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Original Article

Prospective Monitoring Study: Isolating *Legionella pneumophila* in a Hospital Water System Located in the Obstetrics and Gynecology Ward after Eradication of *Legionella anisa* and Reconstruction of Shower Units

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SUMMARY: We previously reported on the sporadic contamination by *Legionella anisa* of shower units and sink taps at Ryukyu University Hospital. Starting in July 2003, the neonatal area underwent an 8-month reconstruction, and in March 2005, the boiler system was replaced. We therefore examined shower water and tap water for the presence of *Legionella* just after replacement of the boiler system. In 3 of the 8 water samples collected from the remodeled area, we isolated *Legionella pneumophila* serogroup 1 and *L. anisa*. Moreover, *L. pneumophila* serogroup 1 was isolated in 4 of the 5 water samples gathered from the unreconstructed area of the same floor. Random amplified polymorphic DNA analysis suggested that a single clone of *L. pneumophila* might exist throughout the floors of the water distribution system. We replaced the shower units at the *Legionella*-positive site, and began flushing the sink-faucets with water heated to 55°C for at least 1 h every morning. As a result, *Legionella* was not subsequently isolated in water samples. In this prospective study, we identified a central contamination by *L. pneumophila* serogroup 1 and showed that flushing with hot tap water was effective to counter this situation.

INTRODUCTION

Studies have shown an association between the presence of *Legionella* in hospital water systems and cases of legionellosis. The level of contamination of hospital water supplies with *Legionella* seems to have a correlation with the incidence of nosocomial legionnaires' disease (1). The number of points in the system testing positive for *Legionella* has been considered to be a better predictor of risk for legionnaires' disease than quantitative levels found at any given point (1,2). However, there have been some hospitals where no cases of nosocomial legionellosis were detected despite contamination of the hot water systems over prolonged periods of time.

In the case of Ryukyu University Hospital, *Legionella anisa* sporadically contaminated shower units and sink taps in the pediatrics ward between 1996 and 1998, and in the obstetrics and gynecology ward, where the neonatal area was located, between 1996 and 2000 (3).

As the following monitoring between January 2001 (the former report) (3) and March 2005 (the present study), we examined these wards 6 times from December 2001 to April 2003 (Table 1).

During the experiment in April 2003, a total of 55 water samples (53 from the shower units and 2 from sink taps), of which 4 samples were from the pediatric ward and 6 samples were from the obstetrics and gynecology ward, were collected from all of the hospital wards. Of the 55, 52 water samples (51 from the shower units and 1 from a sink tap) tested negative for *Legionella*, but 2 shower-water samples and 1 tap-water sample collected from the neonatal area located in the obstetrics and gynecology ward were positive for *L. anisa* (Table 1).

However, the number of isolated *L. anisa* was few (up to 950 colony formig units [cfu]/100 ml), with the exception of one point (neonatal room c, 1,400 cfu/100 ml [December 2001] and 3,000 cfu/100 ml [December 2002]).

The neonatal area of the obstetrics and gynecology ward underwent an 8-month reconstruction between July 2003 and March 2004 (Figure 1, dotted arrow). During this reconstruction all sinks and showers (including the *L. anisa*-positive sites) in the neonatal area were replaced. In March 2005, the boiler system was also replaced.

To prevent nosocomial infection, we considered that it would be important to evaluate the influence of the reconstruction and boiler replacement on the ecology of microbial flora in the hospital water system. We therefore examined the shower water and tap water for the presence of *Legionella* immediately after replacement of the boiler system in the neonatal area of Ryukyu University Hospital.

MATERIALS AND METHODS

Hospital and water samples: Ryukyu University Hospital is a single building with 17 wards and a total of 604 beds. The building receives water from a single municipal supply, and this is then distributed into two hot-water tanks. The hot-water tanks are joined to the boiler system through connecting pipes. Cold water pipes and hot water pipes run side by side. In March and May 2005, just after the boiler replacement was completed, a total of 24 water samples were collected from 6 shower heads located within the neonatal

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Table 1. Long-term follow-up study to detect *Legionella* from each shower unit and sink tap water at Ryukyu University Hospital (from 2001 to 2005)

Ward	(No. of samples tested) / Sampling site	Dec. 2001 (10)	Jul. 2002 (6)	Oct. 2002 (10)	Dec. 2002 (6)	Jan. 2003 (10)	Apr. 2003 (55)	Jun. 2003 - Feb. 2005
Obstetrics and	Neonatal room a S	N	N	N	N	N	N	R
gynecology	Neonatal room b T	N	N	*L. anisa* (10)	*L. anisa* (10)	N	N	R
(4th floor)	Neonatal room c T	*L. anisa* (1400)*	*L. anisa* (140)	N	*L. anisa* (3000)	N	*L. anisa* (360)	R
	Shower room 8 S	N	N	N	N	N	*L. anisa* (90)	ND
	Shower room 9 S	*L. anisa* (150)	*L. anisa* (20)	*L. anisa* (30)	N	N	*L. anisa* (950)	ND
	Shower room 7 S	N	N	N	N	N	N	ND
Pediatrics	NICU d S	N	ND	N	ND	N	N	ND
(6th floor)	NICU e S	N	ND	N	ND	N	N	ND
	Room f S	N	ND	N	ND	N	N	ND
	Room g S	N	ND	N	ND	N	N	ND

*: Numbers show colony forming units (cfu)/100 ml.
S, shower water; T, sink tap water; N, negative (below 10 cfu/100 ml); ND, not done; R, reconstructed (lost).

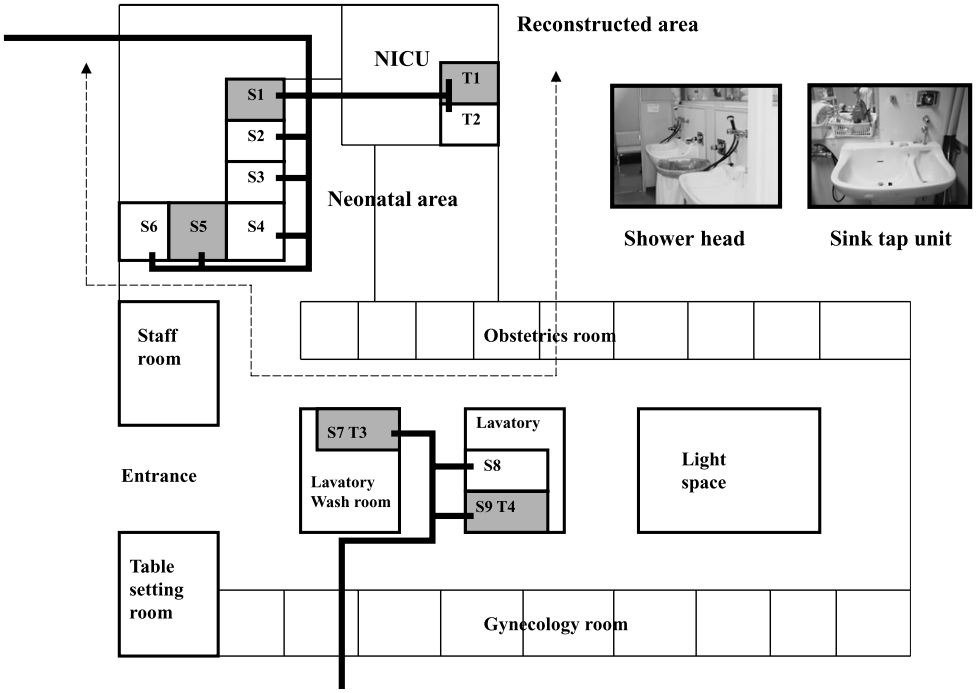

Fig. 1. Contamination of *L. pneumophila* in the obstetrics and gynecology ward. *Legionella* were detected from water of shaded rooms. ←- - -, reconstructed area; ▬: the supply of water to the rooms; cold water pipes and hot water pipes run side by side. S, shower; T, sink tap.

area, 2 sink taps within the neonatal intensive care unit (NICU), 1 shower head and 1 sink tap within the unreconstructed obstetrics ward, 2 shower heads and 1 sink tap within the unreconstructed gynecology ward, and 11 shower heads located in other wards. Water samples were collected in the early morning before the showers and sinks had been used.

Distribution to the neonatal area and unreconstructed obstetrics and gynecology ward was through different water pipe routes from one hot-water tank located on the roof (Figure 1). After reconstruction, the NICU was moved to the neonatal area in 4F from the pediatric ward in 6F. In August 2005, after changing the shower heads at *Legionella*-positive sites and performing a flush-treatment of all showers and sink taps in the hospital, 13 water samples were again collected from the same shower heads and sink taps as in the former sampling within the obstetrics and gynecology ward to test microbiological examination.

Culture method of water samples: A sample volume of 200 ml was collected in sterile bottles for microbiological examination. Each sample was centrifuged at 8,000 *g* for 20 min. The resultant sediment was resuspended in 2 ml of supernatant, from which 1 ml was taken for culturing *Legionella* spp. All samples were examined on the day of collection. We inoculated a 0.2M HCl-KCl buffer (pH 2.2) into an equal volume of the suspension and allowed the mixture to stand at room temperature for 30 min. A one-hundred microliter portion of this buffer-treated suspension was plated on buffered charcoal yeast extract agar supplemented with 0.1% α-ketoglutarate (BCYEα; Oxoid, Hampshire, UK), and modified Wadowsky-Yee (MWY; Oxoid) agar medium for duplication. The preparations were incubated at 35°C for 7 days. Up to 6 *Legionella*-like colonies were collected from each plate and subjected to identification assays.

Identification of the strain isolated: Colonies of *Legionella* were identified by biological profile, including assays of colony morphology, long-wave UV fluorescence, serological slide-agglutination test, oxidase, catalase, hippurate hy-

drolysis, and gelatin liquefaction. Then, the organisms were identified by a microplate DNA-DNA hybridization test (Kyokuto Pharmaceutical, Tokyo, Japan).

DNA fingerprinting: Random amplified polymorphic DNA (RAPD) typing was performed on 6 of the 7 isolates of *L. pneumophila* that were derived from water samples by using primers 1 to 6 with a commercially available kit, Ready-To-Go RAPD Analysis (Amersham Bioscience, Buckinghamshire, UK). In addition, 1 isolate of *L. anisa* was compared to 4 former *L. anisa* strains isolated from the obstetrics and gynecology ward in 2000 (2 strains; 1 from a tap-water sample within the reconstructed area and 1 from a shower-water sample in the unreconstructed area) and in 2002 (2 strains; 1 from a tap-water sample within the reconstructed area and one from a shower-water sample in the unreconstructed area), by using primers 2 to 4 with a Ready-To-Go RAPD Analysis kit. The cycling parameters used were initial denaturation at 95°C for 5 min, followed by 45 cycles each consisting of denaturing at 95°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min. Aliquots of each PCR product were analyzed by 3.0% NuSieve 3:1 agarose (BioWhittaker Molecular Applications, Rockland, Maine, USA) gel electrophoresis in Tris-borate-ethylenediaminetetraacetic acid (TBE) buffer.

Disinfection of the shower units and sink taps: We replaced the shower units at the *Legionella*-positive site. In addition, all shower and sink taps in the hospital were flushed with 55°C water containing 0.5 ppm of free residual chlorine for at least 1 h once a week. All sink taps in the obstetrics and gynecology ward were flushed using hot water (55°C, 0.5 ppm of free residual chlorine) for at least 1 h every morning.

RESULTS

In March and May of 2005, we collected 24 water samples and examined them for the presence of *Legionella*. *Legionella* was not isolated in any of the 11 shower-water samples gathered on the 5th through the 10th floors, including the 6th floor, where the pediatrics ward was located and where *L. anisa* was isolated during monitoring in 1996 and 1998. However, on the 4th floor, 2 of the 6 shower-water samples from the neonatal area, 1 of the 2 tap-water samples from the NICU, the single shower-water sample from the obstetrics ward, the single tap-water sample from the obstetrics ward, 1 of the 2 shower-water samples from the gynecology ward, and the single tap-water sample from the gynecology ward showed positive results (Table 2). Shower rooms 7, 8, and 9 in Table 2 correspond to those in Table 1. Through biochemical, serological and molecular testing, a total of 8 strains that had been isolated from 7 sites were identified as *L. pneumophila* serogroup 1 (7 strains) and *L. anisa* (1 strain). Seven strains of *L. pneumophila* serogroup 1 showed positive reaction by oxidase, catalase, hippurate hydrolysis, and gelatin liquefaction tests. They showed dull-yellow fluorescence under long-wave UV light, and strongly agglutinated with *L. pneumophila* serogroup 1 antiserum by slide-agglutination test. In the molecular testing, they hybridized with the *L. pneumophila*-type strain immobilized by the microplate DNA-DNA hybridization test kit.

Both *L. pneumophila* serogroup 1 (20 cfu/100 ml) and *L. anisa* (20 cfu/100 ml) were isolated from one of the shower heads in the neonatal room. The numbers of isolated *L. pneumophila* serogroup 1 were between 20 cfu/100 ml and 320 cfu/100 ml. In two cases, the numbers of isolated

Table 2. Detection of *Legionella* from shower water and sink tap water of neonatal area and other area in March and May, 2005

Ward	Sampling site	Species of *Legionella* isolated (cfu/100 ml)
Obstetrics	Neonatal room 1 shower	*L. pneumophila* SG 1(20)*, *L. anisa* (20)
(4th floor)	Neonatal room 2 shower	N
Reconstructed	Neonatal room 3 shower	N
area	Neonatal room 4 shower	N
	Neonatal room 5 shower	*L. pneumophila* SG 1(320)
	Neonatal room 6 shower	N
	NICU sink tap water 1	*L. pneumophila* SG 1(40)
	NICU sink tap water 2	N
Unreconstructed	Shower room 7	*L. pneumophila* SG 1(20)
area	Sink tap water 3	*L. pneumophila* SG 1(260)
Gynecology	Shower room 8	N
(4th floor)	Shower room 9	*L. pneumophila* SG 1(60)
	Sink tap water 4	*L. pneumophila* SG 1(120)
Other wards	Shower room 10 (5th floor)	N
(5th-10th floor)	Shower room 11 (6th floor)	N
	Shower room 12 (6th floor)	N
	Shower room 13 (7th floor)	N
	Shower room 14 (7th floor)	N
	Shower room 15 (8th floor)	N
	Shower room 16 (8th floor)	N
	Shower room 17 (9th floor)	N
	Shower room 18 (9th floor)	N
	Shower room 19 (10th floor)	N
	Shower room 20 (10th floor)	N

*: Numbers show higher *Legionella* counts obtained on whether BCYEα agar or MWY agar.
Shower rooms 7, 8, and 9 correspond to those in Table 1.
SG, serogroup; N, negative.

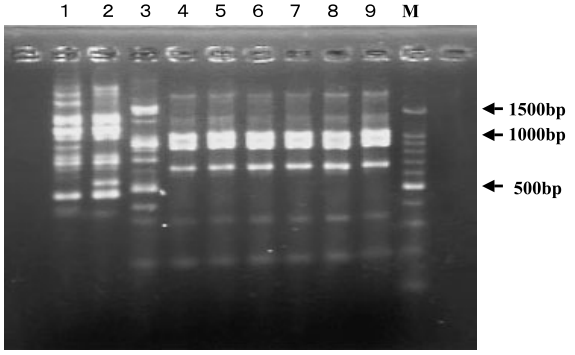

Fig. 2. RAPD pattern of isolated *L. pneumophila* by primer 1 (5′-d[GGTGCGGGAA]-3′). lane 1, *E. coli* BL21; lane 2, *E. coli* C1a; lane 3, *L. pneumophila* ATCC33152; lane 4, *L. pneumophila* from neonatal room 1; lane 5, *L. pneumophila* from neonatal room 5; lane 6, *L. pneumophila* from NICU sink tap water 1; lane 7, *L. pneumophila* from sink tap water 3; lane 8, *L. pneumophila* from shower room 9; lane 9, *L. pneumophila* from sink tap water 4; lane M, molecular weight marker 100-bp ladder (Takara Bio, Shiga, Japan).

Legionella on MWY agar were higher than on BCYEα agar (shower room 9, 60 cfu/100 ml versus 40 cfu/100 ml; sink tap 4, 120 cfu/100 ml versus 100 cfu/100 ml). In other cases, the numbers of isolated *Legionella* on MWY agar were fewer than on BCYEα agar.

Six of 7 isolated *L. pneumophila* strains were examined by RAPD analysis. All 6 strains showed the same pattern by using 5 of 6 primer sets (Figure 2). The exception was primer set 3, by which 2 of 6 strains showed different patterns (data not shown). One *L. anisa* strain was compared to 4 former isolates of *L. anisa*. These 5 *L. anisa* strains showed a similar pattern by using 3 primer-sets applied (data not shown).

In August 2005, after undertaking treatment to disinfect the positive sites, we collected 13 water samples from the obstetrics and gynecology ward. *Legionella* were not isolated in any of the 13 samples.

DISCUSSION

In our previous study between 1996 and 2003, we detected sporadic *L. anisa* contamination in shower units or sink taps of the obstetrics and gynecology ward (4F) and the pediatrics ward (6F), despite other wards having no contamination with *Legionella*. Although it was suspected based on RAPD analysis that a symbiotic biofilm covered the pipes throughout the water distribution system, we replaced the shower units at the *Legionella*-positive site (3,4). In addition, outlets were treated to disinfection. As a result, *Legionella* has not been isolated in any of the newly replaced shower units, despite the indication by Yu and Stout that *L. anisa* is a common colonizer of water distribution systems and that the replacement of shower units is an unreliable method for eradication of *Legionella* (5).

During reconstruction of the neonatal area starting in July 2003, much dust and air route contamination might have occurred at the peripheral faucets. In March of 2005, the boiler system was replaced. Just after the boiler replacement was completed, we examined for the presence of *Legionella*. From 3 of the 8 water samples gathered from the remodeled area, *L. pneumophila* serogroup 1 and *L. anisa* were isolated. Moreover, from 4 of the 5 water samples gathered from the intact area on the same floor (4F), *L. pneumophila* serogroup 1 was isolated. This time, *L. anisa* was not isolated in 3 unreconstructed shower-water sampling sites (showers 7, 8, and 9) from which *L. anisa* had been sporadically isolated between 1999 and 2003, despite *L. pneumophila* serogroup 1 being isolated from 2 shower-water samples (Tables 1, 2).

The number of colonies obtained on selective MWY agar is commonly fewer than that obtained on BCYEα agar, because MWY agar contains antibiotics. However, in the cases of shower 9 and sink tap 4, many bacterial colonies grown on BCYEα agar inhibited the growth of some *Legionella* colonies, and thus the number of *Legionella* colonies observed on BCYEα agar was lower than that oberved on MWY agar.

Six of 7 isolated *L. pneumophila* strains were maintained until this study, and were subjected to RAPD analysis. All 6 strains showed the same pattern by using 5 of 6 primer sets, which led us to suspect that a single clone of *L. pneumophila* might exist throughout the water distribution system. One *L. anisa* strain showed a RAPD pattern similar to that of 4 former isolates of *L. anisa*, indicating that *L. anisa* had colonized within the piping system.

In agreement with the results of this study, a previous study reported the continuous isolation of a single clone of *Legionella* DNA-type in hospital water over a number of years (6). In the present study, we did not confirm the origin of a contamination because no nosocomial legionellosis case occurred during the study period. But the difficulty of using molecular epidemi-ological data to determine the origin of a contamination has been reported in foreign countries based on the fact that the same clone of *Legionella* PFGE-type was isolated from tap water of several hospitals located in a wide area of the Franche-Comté region of France (7), or from the cooling tower water of many buildings located in a wide area of Paris (8). However, we performed DNA profiling tests not based on these references, and not based on the fact that no clinical strains of *L. pneumophila* were isolated during the monitoring period. Pulsed field gel electrophoresis (PFGE) is the gold standard technique due to its accuracy and reproducibility. However, these protocols involve time-consuming DNA preparation, costly reagents, specialized equipment, and degradation by DNase activity in bacteria. In contrast, RAPD has the advantage of speed and simplicity, although its stability and reproducibility depend on the primer sequences chosen and the skill of the individual conducting the experiments (9,10). We therefore chose RAPD analysis and judged its results referring to PFGE analysis criteria, because RAPD had no apparent criteria (10). By the results of RAPD test, we suspected that the isolated *L. pneumophila* consisted of a single clone.

There have been many reports on methods for the prevention of nosocomial legionellosis: (i) passage of 70°C water for 15 min through a cold water pipe system at time intervals of 7 to 49 weeks (11); (ii) keeping the circulating hospital hot water temperature above 55°C (12); (iii) disinfection of hot water-distribution networks by the combination of ozonation (0.3 mg/l) and increased temperature (65°C) (13); and (iv) the combination of silver-copper ionization (0.3 mg/l copper) and increased temperature (13). For the sterile water used for an intensive care unit, it has been suggested that sinks and showers should be equipped with disposable filters having a pore size of 0.2 μm (14). In contrast, Darelid et al. reported that complete eradication of legionellae from hot water systems does not seem to be necessary (12).

Referring to these methods, we replaced the shower units at *Legionella*-positive sites, and performed flushing outlets with 55°C water containing 0.5 ppm of free residual chlorine for at least 1 h once a week. The water temperature of the

hot-water tank located on the roof was heated above 60°C, and the circulating water temperature of the central piping system had to be maintained above 55°C. Consequently, during the experiment in August 2005, *Legionella* was not isolated in the replaced new shower units. It was not possible to replace the sink taps, so we instead flushed them using 55°C water containing 0.5 ppm of free residual chlorine for at least 1 h every morning, and this flushing has continued to the present. As a result, in August 2005, *Legionella* was not isolated in water samples from the sink taps.

In this prospective study, we identified a central water pipe system contamination by *L. pneumophila* serogroup 1 and showed that flushing with hot tap water was effective to counter this situation.

REFERENCES

1. Best, M., Yu, V.-L., Stout, J., et al. (1983): *Legionellaceae* in the hospital water-supply. Epidemiological link with disease and evaluation of a method for control of nosocomial legionnaires' disease and Pittsburgh pneumonia. Lancet, 2, 307-310.
2. Kool, J.-L., Bergmire-Sweat, D., Butler, J.-C., et al. (1999): Hospital characteristics associated with colonization of water systems by *Legionella* and risk of nosocomial legionnaires' disease: a cohort study of 15 hospitals. Infect. Control Hosp. Epidemiol., 20, 798-805.
3. Yamamoto, N., Kubota, T., Tateyama, M., et al. (2003): Isolation of *Legionella anisa* from multiple sites of a hospital water system: the eradication of *Legionella* contamination. J. Infect. Chemother., 9, 122-125.
4. Yamamoto, N., Koide, M. and Saito, A. (2004): Prevention of nosocomial legionellosis: *Legionella anisa*. J. Infect. Chemother., 10, 134.
5. Yu, V.-L. and Stout, J.-E. (2004): *Legionella anisa* and hospital water systems. J. Infect. Chemother., 10, 133.
6. Rangel-Frausto, M.-S., Rhomberg, P., Hollis, R.-J., et al. (1999): Persistence of *Legionella pneumophila* in a hospital's water system: a 13-year survey. Infect. Control. Hosp. Epidemiol., 20, 793-797.
7. Thouverez, M., Godard, C., Leprat, R., et al. (2003): Is pulsed-field gel electrophoresis a valuable tool to identify nosocomial cases of *Legionella pneumophila* disease? J. Hosp. Infect., 55, 254-259.
8. Lawrence, C., Reyrolle, M., Dubrou, S., et al. (1999): Single clonal origin of a high proportion of *Legionella pneumophila* serogroup 1 isolates from patients and the environment in the area of Paris, France, over a 10-year period. J. Clin. Microbiol., 37, 2652-2655.
9. Seo, Y,-S., Lee, S,-H., Shin, E,-K., et al. (2006): Pulsed-field gel electrophoresis genotyping of *Salmonella gallinarum* and comparison with random amplified polymorphic DNA. Vet. Microbial., 115, 349-357.
10. Tenover, F,-C., Arbeit, R,-D., Goering, R,-V., et al. (1995): Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol., 33, 2233-2239.
11. Wagenvoort, J.-H.-T. and Sijstermans, M.-L.-H. (2004): From legionnaire to guerrilla combatant: suppression of *Legionella pneumophila* in a hospital cold water supply. J. Hosp. Infect., 58, 162-163.
12. Darelid, J., Löfgren, S. and Malmvall, E. (2002): Control of nosocomial legionnaires' disease by keeping the circulating hot water temperature above 55°C: experience from a 10-year surveillance programme in a district general hospital. J. Hosp. Infect., 50, 213-219.
13. Blanc, D.-S., Carrara, P.-H., Zanetti, G., et al. (2005): Water disinfection with ozone, copper and silver ions, and temperature increase to control *Legionella*: seven years of experience in a university teaching hospital. J. Hosp. Infect., 60, 69-72.
14. Vonberg, R.-P., Eckmanns, T., Bruderek, J., et al. (2005): Use of terminal tap water filter systems for prevention of nosocomial legionellosis. J. Hosp. Infect., 60, 159-162.