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Relationship between *Legionella* spp. and antibody titres at a therapeutic thermal spa in Portugal

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SUMMARY

The presence of *Legionella* spp. in the water of a Portuguese spa was ascertained during the spa season, between May and November. Simultaneously the prevalance of anti-legionella antibodies in people attending the spa was also investigated. The antibody titres of 172 randomly selected patients and 42 therapists were determined, and compared with a control group of 503 blood donors. Legionellae were present in the spa water at low concentrations, generally lower than 10^3 c.f.u./l. A total of 92 strains representing eight different species or serogroups were isolated; the predominant isolates belonged to *Legionella pneumophila* serogroup 6 and to *L. londiniensis*.

During the study, no clinical cases of Legionnaires' disease were observed, and the antibody titres were generally low in the groups studied. However, the antibody titres of the patients increased slightly during their stay at the spa, approaching the values for the therapists. Mean antibody titres in the groups related with the spa were significantly higher than those in the blood donors against five of the seven legionella antigens tested. The largest number of elevated antibody titres in the exposed groups were to the *L. pneumophila* sg 5 and sg 6 antigens.

INTRODUCTION

Cases of Legionnaires' disease and Pontiac fever have been associated with air conditioning cooling towers [1, 2], portable water systems [3–6], plumbing and water systems in hotels, hospitals [7–10], and homes [11]. Legionellosis acquired

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from artificial water systems is generally due to *Legionella pneumophila* serogroup (sg) 1, which appears to be the most common legionella in these environments [12]. A few outbreaks of Pontiac fever have also been associated with whirlpool baths and spas in the USA [13], where the ethiological agent was *L. pneumophila* sg 6, and in Scotland, where the illness, designated Lochgoilhead fever, was caused by *L. micdadei* [14]. In addition to artificial systems, *Legionella* spp. have been recovered from natural sources such as streams, rivers, lakes [15–17], hydrothermal springs [18, 19], and potting soil [20]. After several cases of unexplained disease were reported in researchers visiting the Mt St Helens blast zone, several species of *Legionella* were isolated from geothermally heated water [21].

In general, warm water seems to promote the growth of many legionellae [22–24], and very high numbers have been isolated from biofilms associated with hot springs, and from hot water plumbing systems [19, 25]. Some therapeutic spas use geothermally heated water for treating rheumatism and respiratory ailments. such as sinusitis and asthma, and this heated water could potentially be a source of legionellosis. Although Joly and colleagues [26] generally recommend against monitoring natural sources for legionellae, he considered hot spring spas to be the 'sole exception'.

As a result of five cases of Legionnaires' disease, caused by *L. pneumophila* sg 1 and sg 3, among patients and therapists at a thermal spa in France, Bornstein and colleagues [27] measured antibody levels in patients and therapists at this therapeutic spa and found elevated levels of antibodies to some species and/or serogroups of legionella. The majority of the organisms isolated from that spa belonged to *L. pneumophila* sgs 3, 1 and *L. dumoffii*. The risk of further cases of legionellosis prompted these authors to recommend careful maintenance of hygiene and clinical surveillance of this type of establishment. Since therapeutic spas are widely distributed in Europe and elsewhere, we initiated a similar study at a thermal spa in Portugal where legionellae (primarily *L. londiniensis* and *L. pneumophila* sg 1) had been isolated from some of the sources, and pipes carrying water to the spa facilities [18]. The purpose of this study was to determine the numbers of culturable legionellae within this Portuguese spa and to compare the antibody levels of patients and therapists with a control group of blood donors from the same area.

METHODS AND MATERIALS

Sampling and isolation of Legionella *spp.*

The therapeutic spa, located in northern Portugal, serves approximately 6000 patients annually from May to November. Water is drawn from nearby thermal (56 °C) and cold springs and is piped to the spa. Patients typically spend 2 weeks at the spa receiving treatments consisting of nasal aerosols, baths, and high pressure water massages. The spa consists of three buildings and the surrounding grounds.

Ten sites were chosen inside the two major buildings, and each site was sampled three times: in May, just before the spa opened; in September; and in November, after the spa closed. Two nasal nebulizers, four bath tub taps, three shower heads,

and one pressurized hose for whole body massage, were sampled. Both water and swab samples were collected.

Water was collected in sterile 10 l plastic containers and then filtered through 142 mm diameter nylon filters (Pall Ultipore N66 or Gelman Supor 200 filters, 0·2 μm pore size). The filter was removed from the filter support, placed into 50 ml of the original water and shaken vigorously for about 1 h. Each sample was spread directly (0·1 ml) on Buffered Charcoal Yeast Extract Medium with α-ketoglutarate (BCYE) containing Glycine–Vancomycin–Polymyxin B-Cycloheximide (GVPC) with replicate samples subjected to acid and heat treatment [28]. The GVPC plates were incubated in a normal atmosphere at 37 °C for up to 14 days. Swabs were placed in 5 ml of sterile distilled water, shaken for at least 30 min, and then treated and plated as for the water samples.

The highest number of suspect legionella colonies on replicate plates were counted from each sampling [18]. All colonies from plates having 10 or less, and between 10 and 20 random colonies from plates having more than 10 colonies, were subcultured onto BCYE, and BCYE lacking cysteine and ferric pyrophosphate. The organisms which grew on BCYE, but failed to grow on BCYE lacking cysteine and ferric pyrophosphate were considered as separate legionella isolates, assigned different isolation numbers and stored at −80 °C in 5% (w/v) yeast extract (Difco) with 15% (v/v) glycerol.

Identification of legionella isolates was performed by indirect immunofluorescence assay (IFA) as described by Veríssimo and colleagues [18]. Strains of *L. pneumophila* sg 1 were subgrouped with the set of monoclonal antibodies (MAb) developed by Watkins and colleagues [29].

Bacteriological quality of the water samples

Samples for the examination of the bacteriological quality of the water were maintained at 4 °C, and examined within 12 h. Samples were filtered through Millipore (type HAWG, 0·45 μm pore size) filters. Faecal coliforms were enumerated on m-FC medium, faecal streptococci on KF-*Streptococcus* agar and *Pseudomonas aeruginosa* on Cetrimide agar and confirmed as described in Standard Methods for the Examination of Water and Wastewater [30]. The heterotrophic plate count (HPC) was performed by the pour plate method on Plate Count Agar incubated at 30 °C for 24 h [30].

Antibody response

A single serum sample was collected from each of the blood donors, patients, and therapists. In order to determine if legionella antibody concentrations increased over time, second and third samples were collected from some patients; second samples were also taken from some of the therapists. For patients, samples were taken when they arrived at the spa, at the end of the 2-week treatment, and then 3 or 4 weeks later. A clinical record sheet was completed at the beginning and end of the 2-week treatment period for each patient.

Serum antibody levels of the subjects were established, by indirect immunofluorescent assay, using 14 prepared antigens of the following seven species or serogroups of legionella: *L. pneumophila* combined serogroups 1–4; *L. pneumophila*

combined serogroups 5 and 6; *L. pneumophila* combined serogroups 7–10; *L. dumoffii*; *L. bozemanii* serogroup 1; *L. bozemanii* serogroup 2; and *L. londiniensis*. Antigens were prepared by the French reference centre for legionellosis in Lyon following the method of Taylor [31] except for *L. londiniensis* which was prepared from thermally inactivated bacteria [32]. These antigens were chosen because they corresponded to the most prevalent isolates in the hydrothermal areas in the vicinity of the spa [18].

Indirect immunofluorescent assays were performed using fluorescein isothiocyanate-labelled antihuman immunoglobulin (Sigma) as the conjugate.

Statistical methods

A value of eight was used for all samples which showed no antibodies to a particular serum [27] and means were calculated for each *Legionella* species group. Mean antibody titres of patients, donors and therapists were compared using analysis of variance techniques (ANOVA); individual pairs of means were then compared using the Tukey-Kramer HSD post hoc test. Probabilities at the $P < 0{\cdot}05$ levels were considered statistically significant.

RESULTS

Distribution of Legionellaceae *within the spa*

In general, the water quality within the spa was relatively free of indicators of faecal contamination. Faecal coliforms were not detected in any samples. Faecal streptococci were detected in 11 of 29 samples, and ranged from $1{\cdot}0 \times 10^1$ to $1{\cdot}3 \times 10^2$ c.f.u./100 ml; *Pseudomonas aeruginosa* was found in five samples (range $1{\cdot}0 \times 10^1$ to $5{\cdot}0 \times 10^1$ c.f.u./100 ml), the heterotrophic plate counts were low ($1{\cdot}2 \times 10^2$ to $8{\cdot}4 \times 10^4$ c.f.u./ml). The water temperature at the source was 56 °C and the pH 8·9. Within the spa the water had a mean temperature of 38·9 °C (minimum 28·7 °C; maximum 41·3 °C).

A series of ten sites within the spa were sampled periodically for the presence of legionella and a total of 92 isolates representing eight different species or serogroups were recovered from 29 water samples and three biofilm swabs. *Legionella pneumophila* sg 6 and *L. londiniensis* were the most common isolates (Table 1). Three strains of *L. pneumophila* sg 1 belonging to the monoclonal antibody subgroup OLDA, and one strain to monoclonal antibody subgroup Bellingham, were also isolated. Legionella concentrations in water were generally very low, and in only one instance did the number of isolates reach 10^3 c.f.u./l (Table 1). The diversity of isolates was highest during the November sampling, and this was statistically higher than the September sampling time ($P < 0{\cdot}05$).

Swabs of biofilms were generally negative for legionella isolates (18/21) although when present, legionella concentrations from swabs were higher than the water concentrations reaching 10^7 c.f.u./l. Positive swab samples were found at three of the four bath tub taps, and the species corresponded largely to those isolated from the water samples.

Two samples of the thermal source consistently yielded *L. londiniensis* (80%) and *L. pneumophila* sg 1 (17%). The strains of *L. pneumophila* sg 1 belonged to the

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Table 1. *Concentration and distribution of* Legionella *spp. detected in the spa during the season*

Sample site	Sampling date	*Legionella* (c.f.u./l*)	*Legionella* isolates
Nebulizer 1	May	ND†	—
	September	ND	—
	November	$5{\cdot}6 \times 10^2$	*L. londiniensis*
Nebulizer 2	May	$5{\cdot}0 \times 10^1$	*L. londiniensis*
	September	ND	—
	November	$3{\cdot}1 \times 10^2$	*L. londiniensis*
Bath tub tap 1	May	$6{\cdot}5 \times 10^2$	*L. pneumophila* sg 6
	September	ND	—
	November	$1{\cdot}3 \times 10^2$	*L. moravica*
Bath tub tap 2	May	$3{\cdot}2 \times 10^3$	*L. dumoffii*
	September	$7{\cdot}1 \times 10^1$	*L. moravica*
	November	$5{\cdot}5 \times 10^2$	*L. moravica*
			L. oakridgensis
			L. pneumophila sg 1 (OLDA)
			L. pneumophila sg 6
Bath tub tap 3	May	$1{\cdot}3 \times 10^3$	*L. moravica*
			L. pneumophila sg 1 (Bellingham)
			L. pneumophila sg 6
	September	ND	—
	November	$4{\cdot}0 \times 10^2$	*L. londiniensis*
Bath tub tap 4	May	$1{\cdot}0 \times 10^2$	*L. pneumophila* sg 3
	September	ND	—
	November	$1{\cdot}0 \times 10^2$	*L. pneumophila* sg 6
Shower head 1	May	ND	—
	September	ND	—
	November	$1{\cdot}8 \times 10^2$	*L. micdadei*
			L. moravica
Shower head 2	May	$2{\cdot}4 \times 10^3$	*L. pneumophila* sg 6
	September	$3{\cdot}3 \times 10^2$	*L. moravica*
			L. pneumophila sg 6
	November	$2{\cdot}7 \times 10^2$	*L. pneumophila* sg 6
			L. spp.
Shower head 3	May	$3{\cdot}3 \times 10^2$	*L. pneumophila* sg 1 (OLDA)
			L. pneumophila sg 6
	September	$2{\cdot}5 \times 10^2$	*L. moravica*
	November	$1{\cdot}0 \times 10^2$	*L. pneumophila* sg 6
Pressurized hose	May	$6{\cdot}7 \times 10^2$	*L. pneumophila* sg 6
	September	$1{\cdot}4 \times 10^2$	*L. pneumophila* sg 6

* c.f.u./l, colony-forming units per litre.
† ND, not detected (samples had low environmental contamination).

monoclonal antibody subgroups OLDA, and Bellingham. The average concentration of legionellae found in the source was $1{\cdot}1 \times 10^3 \pm 8{\cdot}8 \times 10^2$ c.f.u./l.

Antibody response of blood donors, patients and therapists

A total of 717 subjects including randomly selected blood donors (503), patients (172) visiting the thermal spa, and therapists (42) participated in the study. Most of the patients (87·3%) had visited the spa before, and most sought treatment for rheumatism (79·6%) or asthma/sinusitis (28·5%). Of the patients, 95% were

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Table 2. *Distribution of antibody responses to all* Legionella *spp. and serogroups for all blood donors, patients (second sampling), and therapists*

Antibody titres	Legionella antigens: *pneumophila* sg 1–4	*pneumophila* sg 5 and 6	*pneumophila* sg 7–10	*dumoffii*	*bozemanii* sg 1	*bozemanii* sg 2	*londiniensis*
			Donors				
8	438	394	462	493	391	494	499
16	51	67	31	1	3	—	4
32	14	40	10	9	56	7	—
64	—	1	—	—	47	2	—
128	—	1	—	—	6	—	—
			Patients				
8	90	73	80	94	73	88	97
16	7	16	13	—	1	—	—
32	3	5	6	6	10	8	2
64	—	5	1	—	14	2	1
128	—	1	—	—	2	2	—
			Therapists				
8	37	31	33	36	31	38	38
16	3	5	4	1	—	—	—
32	1	4	3	4	4	3	2
64	1	1	—	1	7	1	2
128	—	1	2	—	—	—	—

Table 3. *Percentage of study groups with negative, borderline or positive antibody titres to at least one legionella antigen*

	Donors	Patients: 1st sampling	Patients: 2nd sampling	Therapists
Negative ⩽ 32 *L. pneumophila* ⩽ 64 other *Legionellaceae*	98·8	95·7	89	90·5
Borderline 64–128 *L. pneumophila* ⩾ 128 other *Legionellaceae*	1·2	3·7	11	9·5
Positive ⩾ 256 single simple	—	0·6	—	—

> 40 years old and 71·5% were female; similarly, most of the therapists were > 40 years old (60·1%) and female (70·7%).

Using conventional criteria for serological diagnosis as defined by Wilkinson and colleagues [32], 96·4% of the subjects in this study showed no antibody response to any of the species or serogroups of legionella tested. With one exception, the remaining individuals showed a borderline response to at least one, and some to as many as six of the species or serogroups. The largest number of borderline responses in the subjects were to *L. pneumophila* sg 5–6, nine individuals, and *L. bozemanii* sg 1, eight individuals (Table 2). The antibody titres of the patients were quite low, but increased slightly during their 2-week stay (Table 3); however, these differences were not statistically significant. One patient

Table 4. *Mean antibody titres of donors (controls), patients (second sampling) and therapists*

Species/ serogroup	Mean antibody titres				Pairwise comparisons		
	Donors	Patients	Therapists	*P*	Donors–patients	Donors–therapists	Patients–therapists
L. pneumophila 1–4	9·48	9·28	10·48	NS*	NS	NS	NS
L. pneumophila 5 and 6	11·32	14·48	15·43	0·0075	0·05	NS	NS
L. pneumophila 7–10	8·97	11·04	16·19	0·0000	NS	0·05	0·05
L. dumoffii	8·45	9·44	11·81	0·0000	NS	0·05	0·05
L. bozemanii 1	17·38	20·72	19·62	NS	NS	NS	NS
L. bozemanii 2	8·56	13·44	11·05	0·0000	0·05	NS	NS
L. londiniensis	8·19	9·04	11·81	0·0000	NS	0·05	0·05
Sample size	503	100	42				

* NS, not significant ($P > 0·05$).

had a very high antibody titre to *L. pneumophila* sg 1 on arrival, but was unavailable for a second sampling.

In order to compare differences among groups, we compared the donors with the patients (second sampling) and the therapists (Table 4). We chose the second blood sampling for the patients as this corresponded to the time they completed their treatment at the spa and hence possibly had time to develop some antibody response. In general, there was a tendency for therapists to have higher antibody titres than either patients or blood donors (controls). This was evident for five of seven species or serogroups, and in three instances the differences between therapists–donors and therapists–patients were highly significant (Table 4). For *L. pneumophila* combined serogroups 5 and 6 and *L. bozemanii* 2, blood donors differed significantly from patients, but not therapists. Although there were statistical differences between and among groups of subjects, most antibody titres were quite low.

DISCUSSION

Legionellae have been detected and isolated from many hydrothermal areas around the world [18, 19, 27]. Despite the fact that in some of those areas water is used for therapeutic purposes in spas, little is known about the consequences of the use of the water, specifically the relationships between the legionellas present and the people exposed to these organisms in the spas.

As previously observed in France [27], our study, shows a clear association between exposure to hot spring water and an increase in antibody titres against the majority of species or serogroups of legionellae isolated within the spa. There were significant differences in antibody titres between the blood donor group and the patients and therapists, exposed to the water in the spa. Furthermore, the largest number of elevated (borderline) antibody titres in all exposed groups were to *L. pneumophila* sg 5–6. In fact, *L. pneumophila* sg 6 was the predominant isolate from the spa water.

Nonetheless, the titres were relatively low, in most cases falling into the 'negative' category. However, we show that antibody titres tend to increase with time. When patients arrive at the spa, the titres were quite similar to those of the blood donor group, but after 2 weeks stay, their titres approached those of the therapists. It is also notable that therapists, who have prolonged and continuous exposure in the spa, were generally the group with the highest antibody titres. During this study there were no reported cases of legionellosis, and as far as we are aware, there have never been any cases that could be directly associated with this spa. Nevertheless, we found one patient with very high antibody titres against *L. pneumophila* sg 1 (> 1024).

This patient was a 89-year-old male who attended the spa, 2 weeks a year for nasal nebulization therapy, for the past 30 years. The previous year he was admitted to a local clinic with severe pneumonia about 3 weeks after attending the spa. He received antibiotic therapy with cephotaxime for 10 days and recovered completely. The illness was not diagnosed at the time of admission to the clinic.

The number and the diversity of legionellae in the surrounding areas were higher than those found in the water used at the spa facilities, where the numbers of culturable legionellae were lower. In another study on similar hydrothermal environments, we demonstrated that the highest numbers of legionellae were associated with biofilms rather than water [19], but in the spa reported here the biofilm accumulation appeared to be low, and may have accounted for the low concentrations of legionellae isolated from the water.

The relationships between the concentration of legionellas in water systems and disease is poorly understood [26]. However, Miller and Kenepp [33] considered concentration greater than 10^6 c.f.u./l to be of risk for disease. By this criterion, the concentrations that we found at the spa are of very low risk. Nevertheless, since legionellae are found in the source water, and because disinfection of spa water is strictly forbidden by Portuguese law, it is difficult, if not completely impossible, to remove the legionellae. Therefore, we recommend that cleaning measures be implemented at this and similar spas, particularly to prevent biofilm accumulation. This should maintain the number of legionellas in the water at low levels, and thus reduce the risk of human contamination.

The strains isolated in the spa are not generally considered to be the most pathogenic. This belief, combined with the low numbers detected, may be the main reasons for the apparent absence of disease. Other factors may also contribute to this absence of disease; namely, the majority of the population exposed to the water were females, who may be relatively less susceptible than males to Legionnaires' disease.

In conclusion, although the risk of contracting legionellosis and associated diseases by the population attending spas is generally low, development and implementation of maintenance procedures to ensure adequate hygiene standards are of crucial importance. These measures should include monitoring the environment for legionellas and clinical surveillance of the populations exposed to spa water.

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