases 2, 3, 5 and 6; isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), alanine dehydrogenase (ALD), adenylate kinase (ADK); glucose-6-phosphate dehydrogenase (G6PD), glutamate dehydrogenase (GDH), leucine aminopeptidase (LAP), peptidase (PEP); indophenol oxidase (IPO), leucine dehydrogenase (LED), lysine dehydrogenase (LYD), threonine dehydrogenase (THD).

For each enzyme, distinctive mobility variants designated as electromorphs were numbered in order of rate of anodal migration, from 0–20, (using bromophenol blue as standard of 10 cm migration). An absence of enzyme activity was attributed to a null allele and designated as 0.

Each isolate was characterized by its combination of alleles at the 16 enzyme loci. These profiles, designated by electrophoretic types (ETs) have been arbitrarily designated by different 'code numbers'[9] and are characteristic for the genotype of each bacterial strain.

Electrophoresis of soluble protein extracts (SDS-PAGE)
Soluble proteins were extracted by the modified method of Lema and Brown.[10] SDS-PAGE was performed in 12·5% (w/v) polyacrylamide and run in the 'Phast system' (Pharmacia, Uppsala, Sweden) at 250 V, 10 mA, a modification[11] of the method of Laemmli.

The average similarity between any two *Legionella* protein electrophoretic patterns was assessed by the use of the 'Dice' coefficient:[12]

$$\%S = \frac{\text{number of matching bands} \times 2}{\text{total number of bands in both isolates}}$$

Plasmid profiles
Plasmid extraction was performed by using a modification[13] of the method of Kado and Liu,[14] using 3% alkaline SDS (pH 12·6) at elevated temperature (56°C). The lysate was extracted with phenol chloroform and centrifugation. After this process, the extracts were subjected to electrophoresis in 0·7% agarose gel at 135 V for 90 min.

Molecular weights of plasmids were compared with the four plasmids of *L. bozemanii* strain WIGA (35, 40, 45 and 80 Md).

Results

As shown in Table II, the 12 monoclonal antibodies defined four reaction patterns or subtypes within the common major subgroup Pontiac. All of the strains reacted with MAb 2, which is the MAb most frequently associated with clinical cases.[15] Twelve of the strains studied were identified as subtype Allentown. Two strains isolated from heart transplant patients in 1990 (HSC_7 and HSC_8) belonged to a subtype similar to Benidorm. Strain A_{10}, an environmental isolate from tap water from one of the heart transplant rooms, belonged to a subtype related to Allentown. The only strain responsible for a community-acquired pneumonia was subtype Philadelphia.

Nine subtypes (electrophoretic types designed as 'ETs') were identified by multilocus enzyme electrophoresis. We observed that the same ET was isolated from patients and from water. ET_1, isolated from a heart transplant patient (HSC_1), from a renal transplant patient (HSC_2) and from two immunosuppressed renal patients (HSC_3 and HSC_4) was also found in the hospital water (A_8). ET_4, isolated from a renal patient immunosuppressed

Table II. *Combined monoclonal antibody, enzyme electrophoresis and plasmid analysis of clinical and environmental strains of* Legionella pneumophila *serogroup 1 isolated at Santa Cruz Hospital (1987–1991)*

Date of isolation and strains studied	Monoclonal antibody subtypes of subgroup Pontiac	ET (code number)	Plasmid (Md)
8/87 HSC_1 HT	Allentown	1	—
4/88 HSC_2 RT	Allentown	1	—
6/89 HSC_4 OIS	Allentown	1	—
6/89 HSC_5 OIS	Allentown	4	—
7/89 HSC_3 OIS	Allentown	1	—
8/89 A_6 ISR	Allentown	4	—
12/89 HSC_6 HT	Allentown	5	—
2/90 HSC_9 RT	Allentown	6	—
8/90 HSC_7 HT	st. Benidorm	8	42
10/90 HSC_8 HT	st. Benidorm	8	—
11/90 HSC_{10} HT	Allentown	3	—
3/91 HSC_{11} HT	Allentown	2	–
4/91 A_8 HTR	Allentown	1	–
4/91 A_{10} HTR	st. Allentown	7	–
4/91 A_{11} HTR	Allentown	4	–
12/91 HSC_{13} NIS	Philadelphia	9	87

Clinical strains responsible for nosocomial Legionellosis: HSC_1, HSC_2, HSC_4, HSC_5, HSC_3, HSC_6, HSC_9, HSC_7, HSC_8, HSC_{10}, HSC_{11}.
Clinical strain isolated from a community-acquired infection: HSC_{13}.
Environmental isolates: A_6, A_8, A_{10}, A_{11}.
HT, heart transplant; RT, renal transplant; OIS, other immunosuppressed patients; ISR, immunosuppressed patient's room; HTR, heart transplant room; NIS, non-immunosuppressed patient. st., similar to.

(HSC_5) was also found twice in the water (A_6 and A_{11}). The most discriminative enzymes were esterases, isocitrate dehydrogenase, adenylate kinase and malate dehydrogenase.

Each strain had an identical electrophoretic protein profile (Dice coefficient >80%). Conversely, we observed different electrophoretic profiles in other species of *Legionella* examined.

The results of plasmid analysis of clinical and environmental strains revealed that only two strains contained plasmids. The clinical strain, HSC_{13}, responsible for a community-acquired infection, contained a 87 Md plasmid and strain HSC_7, responsible for an infection in a heart transplant patient, also contained a single plasmid of about 42 Md. All the other isolates were plasmid free (Figure 1).

Discussion

Among the four methods assayed, enzyme electrophoresis was the most discriminative marker. A good correlation was observed between electrophoretic types and the monoclonal antibody subtypes described by Marques *et al.*[16] with a close relation between subtype 'closely Benidorm'

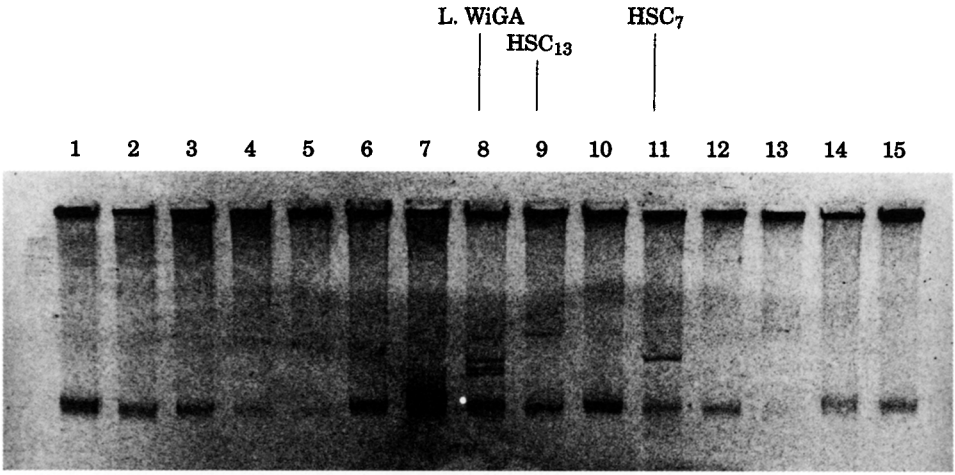

Figure 1. Plasmid profile performed using a modification of Kado and Liu method showing plasmids in HSC_7 (42 Md) and HSC_{13} (87 Md). Molecular weights were compared with those of *Legionella bozemanii* Wiga strain (35,40,45 and 80 Md). Lane 1, A6; 2, A8; 3, A10; 4, A11; 5, HSC_{10}; 6, HSC_{11}; 7, HSC_6; 8, WIGA; 9, HSC_{13}; 10, HSC_1; 11, HSC_7; 12, HSC_4; 13, HSC_5; 14, HSC_8; 15, HSC_9.

and 'ET_8', subtype 'closely Allentown' and 'ET_7' and subtype 'Philadelphia' and 'ET_9'.

The identical electrophoretic profile of soluble proteins in all *L. pneumophila* sg 1 strains examined suggests that, as other authors have already remarked,[17] this technique is not very discriminative for strains belonging to the same species.

Epidemiological correlations of results of this study revealed that monoclonal subtype Allentown was most frequent.[13–18] Analysis by MEE showed that five identical strains (ET_1) were recovered: four of clinical origin, from patients of different departments (in 1989) and one that persisted in the water system of the same hospital two years after control measures were undertaken.

This study demonstrates that water samples contained a phenotypically diverse population of *L. pneumophila* sg 1 but that only some of them were related to clinical isolates. It is possible that the samples were not representative of the whole water system and also that not enough colonies had been examined to permit identification of all phenotypes of *Legionella* that were present, giving rise to misleading results.[18] These difficulties point to the necessity of testing a large number of bacterial colonies when investigating an outbreak source. In theory all colonies from every sample should be examined but as this is not practical, picking of at least three colonies of apparently similar morphology is recommended.

With our technique of plasmid analysis we only found two plasmid-containing strains: HSC_{13}, and HSC_7. The first was distinguished by a

particular MAb subtype and a distinct ET which may reflect the presence of the plasmid. However strains HSC_7 and HSC_8 had the same MAb and the same ET (different from all the others) but only HSC_7 contained a 42 Md plasmid. The absence of plasmids from the other isolates may indicate that they were representatives of the same strain; alternatively these isolates may have lost plasmids during subculture or else have been more resistant to the method of plasmid extraction we used.

From our findings we conclude that multilocus enzyme electrophoresis was the most discriminative method to use as a single epidemiological tool and a good correlation was observed with monoclonal antibody typing.[19,20] These two methods of typing should therefore be applied for subtyping *L. pneumophila* sg 1 strains when investigating their transmission from the environment to patients. Plasmid content and electrophoresis protein profiles used to discriminate different populations of the same serogroup (sg 1) of *L. pneumophila* do not provide conclusive results for epidemiological purposes.

Repeated efforts to eliminate *Legionella* from the hot water by 'superheating' and hyperchlorination proved to be unsuccessful in our hospital as in many others. In hospitals with immunosuppressed patients, the prevention of legionellosis requires constant vigilance with a high standard of infection control and sophisticated engineering design and maintenance.[21]

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