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Detection of *Legionella pneumophila* in wastewater by nested polymerase chain reaction

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SUMMARY

The study of *Legionella* in treated wastewater acquires special importance when this water is used in irrigation by spray, as *Legionella* is transmitted via the inhalation of aerosols and may consequently represent a health risk. In this study, we applied polymerase chain reaction (PCR) amplification as an alternative method to plate culture for detecting *L. pneumophila* in twelve heavily biocontaminated samples from a wastewater treatment plant. Moreover, we studied the efficiency of rapid gel filtration methods and filtration through chelating ion exchange resin in the elimination of PCR inhibitors from wastewater samples. When *Legionella* was investigated by PCR without any previous treatment, no amplification occurred, and when we used chromatographic methods to eliminate PCR inhibitors, nine out of twelve samples became positive. These results indicate the abundant presence of *Legionella* in wastewater, and although the methods used to eliminate PCR inhibitors are effective in the preparation of clean samples, the possible presence of different metal-organic matter compounds, which are not eliminated, may produce false-negative results.

Key-words: Irrigation, *Legionella pneumophila*, Nested PCR, Wastewater, Biocontamination.

INTRODUCTION

Over the last few years, the growing demand for water has resulted in increasing re-utilization of wastewater. One of the most frequent applications of wastewater is in agricultural and landscape irrigation. Watering by spray is one of the most common methods, particularly in gardens and golf courses. This re-utilization of water involves a health risk that is associated with the effect of exposure to the microbial or chemical content of reclaimed water (Kindzierski and Gabos, 1994).

Legionella pneumophila, the aetiological agent of Legionnaire's disease and Pontica fever (Fraser *et al.*, 1977; Glick *et al.*, 1978), is found in natural aquatic habitats (reservoirs, rivers, lakes, etc.) and has found an appropriate ecological niche for itself in potable water systems, cooling tower systems, thermal waters for rehabilitation and other human aquatic environments (Fliermans *et al.*, 1981; Barlett *et al.*, 1983; Coulbourne *et al.*, 1988; Dondero *et al.*, 1980). Human infection occurs through the inhalation of aerosols contaminated by *Legionella*; no interhuman transmission has yet been found.

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Treated wastewater may be an important reservoir for *Legionella* and, although its use in irrigation by spray may be an important health risk, the presence of *Legionella* in this aquatic environment has not yet been studied extensively (Palmer *et al.*, 1993).

The standard method for *L. pneumophila* detection involves isolation in a selective medium. However, several problems are encountered with this method, including the presence of viable but non-culturable cells, loss of viability of bacteria after collection and the time required for culture and confirmation, which can take several days. In addition, the detection of *L. pneumophila* in biocontaminated samples such as wastewater is difficult, since *Legionella* can be inhibited or masked by the rapid or abundant growth of other microorganisms (Hussong *et al.*, 1987; Flesher *et al.*, 1980).

For these reasons, in order to avoid the problems encountered with plate culture, the use of an alternative method is needed. The advantages of polymerase chain reaction (PCR) (Saiki *et al.*, 1988) make it the preferred alternative method. The usefulness of PCR for detecting *L. pneumophila* in potable water has been extensively demonstrated (Starnbach *et al.*, 1989; Mahbubani *et al.*, 1990; Bej *et al.*, 1991; Catalán *et al.*, 1994). Despite the theoretical possibility of using PCR for detecting *L. pneumophila* in wastewater, it has been previously reported that trace amounts of organic matter and heavy metals can inhibit the PCR, giving false-negative results (Steffan *et al.*, 1988; Tsai and Olsen, 1992a).

To overcome this problem, rapid gel filtration methods to separate DNA from extracts containing humic substances, and filtration through chelating ion exchange resins to eliminate metal ions, have been developed (Tsai and Olson, 1992b; Tsai *et al.*, 1993; Abbaszadegan *et al.*, 1993; Straub *et al.*, 1994).

The objectives of this study were to determine the presence of *L. pneumophila* in wastewater by nested PCR, avoiding the problems encountered with plate culture, and to analyse the efficiency of chromatographic methods in the elimination of PCR inhibitors from complex wastewater containing significant industrial and urban inputs.

MATERIALS AND METHODS

Sample collection

Twelve treated wastewater samples were collected from a wastewater treatment plant in Ibi (Spain). This plant receives both industrial and urban inputs and carries out primary treatment of sewage. Each of those twelve samples was collected weekly for three alternate months with an autosampler (ISCO, 2.900).

Culture method

For plate culture, 200 ml of water sample were pre-filtered through a 4-μm pore size, 11-cm diameter glass microfibre filter GF/C(Whatman, Maidstone, England). The filtered water was filtered again through 0.45-μm pore size, 47-mm diameter mixed esters of cellulose membranes (Millipore, Molsheim, France). Filters were placed on buffered charcoal-yeast extract (BCYE) containing 0.1% α-ketoglutarate, adjusted to pH 6.9 with KOH, supplemented with 0.4 g L-cysteine, 0.25 g ferric pyrophosphate per litre of medium (Edelstein, 1982), to which were added 3 g glycine and 1 mg vancomycin, 50,000 U polymyxin B and 80 mg cycloheximide. Finally, plates were incubated at 37°C in a 5% CO_2 atmosphere and observed daily for 7 days.

Alternatively, when abundant growth of hererotrophic bacteria was suspected, as occurs with wastewater, the filter was placed in a sterile 20-ml screw-capped tube with 5 ml of sterile distilled water, and the tube vortex mixed for 5 min to release any microorganism present in the filter. This suspension was diluted 1:10 with 0.2 N HCl-KCl pH 2.2 buffer and after 5 min, 0.1 ml was inoculated on the BCYE medium (Boop *et al.*, 1981).

Elimination of PCR inhibitors by the "spun column" procedure

In order to eliminate PCR inhibitors by a simple method, samples were filtered through Bio-Gel P-

BCYE = buffered charcoal-yeast extract.
mip = macrophage infectivity potentiator.
PCR = polymerase chain reaction.
TOC = total organic carbon.

100 (BioRad Laboratories, Richmond CA) combined with Chelex-100, 100-200 mesh (BioRad) in a "spun column" procedure. An insulin syringe was filled with 0.5 ml of 20% Chelex-100, and then 1 ml of Bio-Gel P-100 was added on top of the Chelex-100. The syringe was inserted into a 50-ml disposable polypropylene graduated conical tube and was centrifuged at 1,500 *g* for 10 min in a swinging-bucket rotor in a benchtop centrifuge. The syringe was refilled with Bio-Gel P-100 and centrifuged again. Finally, 200 µl of sample were layered on top of the column, the column was then centrifuged at 1,500 *g* for 10 min, and the sample recovered in a 1.5-ml Eppendorf tube was placed at the bottom of the 50-ml tube.

With this "spun column" procedure, the volume of sample recovered is insufficient for PCR, total organic carbon (TOC) and metal analysis to be carried out, so for the latter two analyses, purification was carried out by traditional chromatographic treatment in a 15-cm long, 1-cm diameter glass column, and 10 ml of each sample were recovered by gravity instead of by centrifugation. PCR analysis was done on these samples as well.

Analysis of total organic carbon and metals

The levels of organic matter and metals were determined before and after the treatment with Bio-Gel P-100 and Chelex 100. Prior to their analysis, the samples were filtered through a 4-µm pore size, 11-cm diameter glass microfibre membrane.

Total organic carbon (TOC) was determined in a "Shimadzu TOC 5.000" autoanalyser. In order to eliminate inorganic carbon, samples were acidified with 25% phosphoric acid (Merck) to pH 4.0, and purged with the same carrier gas (synthetic air CO_2-free) for 10 min at a flow of 150 ml/min. The limit of determination was 0.2 mg/l.

Metals (boron, barium, calcium, iron, manganese, magnesium, lead, nickel, zinc, aluminium, copper, chromium and strontium) were determined in an inductively coupled plasma "Thermo Jarrell Ash Iris" emission spectrometer equipped with a charge injection device detector and a "cross-flow" nebulizer. Before the metal analysis was carried out, 2 ml of 65% nitric acid (Merck) were added to 100 ml of filtered sample. The determination limit was 0.02 mg/l for all metals.

Detection of *L. pneumophila* by PCR

For PCR analysis, 200 ml of water were filtered through a 4-µm pore size, 11-cm diameter glass microfibre filter; the filtered water was filtered again through 0.45-µm pore size, 47-mm diameter mixed esters of cellulose membrane. Both filters were deposited in a 50-ml tube with deionized water, vortexed, and the water transferred to a new tube and centrifuged at 3.500 *g* for 30 min. The supernatant was discarded; 100 µl of 20% Chelex 100 were added, and finally the sample was heated at 90°C for 10 min.

After the elimination of PCR inhibitors, 10 µl of sample were amplified according to a previously described method (Catalán *et al.*, 1994). Briefly, primers L*mip*920 and L*mip*1548 that amplify a 650-bp sequence of the coding region of the *L. pneumophila* macrophage infectivity potentiator (*mip*) gene were used in the first reaction, and then 2 µl of amplification product were amplified using internal primers L*mip*976 and L*mip*1427, resulting in the detection of a 471-bp sequence. In order to avoid contamination, published guidelines were followed to safeguard against nucleic acid contamination (Kwok and Higuchi, 1989). The study of the specificity of internal primers had been determined in a previous study (Catalán *et al.*, 1994) in which we used DNAs from several closely related bacterial species of *L. pneumophila* as matrices, and no amplified product was obtained.

RESULTS

When *L. pneumophila* was investigated by plate culture in the twelve wastewater samples collected, all the samples produced plate contamination in spite of the acid pretreatment, and subsequently, it was impossible to isolate *Legionella.*

Afterwards, when we studied the presence of *L. pneumophila* in these samples without any previous treatment by nested PCR, no amplification occurred (table II).

Consequently, due to the possibility of the presence of many different PCR inhibitors such as organic matter and metals in the wastewater samples, we studied the levels of these inhibitors before and after the described chromatographic methods.

The studied metals could be classified into two groups. The first included the major constituents of water composition (calcium, magnesium, boron, strontium and barium), whereas the other group included metals present in industrial inputs, especially from chromium-

plating industries (copper, chromium, nickel and zinc). The concentration of all of them was constant in all the samples studied. Results (tables I and II) show that the decontamination treatment enabled the elimination of over 98%, except in the case of iron, ranging between 90% and 80%, and zinc, the elimination of which is poor (between 85% and 20%), especially in samples number one, seven and eleven.

With regard to TOC (table II) elimination rates of over 97% were obtained, with the amount of organic matter in the samples being very small (between 6 and 0.2mg/l), although three samples (samples number one, seven and eleven) showed high levels of TOC after treatment.

When *L. pneumophila* was investigated by PCR in samples that were treated by the "spun column" procedure or by traditional chromatographic treatment, 9 of the 12 samples showed an amplified product of 471 bp (fig. 1), and since the primers were specific for *L. pneumophila*, were considered as positive samples. It is important to underline that the three samples in which the PCR-negative persisted after the treatment were those with the highest TOC, iron and zinc values (samples number one, seven and eleven) (table II).

DISCUSSION

L. pneumophila, the organism responsible for the majority (80%) of legionellosis outbreaks, is ubiquitous in aquatic environments. This study determined the abundant presence of *L. pneumophila* in treated wastewater from a standard treatment plant, and this result is in agreement with those obtained by other authors when detecting *Legionella* in primary influents and primary and secondary effluents of a sewage treatment plant (Palmer *et al.*, 1993).

The resistance of *Legionella* to wastewater treatment had been described previously (Kuchta *et al.*, 1993). Several studies have demonstrated the intracellular presence and growth of *Legionella* in various aquatic protozoa, especially amoebae (Fields, 1993). This intriguing relationship would allow the bacteria to withstand a variety of adverse conditions, such as processing at a wastewater treatment plant.

While *Legionella* was not isolated from any of the samples we studied, other authors encountered 25% of positive samples (1 out of 4 samples), although they indicate that plate culture is the least sensitive method available (Palmer *et al.*, 1993). This difference in *Legionella* recovery may be due to the high level of biocontamination

Table I. Levels of major constituents of water composition in wastewater sample.

	Major constituents (µg/ml)									
	Calcium		Magnesium		Boron		Strontium		Barium	
Sample number	A	B	A	B	A	B	A	B	A	B
1	94.4	0.94	29.2	0.13	2.37	≤0.02	0.47	≤0.02	0.12	≤0.02
2	84.8	0.70	31.5	0.07	2.69	≤0.02	0.45	≤0.02	0.05	≤0.02
3	82.6	0.36	29.3	0.03	2.37	≤0.02	0.42	≤0.02	0.05	≤0.02
4	72.8	0.42	19.1	0.13	1.51	≤0.02	0.37	≤0.02	0.07	≤0.02
5	64.8	0.76	26.4	0.24	2.31	≤0.02	0.35	≤0.02	0.06	≤0.02
6	86.5	0.59	34.8	0.12	2.97	≤0.02	0.46	≤0.02	0.06	≤0.02
7	92.3	0.34	30.8	0.09	1.92	≤0.02	0.48	≤0.02	0.07	≤0.02
8	74.1	1.71	27.7	0.16	2.77	≤0.02	0.36	≤0.02	0.05	≤0.02
9	59.1	0.56	25.9	0.04	2.59	≤0.02	0.34	≤0.02	0.05	≤0.02
10	59.3	0.70	20.7	≤0.02	2.55	≤0.02	0.34	≤0.02	0.05	≤0.02
11	80.0	0.70	27.4	0.11	2.27	≤0.02	0.41	≤0.02	0.20	≤0.02
12	79.8	0.30	27.5	0.05	2.29	≤0.02	0.42	≤0.02	0.04	≤0.02

A = before chromatographic treatment; B = after chromatographic treatment.

Table II. Results of metals, TOC and PCR analysis in wastewater samples.

Sample number	Metals (μg/ml) Iron A	Iron B	Copper A	Copper B	Aluminium A	Aluminium B	Zinc A	Zinc B	Nickel A	Nickel B	Lead A	Lead B	Manganese A	Manganese B	Chromium A	Chromium B	TOC (μg/ml) A	TOC (μg/ml) B	PCR A	PCR B
1	1.26	0.26	2.70	0.09	≤0.02	≤0.02	0.15	0.12	2.56	0.04	0.03	≤0.02	0.10	≤0.02	0.79	≤0.02	256	22.6	–	–
2	1.37	0.06	5.99	≤0.02	0.17	≤0.02	0.81	0.08	4.70	≤0.02	≤0.02	≤0.02	0.09	≤0.02	2.15	≤0.02	223	≤0.2	–	+
3	0.76	0.03	4.47	≤0.02	0.05	≤0.02	0.37	0.03	3.22	≤0.02	≤0.02	≤0.02	0.03	≤0.02	1.82	≤0.02	227	2.7	–	+
4	0.59	0.09	3.26	≤0.02	≤0.02	≤0.02	0.25	0.06	3.15	≤0.02	0.04	≤0.02	0.06	≤0.02	0.91	≤0.02	178	5.4	–	+
5	0.29	≤0.02	1.02	0.03	0.13	≤0.02	0.13	≤0.02	1.07	≤0.02	≤0.02	≤0.02	0.04	≤0.02	0.08	≤0.02	124	≤0.2	–	+
6	1.64	0.19	4.22	0.04	0.18	≤0.02	0.83	0.06	4.02	≤0.02	≤0.02	≤0.02	0.08	≤0.02	1.58	≤0.02	240	6.0	–	+
7	1.09	0.20	1.02	0.04	≤0.02	≤0.02	0.12	0.09	1.74	0.03	0.04	≤0.02	0.12	≤0.02	0.44	≤0.02	226	34.8	–	–
8	1.07	0.06	4.23	≤0.02	0.18	≤0.02	0.66	0.11	3.87	≤0.02	0.03	≤0.02	0.04	≤0.02	3.45	≤0.02	238	≤0.2	–	+
9	0.68	0.03	2.02	≤0.02	0.50	≤0.02	0.35	0.07	0.78	≤0.02	≤0.02	≤0.02	0.03	≤0.02	1.57	≤0.02	232	3.8	–	+
10	0.77	0.05	2.38	≤0.02	0.07	≤0.02	0.36	0.07	1.18	≤0.02	≤0.02	≤0.02	≤0.02	≤0.02	4.40	≤0.02	242	4.9	–	+
11	1.72	0.30	4.34	0.05	≤0.02	≤0.02	0.12	0.09	1.74	0.03	0.04	≤0.02	0.12	≤0.02	0.44	≤0.02	324	12.6	–	–
12	0.69	≤0.02	4.45	≤0.02	0.03	≤0.02	0.27	0.04	3.03	≤0.02	≤0.02	≤0.02	0.03	≤0.02	3.02	≤0.02	230	3.0	–	+

A = before chromatographic treatment; B = after chromatographic treatment.

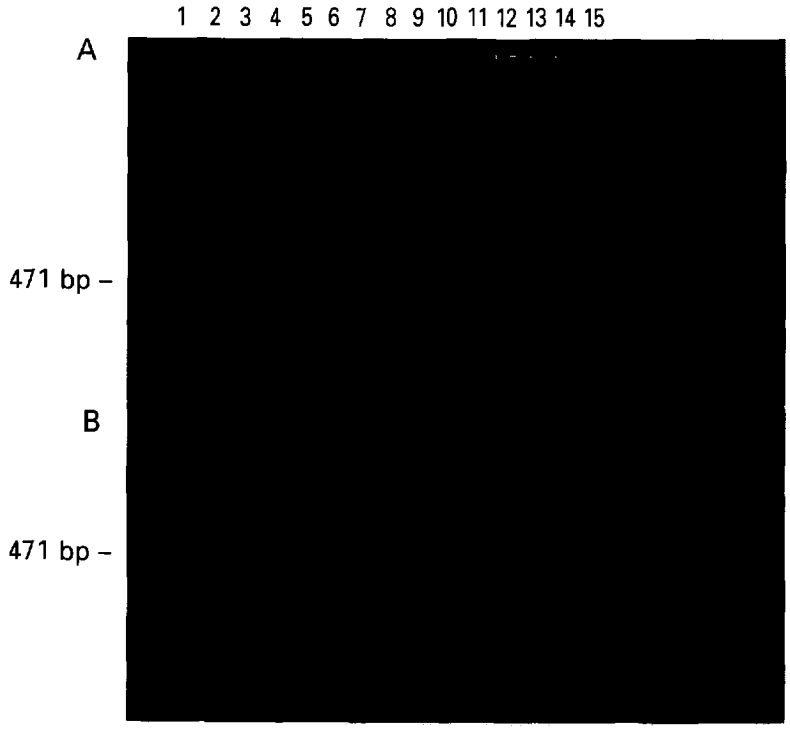

Fig. 1. Agarose gel electrophoresis of *L. pneumophila* nested PCR-amplified products from standard chromatographic treated wastewater (A) and from "spun column" treated wastewater (B).

Lane 1, length marker (*Hae*III-digested pBR322); lane 2, positive control; lane 3, negative control; lane 4 to 15 show PCR-amplified products from samples number 1 to 12.

of samples we studied compared with those encountered by other authors.

In any case, it is evident that plate culture is not an appropriate method for detecting *Legionella* in this kind of sample, since competition from faster-growing organisms and the inhibitory effect of heterotrophic bacteria can overcome *Legionella* in the media.

For this reason, we consider that the application of PCR amplification for detecting *L. pneumophila* in these heavily contaminated samples is interesting.

Our results show that PCR permitted the detection of *L. pneumophila* in 9 out of 12 samples analysed (75%). Palmer *et al.* (1993) encountered 5% positive samples for *L. pneumophila* and 100% for *Legionella* species. These results may be explained by differences in the sensitivity of the method or by the differing contamination of samples.

In consequence, PCR is the preferred method for detecting *L. pneumophila* in biocontaminated samples, as it is rapid, specific and sensitive and avoids the problems encountered with plate culture techniques. Moreover, previously described nested PCR has the advantage of a rapid turnaround time (5-6 h), sensitivity and simplicity, since only amplification and agarose gel electrophoresis are required. In spite of these advantages, it is necessary to increase precautions in order to avoid false-positives due to contamination. Future nested systems may perform the first and nested amplification in the same tube, avoiding sample manipulation.

Since wastewater is a heavily contaminated source, it is necessary to use methods that permit the elimination of PCR inhibitors in order to

avoid false-negative results. Experimentally, it has been demonstrated that by using chromatographic methods, sewage samples which were initially PCR-negative were positive after the treatment (Tsai *et al.*, 1993). In this study, we investigated the efficiency in the elimination of heavy metals and organic matter present in the wastewater samples analysed. Our results confirm that this method permits effective elimination of the heavy metals and organic matter present in the sample, obtaining positive results with samples initially PCR-negative. All the PCR-positive samples presented low levels of TOC and metals after treatment.

Nevertheless, there were some samples with high levels of organic matter and metals (iron and zinc) after the decontamination treatment and, in view of the fact that all these samples were PCR-negative, an inhibitory effect could have been present. This failure in the elimination of inhibitors may be due to the complexity of the sample. The presence of metals from industrial inputs, as well as organic matter of urban origin, can produce a complex association among these metals and the organic matter. These organometallic compounds can present a higher molecular weight than the exclusion limit of the filtration gel. In addition, Chelex-100 cannot retain the metals when they are associated with organic matter (del Castelho, 1991). In consequence, the chromatographic treatment evaluated in this study is effective in the elimination of PCR inhibitors from simple samples, but may fail in the elimination of these substances if the chemical composition of the samples is too complex, a fact which can lead to false-negative results.

Additional studies are needed in order to determine the nature of these PCR inhibitors and to develop rapid and simple methods for their effective elimination.

Finally, as the results show, it is clear that the presence of *L. pneumophila* in wastewater can represent a health risk, although further studies are required to determine this, especially when this water is reused for irrigation by spray (Palmer *et al.*, 1993; Hughes and Steele, 1994). Subsequently, it is necessary to study different measures to preserve public health, although initially, the use of wastewater for irrigation by spray in public gardens and golf courses should be controlled.

Mise en évidence de *Legionella pneumophila* dans les eaux usées, à l'aide de la PCR nichée

La recherche de *Legionella pneumophila* dans les eaux usées traitées prend une importance particulière quand l'eau est utilisée pour l'irrigation par arrosage, étant donné que *Legionella* est transmis via l'inhalation d'aérosols et, par conséquent, peut représenter un risque pour la santé publique. Dans cette étude, nous appliquons pour la détection de *L. pneumophila* l'amplification par PCR (polymerase chain reaction) comme alternative à la culture sur plaques, sur 12 échantillons d'eaux d'irrigation usées dont la contamination biologique est massive. De plus, nous étudions l'efficacité de techniques de filtration rapide sur gel et de filtration sur résines échangeuses d'ions chélatés pour l'élimination des inhibiteurs de la PCR. Quand les échantillons d'eaux usées ne sont pas préalablement filtrés, la recherche de *Legionella* par PCR est négative, mais quand les techniques chromatographiques d'élimination des inhibiteurs de la PCR sont utilisées, 9 échantillons sur 12 deviennent positifs. Ces résultats indiquent la présence de *Legionella* en abondance dans les eaux usées, et bien que les techniques utilisées soient efficaces pour la préparation des échantillons, la présence possible de différents composés métaux-matière organique non éliminés peut donner des résultats faussement négatifs.

Mots-clés : PCR nichée, Eau usée, Irrigation, *Legionella pneumophila,* Contamination biologique.

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