ORIGINAL ARTICLE

Long-Term Effects of Hospital Water Network Disinfection on *Legionella* and Other Waterborne Bacteria in an Italian University Hospital

Beatrice Casini, BSc, MS;[1] Andrea Buzzigoli, BSc;[1] Maria Luisa Cristina, BSc, PhD;[2] Anna Maria Spagnolo, BSc, PhD;[2] Pietro Del Giudice, MD;[3] Silvio Brusaferro, MD;[3] Andrea Poscia, MD;[4] Umberto Moscato, MD;[4] Paola Valentini, BSc, MS;[1] Angelo Baggiani, MD;[1] Gaetano Privitera, MD[1]

OBJECTIVE AND DESIGN. *Legionella* control still remains a critical issue in healthcare settings where the preferred approach to health risk assessment and management is to develop a water safety plan. We report the experience of a university hospital, where a water safety plan has been applied since 2002, and the results obtained with the application of different methods for disinfecting hot water distribution systems in order to provide guidance for the management of water risk.

INTERVENTIONS. The disinfection procedures included continuous chlorination with chlorine dioxide (0.4–0.6 mg/L in recirculation loops) reinforced by endpoint filtration in critical areas and a water treatment based on monochloramine (2–3 mg/L). Real-time polymerase chain reaction and a new immunoseparation and adenosine triphosphate bioluminescence analysis were applied in environmental monitoring.

RESULTS. After 9 years, the integrated disinfection-filtration strategy significantly reduced positive sites by 55% and the mean count by 78% ($P < .05$); however, the high costs and the occurrence of a chlorine-tolerant clone belonging to *Legionella pneumophila* ST269 prompted us to test a new disinfectant. The shift to monochloramine allowed us to eliminate planktonic *Legionella* and did not require additional endpoint filtration; however, nontuberculous mycobacteria were isolated more frequently as long as the monochloramine concentration was 2 mg/L; their cultivability was never regained by increasing the concentration up to 3 mg/L.

CONCLUSIONS. Any disinfection method needs to be adjusted/fine-tuned in individual hospitals in order to maintain satisfactory results over time, and only a locally adapted evidence-based approach allows assessment of the efficacy and disadvantages of the control measures.

Infect Control Hosp Epidemiol 2014;35(3):293-299

In Europe from 2007 to 2008, 28 outbreaks of Legionnaires' disease (involving 98 cases) out of 111 (11.5%) reported by the European surveillance scheme for travel-associated legionnaires' disease were linked to hospitals or healthcare facilities, and 22 of these (78.6%) were attributed to water systems colonized by *Legionella*.[1] The large distribution systems of hospitals and the high volume of hot water storage tanks provide optimal conditions for growth of *Legionella*. Healthcare facilities have a special responsibility for preventing Legionnaires' disease, since the proportion of cases that are fatal tends to be much higher as a result of the presence of patients with predisposing risk factors for the infection and the use of medical devices that can disseminate *Legionella* into the lower respiratory tract.[2] The preferred approach to health risk assessment in evaluating specific risks of exposure to *Legionella* is to develop a water safety plan, which provides a detailed and systematic assessment and prioritization of hazards, and operational monitoring of barriers and control measures.[2-5]

Validation procedures should be established to ensure that the water safety plan is working effectively and meets the health-based targets.[6] Although the preventive strategy proposed by the Centers for Disease Control and Prevention advocates intensive clinical surveillance without routine environmental surveillance, with the exception of transplant units,[7] the extent of *Legionella* colonization of a hospital water system—measured as the percentage of positive sites or the quantitative concentration of *Legionella*—has been found to be a better indicator of the risk of hospital-acquired legionellosis.[8] The presence of *Legionella* in the hospital water supply suggests that patients may be at risk for hospital-acquired pneumonia and triggers the routine implementation of *Legionella* diagnostic tests for patients with symptoms of pneu-

Affiliations: 1. Department of Translational Research, New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy; 2. Department of Health Sciences, University of Genoa, Genoa, Italy; 3. Department of Medical and Biological Sciences, University of Udine, Udine, Italy; 4. Hygiene Institute, Welfare Department of Public Health, Catholic University of Sacred Hearth, Rome, Italy.

Received August 30, 2013; accepted November 12, 2013; electronically published February 3, 2014.

 0899-823X/2014/3503-0014$15.00. DOI: 10.1086/675280

monia, especially for those ones who are at high risk of acquiring the disease.

In this study, we report the experience of an Italian university hospital where a proactive strategy for prevention and control of legionellosis has been applied since 2002. In particular, the results obtained with the application of different methods for disinfecting sanitary hot water distribution system are discussed.

METHODS

Setting

In response to 2 nosocomial cases of Legionnaires' disease (3.2 per 100,000 ordinary admissions) in the Azienda Ospedaliero-Universitaria Pisana, a 1,077-bed teaching hospital, a control and risk management plan was started in March 2002. The plan included (1) active clinical surveillance for *Legionella* infections, (2) standard operating procedures for maintenance and operation of water systems, and (3) monitoring of the water system through systematic water sampling at the endpoints of use.

Owing to a systemic colonization of the water system, a continuous chlorine dioxide treatment of the warm water network started in April 2003 (0.4–0.6 mg/L in the recirculation loop). Where *Legionella* colonization was still detectable and in high-risk areas, point-of-use water filtration was introduced. Starting in November 2010, a monochloramine-based disinfection began in a new building housing the emergency department, a 112-bed ward.

Sample Collection

Between March 2002 and December 2011, 1,015 hot water samples were collected from tap outlets of the hospital water network treated with chlorine dioxide, and 443 samples were collected from point-of-use devices where a filter was fitted. Similarly, a total of 122 (between November 2010 and May 2013) water samples and 100 biofilm samples were collected within the sanitary water network of the emergency department; 6 points for sampling were selected as distal and proximal sites from the location of the continuously producing and dispensing monochloramine device (Sanipur).

Detection of *Legionella* spp. and Identification of Isolates

Legionella bacteria in water and biofilm samples were isolated in accordance with standards procedures.[9,10] Serogrouping was performed by the *Legionella* latex test (Oxoid), and species identification was carried out by sequencing of the *mip* gene.[11] According to temporal and spatial criteria, representative *Legionella* strains were genotyped by sequence-based typing in accordance with the European Working Group for Legionella Infections typing scheme[12] and with pulsed-field gel electrophoresis, as previously reported.[13]

Detection of Viable but Nonculturable *Legionella* by Real-Time Polymerase Chain Reaction and Immunomagnetic Separation with Adenosine Triphosphate Bioluminescence Analysis

To study the induction by monochloramine of viable but apparently nonculturable (VBNC) state, a condition gained by the bacteria in response to stress that determines a state of low metabolic activity, genomic DNA extracted from water samples (QIAamp DNA Mini Kit, Qiagen) was analyzed according to the protocol of the SsoAdvanced SYBR Green Supermix (Bio-Rad), using the CFX96 real-time polymerase chain reaction (PCR) detection system (Bio-Rad) to detect the *mip* gene.[11] Briefly, 12.5 μL of Supermix were added to 5 μL of DNA template in a 25-μL volume, with 0.3 μM of each primer. Reaction conditions were 98°C for 2 min, followed by 40 cycles of 98°C for 2 s, 55°C for 20 s, and 72°C for 20 s. A melt curve was generated by heating from 65° to 95°C with 0.5°C increments.

The suitability of combining immunomagnetic separation (IMS; Dynabeads anti-*Legionella*, Invitrogen) with adenosine triphosphate (ATP) bioluminescence assay (Quench-Gone Aqueous, Aqua-Tools) was assessed according to manufacturer's protocols as a rapid and selective method for the *Legionella* detection. Although the IMS capturing efficiency is largely dependent on cell bead contact time, sample and reagent concentration, and ability of each single bead to capture more than 1 bacterial cell, a strong association was observed between *Legionella* bacteria counts and ATP concentrations applying the method to water samples (5 replicates) contaminated with *Legionella pneumophila* ATCC 33153 within the range of 10^2–10^7 colony-forming units (CFUs)/L (correlation coefficients $R^2 = 0.93$). The assay showed the same 10^2 CFUs/L detection limit indicated in ISO 11731. The specificity of the assay was confirmed by the lack of positive results on water samples contaminated with a variety of other gram-negative bacteria (data not showed).

Detection of Nontuberculous Mycobacteria and Identification of Isolates

The water samples were analyzed for *Mycobacterium* spp. according to the protocol described by Falkinham.[14] Moreover, nontuberculous mycobacteria were searched in biofilm samples, using the same method applied to investigate *Legionella* in biofilm. Following acid-fast staining, DNA was extracted (QIAamp DNA Mini Kit, Qiagen) from colonies grown on Middlebrook 7H10 (Becton Dickinson), and species identification was carried out by PCR restriction enzyme pattern analysis of the gene encoding for the 65-kDa heat shock protein (*hsp65*), according to the protocol published by Telenti,[15] and also by sequencing of *hsp65* gene.[16]

Data Analysis

Repeated-measures ANOVA was performed using Bonferroni's procedure for multiple comparisons.

RESULTS

Efficacy of the Combined Chlorine Dioxide Filtration Strategy

At the beginning of the monitoring program, a systemic colonization of the water network was demonstrated. *Legionella* was isolated from 54 of the 81 (66.7%) sampling points, with a mean count of 3.2×10^4 CFUs/L, ranging from 2.0×10^2 to 6.0×10^5 CFUs/L; in addition, 42 (52%) samples exceeded 10^3 CFUs/L.

Following the start of continuous chlorination with chlorine dioxide in April 2003, the number of positive supply points and the mean bacterial loads decreased progressively, although *Legionella* appeared repeatedly absent in only few sampling points: positive site rate was reduced by 51% (from 66.7% to 32.9%), and the mean count was cut down by 78.2% (from 3.2×10^4 to 2.97×10^3) after 9 years of water chlorination (Figure 1*A*). In December 2006, an accidental event occurred in a water tank of the municipal water distribution plant, causing a sediment buildup in the pipework and increasing water contamination; the event probably contributed to the failure to further reduce *Legionella* colonization through only the use of disinfection. After this event, the hospital water safety plan relying only on chlorine dioxide was modified to include point-of-use filtration (0.2-μm sterile filters for 30 days) as an additional measure in selected wards—such as transplant, hematology, oncology, and intensive care units—to insure complete protection from legionellosis and other waterborne infections for high-risk patients. The integrated disinfection-filtration strategy adopted since 2007, although expensive, allowed hospitals to significantly reduce ($P < .05$) both the mean bacterial loads and the percentage of positive sites (Figure 1*B*) below the threshold level of 10^3 CFUs/L and less than 30% of positive points, as recommended by European and Italian guidelines[10] and by Allegheny County guidelines.[17] Molecular typing identified more than 90% of isolates as *L. pneumophila* sg 1 strain Wadsworth belonging to 2 prevalent clones, the SBT 269 pulsetype 2, and the SBT 657 pulsetype 1, which were isolated in 70% (71/101) and 28% (28/101), respectively, of strains.

Efficacy of Monochloramine Disinfection

In December 2010, before the opening of the emergency department, hyperchlorination shock with 4.00 mg/L of monochloramine for 4 hours and super flushing were performed in order to disinfect the warm water system. Subsequently,

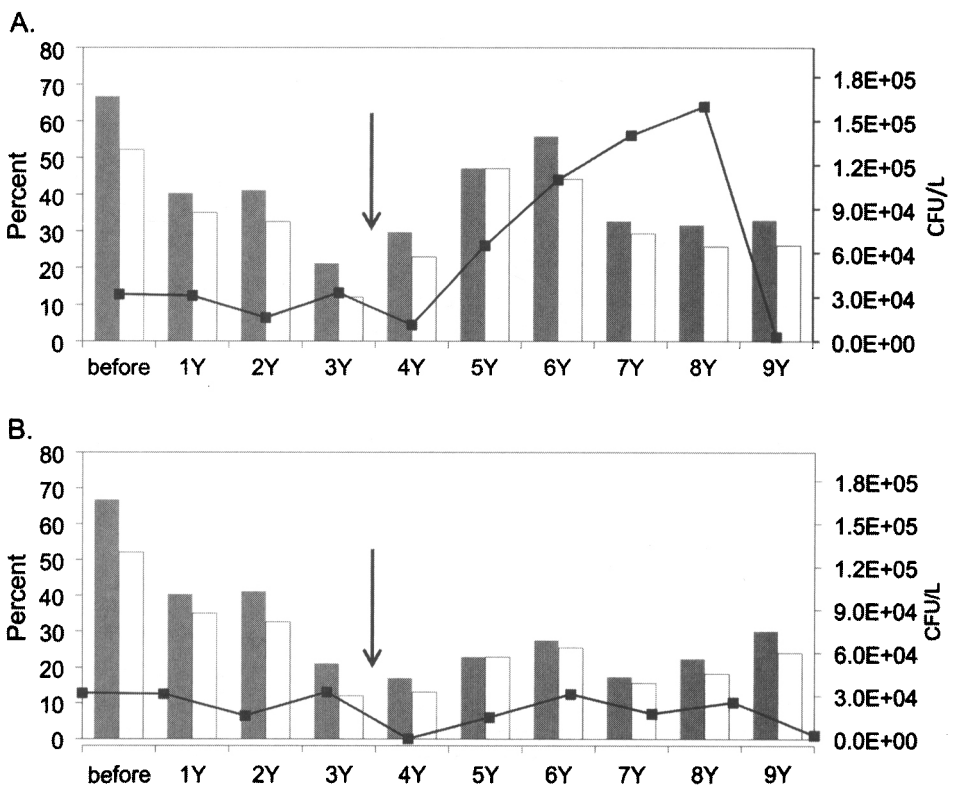

FIGURE 1. Percentage of total positive sites (gray bars), positive sites with bacterial loads greater than 10^3 colony-forming units (CFUs)/L (white bars), and mean count (line) of *Legionella* spp. before and after chlorine dioxide disinfection in 1,015 unfiltered samples (*A*) of 1,458 total, including point-of-use filtration hot water samples (*B*). Arrows indicates accidental event that occurred at the Municipal water treatment plant, which caused the increase in water contamination.

https://doi.org/10.1086/675280 Published online by Cambridge University Press

the dosage was regulated to obtain a continuous chlorination to 2 mg/L. Over the course of the investigation, the average observed distal site NH_2Cl concentration was 1.93 ± 1.04 mg/L. While at the initial monitoring phase before the start of the monochloramine disinfection, all 6 sites were positive for *L. pneumophila* sg 1 ST269, with a mean count of $7.2 \times 10^3 \pm 5.3 \times 10^3$ CFUs/L; no sample was positive after treatment. However, the ST269 strain was cultured in 2 instances (May 2011 and October 2012) as a consequence of a failure of the monochloramine generator device, during which the release of disinfectant was interrupted for around 24 hours. In these occasions, *Legionella* was isolated in all 6 sites, with mean counts of $3.7 \times 10^4 \pm 3.5 \times 10^4$ and $1.4 \times 10^5 \pm 1.3 \times 10^5$ CFUs/L, respectively (Figure 2). All samples became negative as soon as the system returned to operation. *Legionella* was recovered in only 17% (14/82) of biofilms and was always associated with positive water samples.

On the basis of the observation of the VBNC state induced on *Legionella* by monochloramine treatment,[18] we applied real-time PCR analysis on samples where *Legionella* was not cultivated; 13 of 24 (54%) samples were positive, with a mean load of $2.3 \times 10^3 \pm 3.4 \times 10^3$ genomic units/L. Since real-time PCR analysis does not allow determination of whether the nucleic acid belongs to intact cells, the IMS-ATP test was applied to a total of 69 samples collected from the emergency department. The results showed a good association between ATP values and CFUs/L, but high values of ATP were observed in the absence of *Legionella* growth (Figure 3). When the *Legionella* mean counts were high, because of the monochloramine generator failure, ATP values were similarly high ($1.2 \times 10^3 \pm 3.2 \times 10^2$ picograms/L). In regard to the negative samples, the assay still revealed high values of ATP, ranging from 6.3×10^3 to 2.6×10^1 picograms/L (mean, $8.2 \times 10^2 \pm 1.1 \times 10^3$ picograms/L). In our opinion, considering the high sensitivity and specificity of the method, we assume that false positive results by the IMS-ATP assay application were due to the presence of metabolically active but nonculturable *Legionella* cells rather than to the presence of other bacterial species brought down by the IMS.

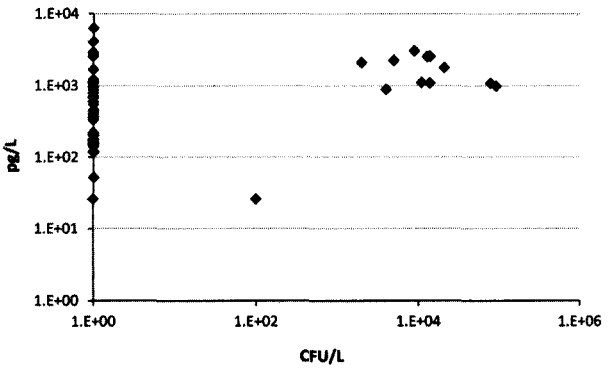

FIGURE 3. Relation between intracellular adenosine triphosphate concentrations (picograms/L) and *Legionella* counts (colony-forming units [CFUs]/L).

After the introduction of the monochloramine disinfection, an increase in the positive sites for nontuberculous mycobacteria and a significant rise in their mean bacterial loads (from $1.9 \times 10^1 \pm 2.0 \times 10^1$ to $1.4 \times 10^3 \pm 2.6 \times 10^3$ CFUs/L; $P < .05$) were observed as long as the concentration remained equal to 2 mg/L. Sixty-eight water samples were analyzed, 29 of which were positive for nontuberculous mycobacteria (43%). Nontuberculous mycobacteria were not isolated from 35 water samples and 29 biofilm samples analyzed following the increase in monochloramine concentration to 3 mg/L (Figure 2). The predominant species identified by PCR restriction enzyme pattern analysis and sequencing of the gene *hsp65* was *Mycobacterium gordonae* (86% of samples), followed by *Mycobacterium mucogenicum* (7% of samples).

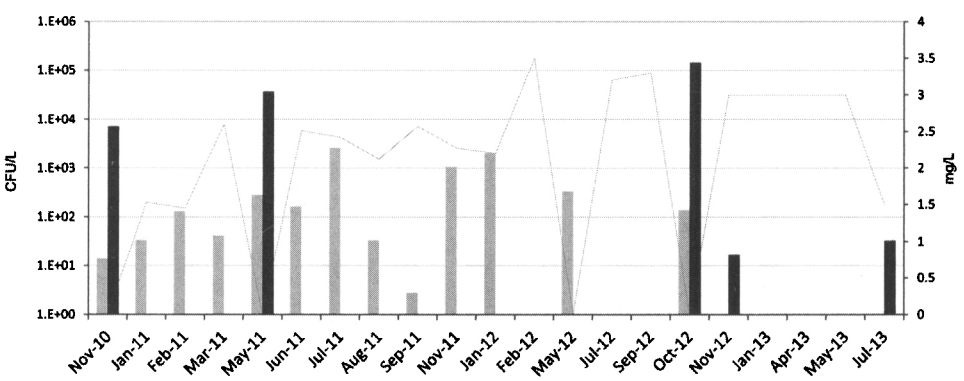

FIGURE 2. Mean loads (colony-forming units [CFUs]/L) of *Legionella* spp. (black bars) and nontuberculous mycobacteria (gray bars) before monochloramine treatment of the hot water system (November 2010) and after application of different doses of monochloramine (line; January 2011–July 2013). In each month, the sampling sites and the number of water samples were the same for both *Legionella* and nontuberculous mycobacteria analysis.

https://doi.org/10.1086/675280 Published online by Cambridge University Press

DISCUSSION

On the assumption that complete eradication of *Legionella* from water network systems seems impossible to achieve, despite extended disinfection,[19-21] the results obtained by an appropriate strategy of environmental surveillance and water risk assessment may be useful in the decision making for the control of *Legionella* colonization. In particular, healthcare facilities with high-complexity organizational structures, where large and complex water distribution systems represent ideal reservoirs for multiplication of *Legionella*, a comprehensive, multibarrier program is necessary to ensure the safety of water, in particular for high-risk patients with increased risk of infection.[5] Age, material of water pipes, or intrinsic structural defects that influence the water flow inside the water network may reduce the disinfectant efficacy and therefore be the cause of rapid biofilm development and multiplication of *Legionella*.[22-24]

A standardized approach, as directed by the Allegheny County Health Department guidelines, recommends that hospitals routinely perform environmental surveillance and consider disinfection of the water supply if the water system is heavily colonized with *Legionella*.[17] Although the first comprehensive review of disinfection methodologies was published in 1990[25] and recently in 2011,[8] no evidence-based recommendation can still be made for all potentially applicable treatments to the hospital water network, so that most of the guidelines and also the Centers for Disease Control and Prevention guidelines suggest validation of decontamination procedures by collecting specimens for culture at 2-week intervals for 3 months after treatment[7] to ensure the institution's safety practices.

Any disinfection method needs to be fine-tuned to obtain satisfactory results in individual hospitals over prolonged time periods (control of *Legionella* contamination and no legionellosis cases), and only an evidence-based approach allows clear understanding of the efficacy and disadvantages of the control measures and whether other strategies must be put in place.

In our study, the results obtained with the monitoring program applied in the university hospital allow us to affirm that *Legionella* was ubiquitous and persisting despite chlorine dioxide disinfection, and only after the adoption of an integrated disinfection-filtration strategy was it possible to efficiently control the water quality.

The application of corrective actions, such as water disinfection, is aimed at maintaining undetectable levels of Legionella at the endpoint of use and at controlling the colonization of the water network system, in particular in high-risk areas accommodating patients immunosuppressed or with predisposing conditions, where the water from the outlet should be free of Legionella.[2]

High-risk patients should benefit from the exclusive use of water that meets a higher standard for microbiologic quality, and if disinfection systems are not able to guarantee this quality, the application of filters to the terminal points of use may be cost effective when restricted to higher-risk wards, in comparison with other interventions that involve complex installation and/or high operating costs (such as increasing water temperature). Continuous long-term use of filters is not recommended[26] because in all cases it is possible for bacteria to colonize or grow through the filter material, so that the filters must be changed at appropriate time intervals in accordance with the manufacturer's recommendations.

In accordance with previous observations that indicated the stability of *Legionella* genotypes despite repeated cycles of chlorination,[21,27,28] our results seem to show that higher fitness strains can be selected and become prevalent, since the SBT 269 pulsetype 2 clone was isolated more frequently in the water supply of the hospital throughout 9 years of environmental monitoring. Moreover, the analysis of chlorine susceptibility demonstrated greater tolerance to chlorine from strains isolated after the adoption of chlorination (B.C., A.B., P.V., et al, personal communication, 25th Meeting of the European Working Group for *Legionella* Infections, Copenhagen, September 2010).

Although the standard method for environmental surveillance of *Legionella* is the culture technique,[9] since this method allows assessment of the real risk of infection, some limitations—such as prolonged incubation periods or inability to detect the VBNC state—require the development of alternative analysis protocols enabling preventive and corrective actions with a higher level of timeliness, effectiveness, and economy than those provided by standard methods.

Recently, for this purpose, new methods for the water risk assessment have been introduced. Alternative approaches, such as PCR methods, which have been included in French guidelines,[29] seem to represent an alternative method able to also detect the presence of VBNC *Legionella*.[30] The measurement of intracellular ATP may be useful for detecting microbial contamination of water,[31] but this method lacks specificity for *Legionella*; nevertheless, combining immunoseparation and ATP detection as proposed in this work could be an attractive approach as a rapid, specific, and cost-saving detection method. These methods were applied to assess the efficacy of the new disinfection treatment based only on monochloramine treatment of the hot water distribution system. Monochloramine appeared more effective than chlorine dioxide, probably because of its stability and greater effectiveness against biofilms.

The shift to monochloramine, however, apparently eradicated *Legionella* from only water and biofilm samples, since the colonization by the same ST269 clone promptly reemerged when the concentration of disinfectant was not optimized. This event could be explained by the induction of VBNC forms of *Legionella*,[18] since the presence of genomic DNA was demonstrated by real-time PCR in water samples negative for *Legionella* by culture and intact *Legionella* cells were detected by the measurement of intracellular ATP.

VBNC cells are able to resuscitate to the metabolically active and culturable state, further increasing the public health

importance of these forms.[32] Considering that doses greater than 1 mg/L of monochloramine seemed to damage cells that are unable to resume repair and thus progressively degenerate,[18] it remains to be evaluated whether the resuscitation of VBNC cells from water samples is possible.

Our study is the first report evaluating monochloramine disinfection applied to hospital hot water system over an extended period of treatment (26 months), where a good efficiency of the disinfection in controlling the colonization by *Legionella* is demonstrated. Satisfactory results in controlling *Legionella* were also reported by Flannery[33] in municipal water supplies, while in a recent study, Marchesi[34] described a decrease in *Legionella* colonization of hospital hot water systems following 1-year application of monochloramine treatment, although no significant effect was observed in colonization by other waterborne bacteria, such as *Pseudomonas* spp. In our opinion, further investigations should be conducted also on other potential waterborne pathogens, especially nontuberculous mycobacteria, which have been detected more frequently in water network systems after monochloramine treatment.[23, 35-37] In our study, an increase in the positive sites and the mean bacterial loads was observed as long as monochloramine concentration was 2 mg/L, but increasing the concentration up to 3 mg/L, no mycobacterial species were isolated by culture. *M. gordonae* and *M. mucogenicum*, the species more frequently isolated, have been associated with nosocomial outbreaks, where in some instances the hospital water was identified as the source of infection.[37-38]

The data obtained from this study suggest that no disinfection method is completely safe and free from side effects, so that the risk related to water supply in hospitals and other healthcare facilities should be periodically assessed. Only water safety plans based on locally adapted interventions and continuous surveillance may be effective in preventing nosocomial *Legionella* infection, such as in our hospital, where no cases of infection have been observed over the past 11 years.

ACKNOWLEDGMENTS

Statistical analyses were done by Dr. Mario Miccoli. Dr. Alessandra Vecchione and Dr. Michele Totaro provided assistance with data acquisition.

Financial support. This work was supported by the Italian Ministry of University and Research (project PRIN 20084ZLAX3). Additional support for this study was provided by Sanipur (Brescia, Italy).

Potential conflicts of interest. This study was supported by Sanipur, which was not involved in study design, data analysis, or data presentation. All authors report no conflicts of interest relevant to this article. All authors submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest, and the conflicts that the editors consider relevant to this article are disclosed here.

Address correspondence to Beatrice Casini, BSc, MS, Department of Translational Research, New Technologies in Medicine and Surgery, University of Pisa via S. Zeno 35-39, 56127 Pisa, Italy (beatrice.casini@med.unipi.it).

REFERENCES

1. Joseph CA, Ricketts KD; on behalf of the European Working Group for *Legionella* Infections. Legionnaires' disease in Europe 2007–2008. *Euro Surveill* 010;15(8):pii=19493. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19493.
2. World Health Organization (WHO). Legionella *and the Prevention of Legionellosis.* Geneva: WHO, 2007.
3. World Health Organization (WHO). *Guidelines for Drinking-Water Quality.* Vol 1. *Recommendations.* Geneva: WHO, 2004.
4. Freije MR. Formulating a risk reduction strategy for waterborne pathogens in hospital water systems. *Am J Infect Control* 2005; 33:S50–S53.
5. Exner M, Kramer A, Lajoie L, et al. Prevention and control of health care–associated waterborne infections in health care facilities. *Am J Infect Control* 2005;33:S26–S40.
6. World Health Organization (WHO). *Water Safety Plan Manual: Step-by-Step Risk Management for Drinking-Water Suppliers.* Geneva: WHO, 2009.
7. Centers for Disease Control and Prevention. Guidelines for preventing health-care-associated pneumonia, 2003: recommendations of CDC and the Healthcare Infection Control Practices Advisory Committee. *MMWR Morb Mortal Wkly Rep* 2004; 53(RR03):1–36.
8. Lin YE, Stout JE, Yu VL. Controlling *Legionella* in hospital drinking water: an evidence-based review of disinfection methods. *Infect Control Hosp Epidemiol* 2011;32:166–173.
9. International Organization for Standardization (ISO). *ISO 11731: Water quality: detection and enumeration of* Legionella. Geneva: ISO, 1998.
10. Superior Institute of Health. *Italian Guidelines for Prevention and Control of Legionellosis.* G.U. 5 May, 103, 2000.
11. European Working Group for Legionella Infections. *Sequence-Based Identification of* Legionella *Using the Macrophage Infectivity Potentiator* (mip) *Gene.* London: Health Protection Agency, 2013. http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/1195733805138. Accessed March 28, 2013.
12. European Working Group for Legionella Infections. *M13 Modified Sequence-Based Typing (SBT) Protocol for Epidemiological Typing of* Legionella pneumophila. London: Health Protection Agency, 2012. http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php. Accessed March 28, 2013.
13. Casini B, Valentini P, Baggiani A, et al. Comparison of two molecular methods used for subtyping of *Legionella pneumophila* 1 strains isolated from a hospital water supply. *Water Sci Technol* 2008;58:683–688.
14. Falkinham JO, Norton CD, Le Chevallier MW. Factors influencing numbers of *Mycobacterium avium, Mycobacterium intracellulare,* and other mycobacteria in drinking water distribution systems. *Appl Environ Microbiol* 2001;67:1225–1231.
15. Telenti A, Marchesi F, Balz M, et al. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* 1993;31:175–178.
16. Plikaytis BB, Crawford JT, Woodley CL, et al. Rapid, amplification-based fingerprinting of *Mycobacterium tuberculosis. J Gen Microbiol* 1993;139:1537–1542.
17. Squier CL, Stout JE, Krsytofiak S, et al. A proactive approach to prevention of health care–acquired Legionnaires' disease: the

Allegheny County (Pittsburgh) experience. *Am J Infect Control* 2005;33:360–367.
18. Alleron L, Merlet N, Lacombe C, et al. Long-term survival of *Legionella pneumophila* in the viable but nonculturable state after monochloramine treatment. *Curr Microbiol* 2008;57:497–502.
19. Rangel-Frausto MS, Rhomberg P, Hollis RJ, et al. Persistence of *Legionella pneumophila* in a hospital's water system: a 13-year survey. *Infect Control Hosp Epidemiol* 1999;20:793–797.
20. Stout JE, Yu VL. Experiences of the first 16 hospitals using copper–silver ionization for *Legionella* control: implications for the evaluation of other disinfection modalities. *Infect Control Hosp Epidemiol* 2003;24:563–568.
21. Scaturro M, Dell'Eva I, Helfer F, et al. Persistence of the same strain of *Legionella pneumophila* in the water system of an Italian hospital for 15 years. *Infect Control Hosp Epidemiol* 2007;28: 1089–1092.
22. Wang H, Masters S, Hong Y, et al. Effect of disinfectant, water age, and pipe material on occurrence and persistence of *Legionella, Mycobacteria, Pseudomonas aeruginosa,* and two amoebas. *Environ Sci Technol* 2012;46:11566–11574.
23. Cooper IR, Hanlon GW. Resistance of *Legionella pneumophila* serotype 1 biofilms to chlorine-based disinfection. *J Hosp Infect* 2010;74:152–159.
24. Spagnolo AM, Cristina ML, Casini B, et al. *Legionella pneumophila* in healthcare facilities. *Rev Med Microbiol* 2013;24:70–80.
25. Muraca PW, Yu VL, Goetz A. Disinfection of water distribution systems for *Legionella*: a review of application procedures and methodologies. *Infect Control Hosp Epidemiol* 1990;11:79–88.
26. Department of Health (DH) Estates and Facilities Division. *Health Technical Memorandum 04-01: The Control of* Legionella, *Hygiene, "Safe" Hot Water, Cold Water and Drinking Water Systems (Part A and Part B).* Leeds: DH Estates and Facilities Division, 2006.
27. Cooper IR, White J, Mahenthiralingam E, et al. Long-term persistence of a single *Legionella pneumophila* strain possessing the *mip* gene in a municipal shower despite repeated cycles of chlorination. *J Hosp Infect* 2008;70:154–159.
28. Perola O, Kauppinen J, Kusnetsov J, et al. Persistent *Legionella pneumophila* colonization of a hospital water supply: efficacy of control methods and a molecular epidemiological analysis. *Acta Pathol Microbiol Immunol Scand* 2005;113:45–53.
29. Association Française de Normalisation (AFNOR). *Qualité de l'Eau: Détection et Quantification des* Legionella *et/ou* Legionella pneumophila *par Concentration et Amplification Génique par Réaction de Polymérisation en Chaîne (PCR).* Saint-Denis: AFNOR, 2006. XP T90-471.
30. Lee JV, Lai S, Exner M, et al. An international trial of quantitative PCR for monitoring *Legionella* in artificial water systems. *J Appl Microbiol* 2011;110:1032–1044.
31. Denys JC, Deniau D. Prévention des risques liés à l'eau dans les établissements de santé de la Réunion. *Hygiènes* 2011;19:379–386.
32. Wood TK, Knabel SJ, Kwan BW. Bacterial persister cell formation and dormancy. *Appl Environ Microbiol* 2013;79:7116–7121.
33. Flannery B, Gelling LB, Vugia DJ, et al. Reducing *Legionella* colonization of water systems with monochloramine. *Emerg Infect Dis* 2006;12:588–596.
34. Marchesi I, Bargellini A, Cencetti S, et al. Control of *Legionella* contamination in a hospital water distribution system by monochloramine. *Am J Infect Control* 2012;40:279–281.
35. Pryor M, Springthorpe S, Riffard S, et al. Investigation of opportunistic pathogens in municipal drinking water under different supply and treatment regimes. *Water Sci Technol* 2004;50: 83–90.
36. World Health Organization. *Pathogenic Mycobacteria in Water: A Guide to Public Health Consequences, Monitoring and Management.* London: IWA, 2004.
37. Fernandez-Rendon E, Cerna-Cortes JF, Ramirez-Medina MA, et al. *Mycobacterium mucogenicum* and other non-tuberculous mycobacteria in potable water of a trauma hospital: a potential source for human infection. *J Hosp Infect* 2012;80:74–76.
38. Billinger ME, Olivier KN, Viboud C, et al. Nontuberculous mycobacteria–associated lung disease in hospitalized persons, United States, 1998–2005. *Emerg Infect Dis* 2009;15:1562–1569.