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ORIGINAL ARTICLE

Persistence of chlorine-sensitive *Legionella pneumophila* in hyperchlorinated installations

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Abstract

Aims: To study the persistence of *Legionella* over time in different disinfected facilities and analysing whether failures in bacterial eradication could be the result of a decrease in the susceptibility of the persistent strains to subsequent treatments.

Methods and Results: A long-term environmental surveillance was carried out in three different facilities associated with cases of Legionnaires' disease (a hospital, a fishing boat and a hotel). Despite several hyperchlorination episodes, amplified fragment length polymorphism, pulsed field gel electrophoresis-*Sfi*I and arbitrarily primed polymerase chain reaction methods demonstrated that a specific clone of *L. pneumophila* serogroup 1 was able to survive for 17, 5 and 10 years in the hospital, fishing boat and the hotel, respectively. Isolates from different years from the same facility showed similar minimal inhibitory concentration and minimal bactericidal concentration values against eight different disinfectants.

Conclusions: Hyperchlorination over long periods of time did not prevent the persistence of *L. pneumophila*. The lack of effectiveness did not appear to be the result of a decreased susceptibility of *Legionella* to chlorine. Hyperchlorination did not modify *in vitro* susceptibility of *Legionella* to other disinfectants to which the bacteria had not previously been exposed.

Significance and Impact of the Study: Persistent *Legionella* in treated installations remain sensitive to disinfectants; hence, new strategies of treatment, different from hyperchlorination, should be developed to achieve bacterial eradication.

Introduction

Legionnaires' disease (LD) is a common form of severe pneumonia caused by *Legionella*. It emerged in the second half of the 20th century owing to changes in the environment caused by man. The disease develops when susceptible individuals inhale aerosols from man-made water supply plants contaminated with the bacteria. *Legionella pneumophila* serogroup (sg) 1 is the group most frequently associated with human infections (Marston *et al.* 1994) and the one most frequently isolated from the environment.

Although most cases of LD are sporadic (PHLS 2000), there have been outbreaks involving a wide variety of facilities, including cooling towers (Keller *et al.* 1996; Castellani-Pastoris *et al.* 1997; García-Fulgueiras *et al.* 2003), water-distribution systems (mainly hot water) in hospitals (Sabria and Yu 2002), hotels (Joseph *et al.* 1996) and ships (Caylà *et al.* 2001; Regan *et al.* 2003) or whirlpool spas (Den Boer *et al.* 2002) and fountains (Correia *et al.* 2001).

Legionellosis is generally considered a preventable illness, as controlling or eliminating the bacterium in installations would prevent cases breaking out. However, the

presence of *L. pneumophila* in hospital water supplies is a well-known risk factor for nosocomial pneumonia (Perola *et al.* 2005). The persistence of epidemic strains has been demonstrated in hospital water systems which are LD associated, despite being disinfected several times (Chang *et al.* 1996; Berthelot *et al.* 1998; Kool *et al.* 1998). Rangel-Frausto *et al.* (1999) demonstrated the survival of *L. pneumophila* for 13 years, causing nosocomial infections over this time (Rangel-Frausto *et al.* 1999). The bacteria were also isolated from the hospital hot water-distribution supply of 11 private health-care facilities in Italy over a period of 1 year (Legnani *et al.* 2002). Hospitals are ideal locations for the transmission of LD, where a large number of individuals are at risk; plumbing systems are frequently old and complex, favouring amplification of the organism; and water temperatures are often reduced to prevent scalding of patients (Mandel *et al.* 1993).

Water-distribution systems in hotels are also frequently colonized by *Legionella* (Borella *et al.* 2005), and they are the most frequent sources of cases of travel-associated LD (Joseph *et al.* 2004). One of the reasons may be that large facilities provide a more hospitable environment for the bacteria than small facilities, because the more extensive piping network provides a larger surface area with variable temperatures and bio-film accumulation (Yu 2002). *Legionella pneumophila* is also isolated relatively frequently from ship's water systems where more than 100 cases have been detected, mainly on cruise ships (Fiore *et al.* 1998; Rowbotham 1998) and there have also been outbreaks on cargo ships (Caylà *et al.* 2001). LD cases and *Legionella* survival over time have mainly been evaluated in hospitals, and there are no published studies of cases in hotels and ships.

Factors affecting the persistence or disappearance of *Legionella* are insufficiently known (Lück *et al.* 1994). One factor could be treatments which are not sufficiently effective against bacteria for different reasons: a facility's intricate piping system that can hinder the effectiveness of thermal or chemical disinfections (Wiedenmann *et al.* 2001); the presence of protozoa and biofilms that protect bacteria against disinfectant activity and support its growth (Kilvington and Price 1990; Donlan 2002); the short-term effect of disinfectants; a possible development of resistance to disinfectants, as in the case of other bacteria (Nishihara *et al.* 2000); or the lack of information available on many disinfectants regarding effective doses and exposure times, mechanisms, secondary effects, resistances, activity in biofilms, etc.

Chlorine is the best-known disinfectant, and it has been widely used to disinfect water systems, cooling towers and others. However, hyperchlorination has proved to be ineffective in eradicating *Legionella* from water systems in hospitals and re-colonization of installation can take place within weeks or months (Heimberger *et al.* 1991; Biurrun *et al.* 1999; Borella *et al.* 2000; Hosein *et al.* 2005). Hyperchlorination effectiveness against the persistence of the bacteria in other kinds of installations, such as hotels or ships, is not well documented. Moreover, possible resistance or changes in *Legionella* susceptibility to chlorine as a consequence of several hyperchlorination treatments of water systems have never been studied. Decrease in the susceptibility of the bacteria to other kinds of disinfectants as a consequence of previous contact with chlorine has not been analysed neither.

The aim of this study is to evaluate the influence of disinfection treatments over the persistence of *Legionella* in water-distribution systems. The proposed objectives were: (i) long-term environmental surveillance of the water system in the three LD-associated facilities (hospital, fishing boat and hotel). In each facility, the presence of *Legionella,* the disinfection treatments applied and the appearance of LD cases were analysed. (ii) To analyse the susceptibility of *L. pneumophila* sg 1 strains isolated over time (years) from these hyperchlorinated water systems to chlorine, in order to assess whether previous contact with the disinfectant reduced the *Legionella* susceptibility to further treatments. In addition, studies were undertaken to assess whether previous episodes of hyperchlorination decreased the susceptibility of these strains to other disinfectants that had never been in contact with the bacteria.

Materials and methods

Environmental surveillance and control measures

The three facilities included in this study were associated with LD cases caused by *L. pneumophila* sg 1. The water-distribution systems in these facilities were identified as the source of infection and were decontaminated several times over the years by hyperchlorination. Environmental research was carried out over several years and water samples were taken to evaluate the effectiveness of the control measures.

In 1984, an LD outbreak caused by *L. pneumophila* sg 1 was detected in a hospital and cases were reported and diagnosed by serology. An environmental study of the water-distribution system was carried out between 1984 and 2002. Twenty-one samplings were taken, each consisting of 3–21 samples. A total of 186 water samples were collected for *Legionella* testing from showers and faucets of hot and cold water in different rooms (some victim related), kitchen, laundry, hairdressing salon, etc. During this period of time, new cases of legionellosis appeared (two in 1991, six in 1993 and two in 1994), but there were no clinical isolates. Control measures were carried

out: at least four hyperchlorinations were applied (one in 1984, two in 1994 and one in 1995) and repairs were made twice (in 1992 and 1993).

In October 1997, an LD case related to a fishing boat was diagnosed. The victim was the skipper but there was no clinical isolate. The boat's three tanks were disinfected with chlorine dioxide in November 1997. Control measures were undertaken and no more cases occurred. After this data, the three tanks were hyperchlorinated with 0·5 l sodium hypochlorite per 1000 l water for 2 h. After that, all taps and showers were opened until the tanks were emptied (about 2 h). During fishing expeditions the control measures applied were: no tank water was used for drinking; washing water was maintained at a temperature below 20°C; shower and tap heads were removed for boiling (20 min) at least once and shower rooms aired as much as possible each time after use. Environmental surveillance was carried out between 1998 and 2002, and 15 samplings were taken, three per year, consisting of one to four samples each. A total of 60 water samples for *Legionella* testing were collected from the three water tanks, the general shower, the general bathroom, the kitchen and the victim's cabin. In February 1998, the three tanks were disinfected again with sodium hypochlorite (50/106) for 2 h. After disinfection, the same control measures were continued until February 2000, when the tanks were again hyperchlorinated using sodium hypochlorite (1/1000) for 24 h. The same control measures were then applied until 2002. In 2003, environmental surveillance ended owing to the boat's change of ownership.

In September 1992, an LD case associated with a hotel was reported, and a clinical isolate was obtained. An environmental study was carried out between 1992 and 2001 and 17 samplings with one to five samples each were taken. A total of 57 water samples were collected from taps and showers of different rooms (hot and cold water) and from the antifire system, for *Legionella* testing. New related LD cases appeared in 1994 (three cases in May, November and December), 1995 (one case in September), 1996 (two cases in January and July), 1998 (two cases in March and April), and 1999 (one case in June). At least seven hyperchlorination treatments were applied (two in August and the other in October 1992, one with 10 ppm of chlorine for 5 h in June and another in December 1994, one in September 1995 and the last one with 20 ppm of chlorine for 8 h in April 2001). Moreover, some repairs were made to the facility in May 1998 to separate water for human consumption from industrial water. The last hyperchlorination was applied in March 2001 before the hotel was re-opened in April.

Isolation and identification of *Legionella* spp.

A total of 186, 60 and 57 water samples were collected, at irregular time intervals, over 17, 5 and 10 years from the hospital, fishing boat and hotel, respectively. One litre of water was collected in sterile containers containing sodium thiosulfate. Water samples were basically processed as the International Standard ISO 11731/1998 describes for *Legionella* isolation. Hundred-fold concentrated by filtration through 45-mm diameter sterile cellulose ester membrane filters (0·22 μm pore size); the filters were cut into pieces, suspended in 10 ml of the analysed water and mixed by vortexing to disperse bacterial cells. The concentrates were divided into three aliquots. The first aliquot (0·2 ml) was subjected to heat treatment (50°C for 30 min) and the second (0·5 ml) to acid treatment (pH 2·2 for 5 min). The third aliquot (0·1 ml) was plated directly for culture. Subsequently, 0·1 ml of each aliquot was plated on modified Wadowsky Yee agar (MWY; Oxoid, Basingstoke, UK) (Edelstein 1982). The agar plates were then incubated at 35°C for 15 days, and inspected every 3 days. Identification of *Legionella* isolates was based on colony characteristics and there was no growth on L-cysteine-deficient buffered charcoal yeast extract agar supplemented with α-ketaglutarate (BCYE-α; Oxoid). Species and serogroup identification of *Legionella* isolates was performed using immunofluorescence (IF) with rabbit antisera, including 14 serogroups of *L. pneumophila* and another nine species, as previously described (Pelaz *et al.* 1992). *Legionella pneumophila* serogroup 1 isolates were subtyped by IF using the International Panel of Monoclonal Antibodies (Joly *et al.* 1986).

Legionella pneumophila sg 1 strains

Legionella pneumophila sg 1 strains from each water-distribution system, all of them sharing identical MAb reactions, were analysed by genotyping methods and susceptibility tests. Altogether, 22 isolates of *L. pneumophila* sg 1 were isolated: 11 from the hospital, collected between 1984 and 2000; seven from the fishing boat, collected between 1998 and 2002; and four from the hotel, collected between 1992 and 2001. Bacterial isolates were stored in skimmed milk at −70°C for further use, after being grown on BCYE-α agar plates for 48 h in an incubator at 35°C.

Genotyping methods

Twenty-two selected *L. pneumophila* sg 1 strains were compared by three molecular typing methods: (i) amplified fragment length polymorphism (AFLP) was performed according to the standardised EWGLI (European

Working Group for Legionella Infections) protocol for epidemiological typing of *L. pneumophila* SG 1 (Fry *et al.* 2000). AFLP patterns were compared with 70 representative strains included in the Spanish AFLP type collection previously defined (Baladrón and Pelaz 2002). (ii) Pulsed field gel electrophoresis (PFGE) was performed as described using *Sfi*I as restriction enzyme (Lück *et al.* 1994). PFGE patterns were compared with 40 representative strains included in the Spanish PFGE type collection. (iii) Arbitrarily primed polymerase chain reaction (AP-PCR) was performed as previously described using the M13 forward primer (Gomez-Lus *et al.* 1993). Gels were stained with ethidium bromide, visualized using UV transillumination and photographed prior to analysis. BIONUMERICS software (Bio-Rad) was used for AFLP and PFGE gel analysis, using UPGMA and Dice coefficient to designation of types.

Disinfectants

Eight products registered in the Spanish Health Ministry's Biocides Register were studied as disinfectants in cooling tower treatments against *Legionella.* Two oxidizing agents were tested: sodium hypochlorite (A) and a combination of hydrogen-peroxide/silver nitrate (B). Six nonoxidizing agents were included: four quaternary salts [didecyl-dimethyl-ammonium chloride (C) and benzalkonium chloride (D), such as ammonium salts, tributyl-tetradecyl-phosphonium chloride (E) and tetrahydroxymethyl-phosphonium sulfide (F), such as phosphonium salts], the halogenated amide 2,2-dibromo-nitropropionamide (G), and the heterocyclic ketone chloro-methyl-isothiazolone (H). All products were kindly supplied by the manufacturers, except sodium hypochlorite, which was bought in a chemical store. Stock solutions were prepared in distilled water and sterilized by filtration before use.

Susceptibility tests

After molecular analysis, activities of eight disinfectants against 22 selected *L. pneumophila* sg 1 strains were tested. A type culture collection strain (ATCC 33152) was also included as control strain because it had not been treated with chlorine. The following tests were undertaken:

Minimal inhibitory concentration (MIC)
This was determined by a conventional macrodilution method in buffered yeast extract (BYE) broth (NCCLS 1990). Bacteria were grown on BCYE-α agar for 3 days at 37°C were suspended at (1–3) × 10^8 CFU ml^{-1} (0·2 OD; λ_{420nm}) in BYE broth containing a series of twofold dilutions of each disinfectant (2–2048 ppm). The MIC value (expressed as ppm) was the lowest concentration of disinfectant without visible growth after 48 h of incubation at 30°C. MIC_{50} and MIC_{90} were calculated with several isolates from the same installation. Negative controls were included following the same method but without disinfectant. Each assay was repeated twice.

Minimal bactericidal concentration (MBC)
This was calculated following the described method (NCCLS 1987). Briefly, aliquots of treated cultures with no visible growth in the MIC assays were plated onto BCYE-α and incubated for 48 h at 35°C for total viable counts (TVC). MBC values, expressed as ppm, corresponded to the lowest concentration of disinfectant where viable counts were lower than 0·1% of untreated samples. Each assay was repeated twice.

Results

Environmental surveillance

Hospital
A total of 186 hospital water samples were analysed over a period of 17 years. 33·3% (62/186) of water samples contained *Legionella*, and of these, 45·1% (28/62) were *L. pneumophila* sg 1 (Philadelphia or Allentown MAb type). After 13 LD cases in 1984, *L. pneumophila* sg 1 was recovered from the nine water samples tested, including both hot and cold water tanks. The water system was then treated with chlorine and the next 10 water samples were negative for *Legionella*. No LD cases occurred for 6 years. However, two LD cases were notified in 1991, six in 1993 and two in 1994. During this time, the sequence of events was identical: (i) detection of LD cases followed by cultures positive for *L. pneumophila* sg 1; (ii) application of treatment followed by negative cultures and absence of cases for a brief period of time. Finally, in 1995, all 13 water samples were again positive for *L. pneumophila* sg 1 in the absence of LD cases. Chlorine treatment then rendered the premise negative and no more cases were detected in the following 2 years.

Fishing boat
Following the notification of one LD case in 1997, four out of five water samples collected from the fishing boat water system were found to be positive for *L. pneumophila* sg 1. Although there were no new LD cases, environmental surveillance was carried out since 1998 and a total of 60 water samples were analysed over 5 years. In total, 75% (45/60) of water samples was positive for *Legionella*, and of these, 91·1% (41/45) was *L. penumophila* sg 1 (Benidorm MAb type). Despite the application of hyperchlorination treatments and the use of appropriate

control measures, the same strain was recovered from the water tanks, the general shower, the general bathroom, the kitchen and the victim's cabin several times until the end of 2002.

Hotel

After the first LD case was notified in 1992, one out of five water samples collected from the water system of the hotel was positive for *L. pneumophila* sg 1. A culture from the patient was identified as *L. pneumophila* sg 1 (Benidorm MAb type). A total of 57 samples from the water system were analysed over the next 10 years and 15·6% (9/57) contained *L. pneumophila* sg 1 (1 Benidorm MAb type). *Legionella pneumophila* sg 1 was recovered from taps and showers of different rooms (hot and cold water) and from the antifire system until 1995. Nine LD cases related to the hotel were notified until June 1999 (as detailed earlier); despite hyperchlorination being applied each time, new cases were notified. An identical sequence of events was also noted: (i) detection of LD cases followed by cultures positive for *L. pneumophila* sg 1; (ii) application of treatment followed by negative cultures and absence of cases for a brief period of time. A last hyperchlorination was applied in March 2001 before the hotel was re-opened in April.

Genotyping of *Legionella pneumophila* sg 1 strains

The AFLP (a), PFGE (b) and AP-PCR (c) profiles were obtained comparing *L. pneumophila* sg 1 strains from the hospital, boat and hotel water systems (Figs 1, 2 and 3, respectively). *Legionella pneumophila* sg 1 isolates collected from each water system showed identical molecular characterization. Eleven environmental *L. pneumophila* sg 1 isolates collected over a period of 17 years from the hospital were characterized as: Philadelphia or Allentown MAb type, CNM 002 AFLP type and CNM 043 PFGE type, sharing similar AP-PCR profiles (Fig. 1). Seven environmental *L. pneumophila* sg 1 isolates collected over 5 years from the fishing boat were characterized as follows: Benidorm MAb type, CNM 033 AFLP type and

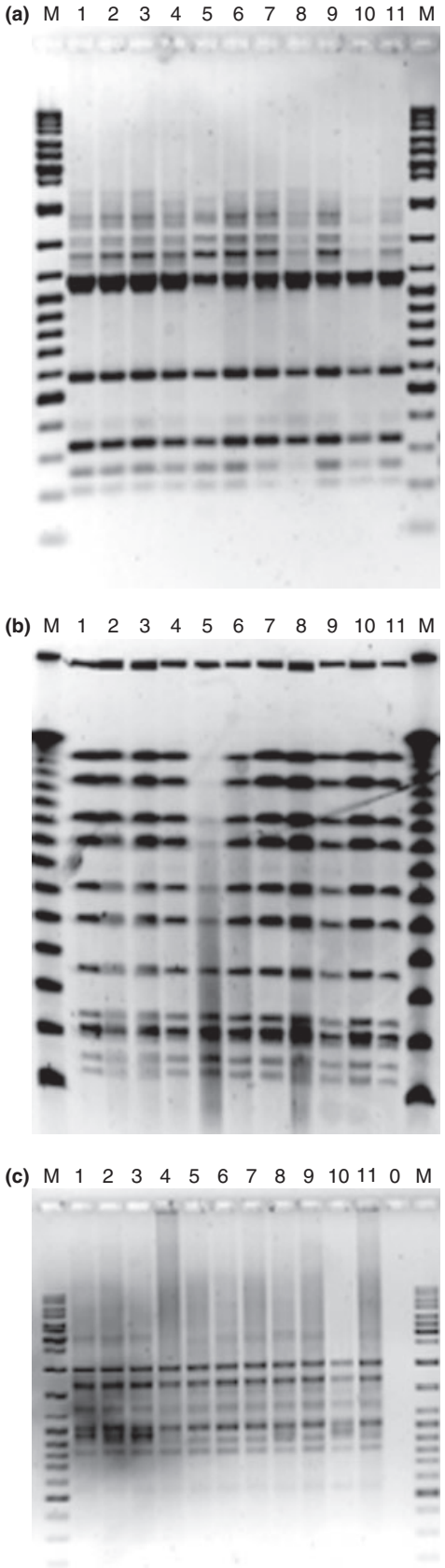

Figure 1 Gels containing *Legionella pneumophila* sg 1 (Philadelphia/Allentown MAb type) strains from the hospital isolated in: 1984 (lane 1), 1991 (lanes 2, 3), 1993 (lane 4), 1994 (lanes 5, 6), 1995 (lanes 7, 8), 2000 (lanes 9–11). CNM 002 amplified fragment length polymorphism (AFLP) type (a), CNM 043 pulsed field gel electrophoresis (PFGE) type (b), arbitrarily primed polymerase chain reaction (AP-PCR) pattern (c). Lane M, molecular weight marker (100–10 000 pb), Ladder Mix, MBI Fermentas (a); lane M, Concatemers of fago λ (48·5–1000 kpb), Boehringer–Mannheim (b). Lane M, molecular weight marker (70–12 200 pb), Boehringer–Mannheim and lane 0, negative control (c).

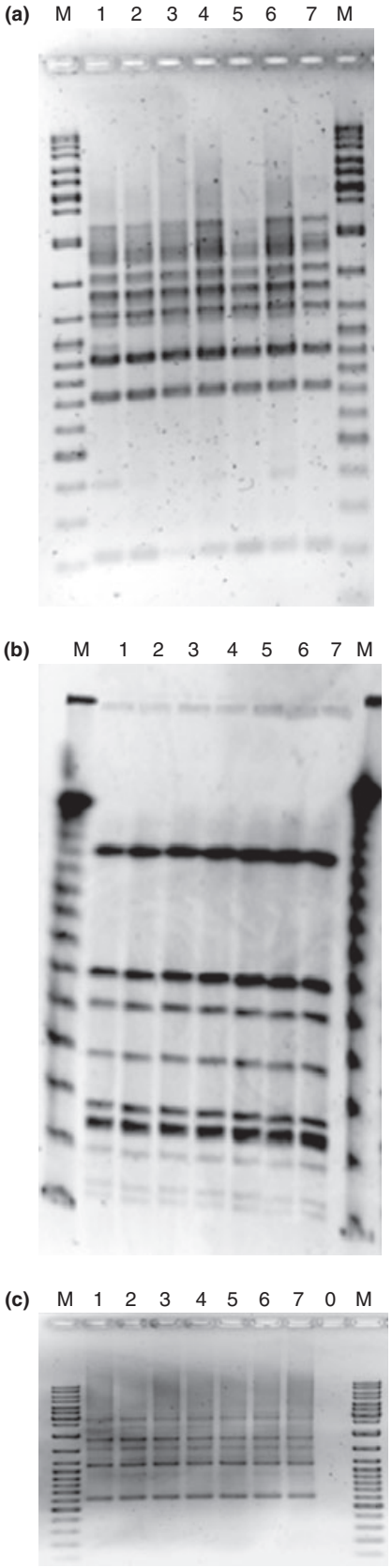

Figure 2 Gels containing *Legionella pneumophila* sg 1 (Benidorm MAb type) strains from the fishing boat, isolated in: 1998 (lanes 1, 2), 1999 (lanes 3, 4), 2000 (lanes 5, 6), 2002 (lane 7). CNM 033 amplified fragment length polymorphism (AFLP) type (a), CNM 026 pulsed field gel electrophoresis (PFGE)-*Sfi*I type (b), arbitrarily primed polymerase chain reaction (AP-PCR) pattern (c). Lane M, molecular weight marker (100–10 000 pb), Ladder Mix, MBI Fermentas (a); Lane M, Concatemers of fago λ (48·5–1000 kpb), Boehringer–Mannheim (b). Lane M, molecular weight marker (70–12 200 pb), Boehringer–Mannheim and lane 0, negative control (c).

CNM 026 PFGE type, sharing identical AP-PCR profiles (Fig. 2). Four environmental *L. pneumophila* sg 1 isolates collected over a period of 10 years from the hotel were characterized as: Benidorm MAb type, CNM 045 AFLP-type and CNM 042 PFGE type, and shared an identical AP-PCR profile (Fig. 3). The hospital, the fishing boat and the hotel were therefore colonized by a specific *L. pneumophila* sg 1 clone that had persisted over the years despite previous hyperchlorination processes.

Susceptibility of Legionella pneumophila sg 1 strains

The MIC and MBC values obtained with eight disinfectants against *L. pneumophila* sg 1 isolates included in the molecular study are summarized in Table 1. Susceptibility was very similar between the ATCC strain and strains from different facilities and between isolates from different years and the same facility. For each disinfectant, the MIC_{50} and MIC_{90} values were similar. The MBC values increased no more than three two-fold dilutions compared with the MIC values. The MIC and MBC values obtained with chloro-methyl-isothiazolone (H), tributyl-tetradecyl-phosphonium chloride (E), didecyl-dimethyl-ammonium chloride (C) and 2,2-dibromo-nitropropionamide (G) were between 2 and 16 ppm, whereas with the rest of the disinfectants, MIC and MBC values were at least four two-fold dilutions higher (between 256 and 1024 ppm). Therefore, all isolates were more susceptible to the inhibitory and bactericidal activity of the amide, the isothiazolone and both quaternary salts. The MIC and MBC values were lower than the concentration recommended by manufacturers in the treatment of the facilities with tributyl-tetradecyl-phosphonium chloride (E), didecyl-dimethyl-ammonium chloride (C), benzalkonium chloride (D) and chloro-methyl-isothiazolone (H). However, the MIC and MBC values of tetrahydroximethyl-phosphonium sulfide (F) and 2,2-dibromo-nitropropionamide (G) were similar to the doses recommended for disinfection treatments. Regarding the oxidizing agents, the MIC and MBC values of hydrogen-peroxide/silver nitrate (B) were slightly higher than the concentrations usually applied in the treatment of facilities, while much higher in the case of sodium hypochlorite (A).

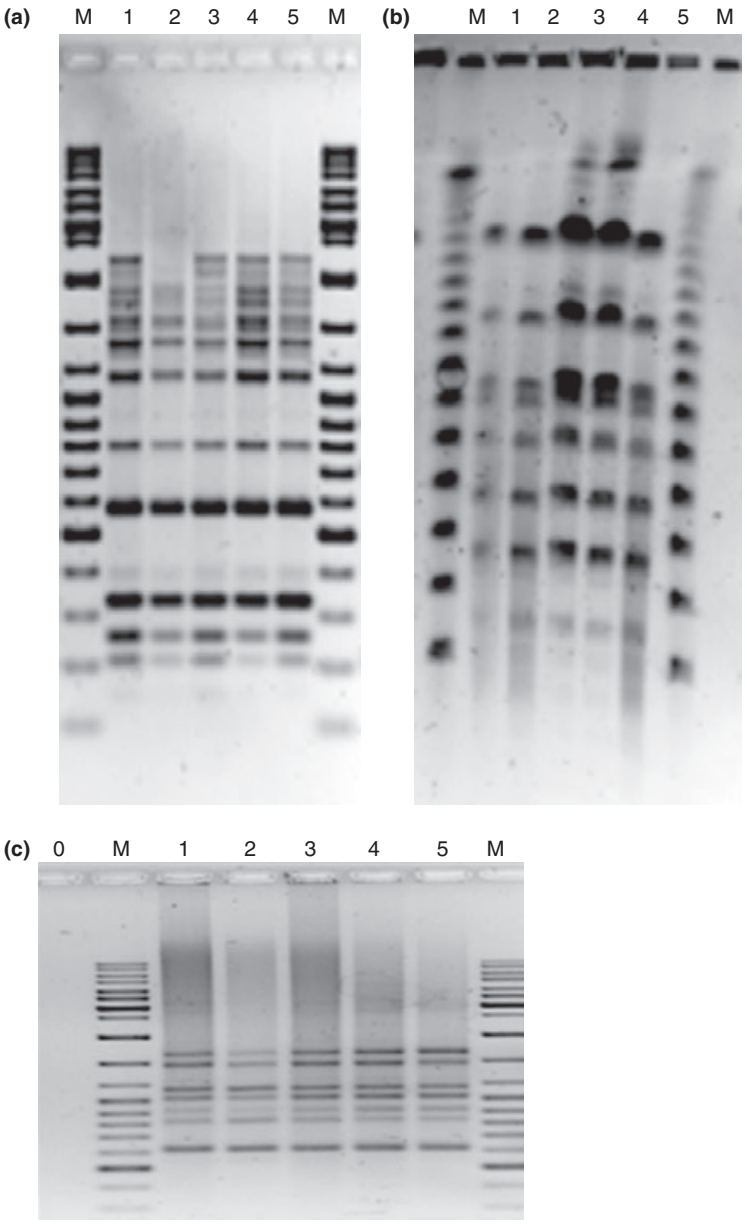

Figure 3 Gels containing *Legionella pneumophila* sg 1 (Benidorm MAb type) strains from the hotel, isolated in: 1992 (lanes 1, 2), 1994 (lane 3), 1995 (lane 4), 1998 (lane 5). CNM 045 amplified fragment length polymorphism (AFLP) type (a), CNM 042 pulsed field gel electrophoresis (PFGE)-*Sfi*I type (b), arbitrarily primed polymerase chain reaction (AP-PCR) pattern (c). Lane M, molecular weight marker (100–10 000 pb), Ladder Mix, MBI Fermentas (a); lane M, Concatemers of fago λ (48·5–1000 kpb), Boehringer–Mannheim (b). Lane M, molecular weight marker (70–12 200 pb), Boehringer–Mannheim and lane 0, negative control (c).

Discussion

The environmental research in this study shows three water-distribution systems contaminated with one strain of *L. pneumophila* sg 1 (MAb 2 positive), which persisted for many years. This persistent contamination could have been due to re-colonization by new strains. However, all isolates collected from the same facility showed identical molecular patterns with the three genotyping methods used. Genotyping methods, such as AFLP or PFGE, have been successfully used in epidemiological research into LD (Pruckler *et al.* 1995; García-Fulgueiras *et al.* 2003). In this research, genotyping methods were also useful to detect persistent clones of *L. pneumophila* involved. These methods could be an effective tool when environmental surveillance is carried out in water-distribution systems in LD-related facilities.

Water-distribution systems included in this study were hyperchlorinated several times over the period of the study. Despite these treatments, a *L. pneumophila* sg 1 clone was able to survive in the installation, persisting for 17 years in the hospital, 5 years in the fishing boat and 10 years in the hotel. Copper–silver ionization, bromine, super-heating or ultraviolet light units are some of the treatments commonly recommended for disinfection of

Table 1 Minimal inhibitory concentration (MIC_{50} and MIC_{90}) and minimal bactericidal concentration (MBC), expressed in ppm, of disinfectants against 22 *Legionella pneumophila* sg 1 isolates

	Hospital (1984–2000)			Fishing boat (1998–2002)			Hotel (1992–1998)		
Disinfectant	MIC_{50}	MIC_{90}	MBC	MIC_{50}	MIC_{90}	MBC	MIC_{50}	MIC_{90}	MBC
Chloro-methyl-isothiazolone (H)	4	4	8–16	2	2	8	2	2	8–16
Tributyl-tetradecyl-phosphonium chloride (E)	2	4	16–32	2	2	16–32	2	4	16–32
Didecyl-dimethyl-ammonium chloride (C)	8	8	8–16	4	4	32	8	8	16–32
2,2-Dibromo-nitropropionamide (G)	16	16	16	8	16	32	8	8	16
Hydrogen-peroxide/silver nitrate (B)	256	256	256–512	256	256	256	256	256	256–512
Tetrahidroximethyl-phosphonium sulfide (F)	256	256	512	128	128	512	256	256	512
Sodium hypochlorite (A)	1024	1024	2048–4096	1024	1024	4096	1024	1024	4096
Benzalkonium chloride (D)	512	512	>4096	128	256	2048	256	512	2048–4096

water systems. These treatments have been reported to be ineffective in the eradication of bacteria (Liu *et al.* 1995; Biurrun *et al.* 1999; Borau *et al.* 2000; Prince *et al.* 2002). Re-colonization with *L. pneumophila* sg 1 from the centralized hot water-distribution system in a hospital, involved in an outbreak of nosocomial LD, took place 2 months after shock heating and hyperchlorination of water (Borella *et al.* 2000). Rapid re-colonization in other hospitals, despite shock hyperchlorination, and new cases of hospital-acquired LD were also reported (Heimberger *et al.* 1991). Other studies also demonstrated persistent contamination by *L. pneumophila* sg 1 (Borella *et al.* 2000) and other serogroups (Perola *et al.* 2002) in hospital water systems that had been previously disinfected and was associated with LD cases. Our results showed that hyperchlorination failed to eradicate *Legionella* not only in the hospital, but also in the hotel and in the fishing boat.

Environmental surveillance of the three water systems in this study demonstrated the absence of *Legionella* after each disinfection treatment, but only for a few months. Over time, the same *L. pneumophila* sg 1 clone was again detected and new LD cases were notified in two of the systems. Therefore, our results confirm that once a water system is contaminated with *Legionella*, eradication of the organism is very difficult, and cases of LD are likely to occur, despite the disinfection measures undertaken. Darelid *et al.* proposed that, despite continuing colonization, control of nosocomial LD is possible by keeping the circulating hot water temperature above 55°C, together with careful clinical surveillance (Darelid *et al.* 2002). In our study, persistent *Legionella* colonization in the fishing boat water system was demonstrated over years in spite of successive hyperchlorination treatments. In this case, other control measures were taken, including: maintaining water tanks hyperchlorinated and not using this water for drinking; removing shower and tap heads to minimize aerosols; and airing shower rooms as much as possible after use. Control measures were effective and no more LD cases occurred. Along these lines, other authors also consider that emphasis should be placed on the control of infection rather than eradication of legionellae, using appropriate criteria including choice of disinfectant and disinfectant dosage method (Bentham 2001).

To evaluate whether chlorine was a good choice for treating facilities over the years, we determined the susceptibility of the isolates to this disinfectant and compared them with other disinfectants frequently used against *Legionella* in cooling towers, which have never been in contact with the bacteria. Our results show that the disinfectants with better inhibitory and bactericidal activity against persistent strains were the heterocyclic ketone (H), two of the four quaternary salts tested – one phosphonium salt (E) and one ammonium salt (C) – and the halogenated amide (G). The other disinfectants show an inhibitory or bactericidal activity at higher doses. All disinfectants were active at concentrations used for treating the facilities, with the exception of chlorine, applied as sodium hypochlorite, which was the least effective disinfectant. ATCC 33152 strain (never exposed to disinfectants) was also poorly susceptible to chlorine meaning that the low susceptibility of isolates to this disinfectant was not because of previous treatments.

Cross-resistance between antibiotics has been described for *L. pneumophila* 1 (Dowling *et al.* 1985). Some biocides can contribute to the development of microbial resistance mechanisms (Braoudaki and Hilton 2004). Bacteria subjected to chlorine stress revealed a decrease in their susceptibility to other kinds of disinfection treatments (Folsom and Frank 2000; Taormina and Beurchat 2001). Taking these observations into account, another factor that could explain the persistence of *Legionella* in installations is the development of resistance to the same and other disinfectants as a consequence of repeated treatments over time. Interestingly, this study demonstrates how all *L pneumophila* sg 1 isolates recovered in different years from the same water system showed similar

susceptibility to chlorine or other disinfectants. Therefore, the stress caused in each clone by successive hyperchlorination treatments does not modify the susceptibility *in vitro* of bacteria to new treatments with chlorine or other kinds of disinfectants. On the other hand, the presence of other flora and its interaction with *Legionella* could also favour the persistence of the bacteria in the environment and to decrease its susceptibility to chlorine treatments (Kilvington and Price 1990). Although amoeba detection was not included in this study, even in this case, bacterial susceptibility was not modified in any studied installation.

In conclusion, this study demonstrates that hyperchlorination does not resolve the persistence of the bacteria in water systems in hospitals, hotels and fishing boats. These results show how one particular clone of *L. pneumophila* sg 1 (MAb 2 positive) may persist for many years, and LD cases may occur. Where prevention and control measures require to be applied in water-distribution systems, emphasis should be focussed on the control of infection rather than eradication of legionellae. Our data also demonstrate that failure to eradicate the bacteria was not the result of a decrease in *Legionella* susceptibility to chlorine, despite several treatments. Moreover, hyperchlorination does not modify *in vitro* susceptibility to other disinfectants to which the bacteria have never been previously exposed.

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