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Legionella spp., amoebae and not-fermenting Gram negative bacteria in an Italian university hospital water system

Pasqualina Laganà[1], Gabriella Caruso[2], Davide Piccione[1], Maria Eufemia Gioffrè[1], Raffaella Pino[1], Santi Delia[1]

[1] *Department of Biomedical Sciences and Morphological and Functional Images, University of Messina, Italy*
[2] *Italian National Research Council, Institute for Coastal Marine Environment, Messina, Italy*

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Abstract

Introduction. In hospital and other health care facilities, contamination of water systems by potentially infectious microorganisms, such as bacteria, viruses and protozoa, is a source of nosocomial infections, which may originate from colonization of water pipes, cooling towers, spa pools, taps, showers and water supplies.

Objective. The study focuses on the occurrence of *Legionella* spp., free-living amoebae and non-fermenting Gram-negative microorganisms in a University hospital water system located in the town of Messina (Sicily, Italy), which had never been examined previously.

Materials and Methods. From January 2008 – March 2009, hot tap water samples were collected from 10 wards. *Legionella* spp. recovered on selective culture medium were identified by microagglutination latex test; free-living amoebae were cultured using *Escherichia coli* as a food source. Non-fermenting Gram negative microorganisms were identified by API 20 NE strips.

Results. *Legionella* spp. were found in 33.33% of the samples. *L. pneumophila* serogroup 1 was recovered from the Laboratory Diagnostic and Anaesthesia-Neurology Wards, with a peak of 3.5×10^4 cfu/L in May 2008. *L. pneumophila* serogroups 2–14 were found in the Othorhinolaryngology, Pathologic Anatomy, Paediatrics and Surgery Wards, and peaked (4×10^4 cfu/L) in April 2008. Pseudomonadaceae and Hyphomycetes were also detected. *Legionella* spp. were recovered from samples positive for non-pathogenic amoebae *Hartmannella* spp.

Conclusion. This first study of a Messina hospital water system suggested potential health risks related to the detection of *Hartmannella* spp., as reservoirs for *Legionella* spp., and *Pseudomonas aeruginosa*, a Gram negative non-fermenting bacterium frequently causing nosocomial pneumonia. The urgent need for monitoring programmes and prevention measures to ensure hospital water safety is stressed.

Key words

Legionella, amoebae, Gram-negative bacteria, hospitals, water systems

INTRODUCTION

Contamination of water systems by potentially infectious microorganisms, such as bacteria, viruses and protozoa, is recognised as a source of nosocomial infections [1, 2, 3]. In hospital and other health care facilities, waterborne diseases may originate from the bacterial colonization of water pipes, cooling towers, spa pools, taps, showers and water supplies [4, 5, 6, 7, 8, 9, 10, 11]. There are several reports concerning the epidemiological surveillance of pathogenic bacteria in Italian hospitals [12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22]. The National Surveillance System (ISS, Rome) reported that during 2011 in Italy nosocomial cases of legionellosis were 65 (6.4% of totally reported cases), of which 33 (50.8%) were of certain nosocomial origin and 32 (49.2%) of probable nosocomial origin [23]. Water systems represent suitable environments for the growth and multiplication of *Legionella* spp., Gram-negative bacteria which survive to different pHs and temperatures [24, 25, 26]. *L. pneumophila* is the main causative agent of legionellosis, considered among the 30 emerging infective diseases [24, 27]. The search for these microorganisms in hospital water systems is of utmost relevance for health risk prevention [2, 24]. Aquatic biofilms represent ecological niches for *Legionella* spp. survival and multiplication [24, 26, 28]. Moreover, protozoa can be important legionellae hosts in natural, hospital and domestic environments; viable but non-culturable *Legionella* within amoebic cysts can contribute to hospital water contamination [28, 29]. Protozoa may increase bacterial infectivity for mammalian cells *in vitro*, and resistance to biocides and antibiotics [30]. Therefore, amoebae play a crucial role in the pathogenesis of *Legionella* spp. and to develop successful prevention strategies [25, 31].

In southern Italy, surveillance of *Legionella* spp. was carried out in the Apulian [27, 32, 33] and Sicilian [16] regions. Limiting our attention to the latter, in the hot water distribution systems of three hospitals located in Catania, *L. pneumophila* was found at variable concentrations (0–10^4 colony forming units/litre, cfu/L) depending on the hospital buildings; decontamination procedures were found to reduce *Legionella* concentration only temporarily.

Objectives: The presented research focuses on the occurrence of *Legionella* spp., free-living amoebae and non-fermenting Gram-negative microorganisms in a University hospital

Address for correspondence: Pasqualina Laganà, Department of S.B.I.MO.F. University of Messina, Italy
e-mail: plagana@unime.it

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water system located in the town of Messina (Sicilian region), which has never been examined previously for these microorganisms.

MATERIALS AND METHOD

Sampling. From January 2008 – March 2009, 66 samples drawn from the water distribution system of the 'G. Martino' University hospital (Messina, Italy) were examined for *Legionella* spp., amoebae and non-fermenting Gram-negative microorganisms. Monthly samplings were performed in 10 different buildings and wards (Tab. 1). Incoming cold groundwater is provided by the municipality and disinfected with chlorine dioxide; the water reaches the hospital by means of a single pipeline which leads to a centralized tank where the water is stored. The water does not undergo further chlorination after it is gathered from the Messina town pipeline. From the centralized tank, the cold water is distributed to each building by electric-motor pumps which send it through a pipeline that runs across the basements of all the buildings. Under each building there is a boiler which produces heated water (average temperature approximately 13–48 °C) that climbs up again to supply the wards located on each floor. Samples of heated water were collected at the start of daily activities from taps using 1 L-sterile glass bottles. In order to obtain a sampling representative of the hygienico-sanitary conditions, care was taken to sample all the floors, and the wards located both on the left and on the right sides inside each building.

Isolation and identification of *Legionella* spp. To recover *Legionella* spp. from water samples the standard procedures reported in the Italian Guidelines for the prevention and control of legionellosis (Gazzetta Ufficiale della Repubblica Italiana n.103, May 5, 2000) were used. 1 L water samples were concentrated to 10 mL through 0.2 µm porosity membrane filters and incubated at 50 °C for 30 min in a thermostatic bath. Concentrated and unconcentrated samples were spread on duplicate plates of Buffered Charcoal-Yeast Extract (BCYE) Agar Base Medium (Oxoid Ltd., Milan, Italy), incubated for 10 days at 36–37 °C in a moist chamber with 2.5% CO_2, the suspected colonies were isolated and confirmed as *Legionella* spp. after screening their inability to grow on a culture medium without cysteine. *Legionella* spp. counts were reported in colony forming units/liter (cfu/L) according to the number of colonies per plate and to the dilutions performed on the original sample. The isolates were further identified as *Legionella pneumophila* serogroup 1, *Legionella pneumophila* serogroups 2–14, or *Legionella* spp using the microagglutination *Legionella* Latex Test Kit (Oxoid).

Isolation and identification of free-living amoebae. The same samples analysed for *Legionella* spp. were filtered and the collected particles were eluted, or were centrifuged and the pellet re-suspended in Page's amoebae saline solution (2.5 mmol/L NaCl, 1 mmol/L KH_2PO_4, 0.5 mmol/L Na_2HPO_4, 40 µmol/L $CaCl_2$. $6H_2O$ and 20 µmol/L $MgSO_4$. $7H_2O$). A sub-culture of the suspension was also made on non-nutrient agar plates with *Escherichia coli* (NCTC 9001) as a food source, incubated at 32 °C [34]. Trofozoites plaques were sub-cultured on a microtitre plate in Page's saline solution, incubated at 32 °C for 1–3 hours and examined by microscope.

Isolation and identification of non-fermenting Gram negative microorganisms. To isolate non-fermenting Gram negative microorganisms, both belonging to bacteria and eukaryotes (*Hyphomycetes*), 100 mL of water were filtered through 0.45 µm porosity cellulose membranes, placed on Tryptic Soya Agar (TSA, Oxoid) and Cetrimide Agar (Oxoid) plates, incubated for 24 hours at 37 °C. The isolates were identified to the species or genus level by API 20 NE profiles (bioMérieux, Marcy l'Etoile, France). Hyphomycetes were identified based on their colony morphology in culture media.

Statistical analysis. SigmaStat software V3.0 was used for analysis of variance (ANOVA) on logarithmic-transformed data, to assess significant differences in bacterial concentrations among the wards. Spearman's correlations were performed between *Legionella* counts and water temperatures, or among *Legionella* serotypes. Cluster analysis by PRIMER 6 software version 6β R6 (Marine Laboratory, Plymouth, UK) was performed on temperature and *Legionella* spp. values.

RESULTS

Bacteriological monitoring. Over 30% of the examined samples were *Legionella* spp. positive (Tab. 1). *Legionella* spp. were recovered mostly from Wards E (Anaesthesia and Intensive care, Neurology, Neurosurgery, Orthopaedics) and F (Surgery), while they were scarcely found in Wards A (Obstetrics and Gynaecology) and NI (Paediatrics). No *Legionella* spp. were isolated from Wards C (Internal Medicine), H (Oncology, Dermatology, Contagious Diseases, Occupational Medicine, Pneumology) and W (Ophthalmology and Psychiatry).

Table 1. Messina hospital buildings with the respective wards. The number and the percentage of the total of the samples positive for *Legionella* spp. are reported

Hospital buildings	Wards	Sampling time	Total number of samples	Number (and % of the total) of samples positive for *Legionella* spp.
A	Obstetrics and Gynaecology	Jan-'08	10	1 (10.0)
B	Otorhinolaryngology Eye diseases	Feb-'08	8	3 (37.5)
C	Internal Medicine	Mar-'08	10	0 (0.0)
D	Pathologic anatomy	Apr-'08	10	5 (50.0)
E	Anaesthesia and Intensive care; Neurology; Neurosurgery; Orthopedics	May-'08	5	5 (100.0)
F	Surgery division	Mar-'09	5	4 (80.0)
G	Laboratory Diagnostic	Mar-'09	6	3 (50.0)
NI	Pediatrics	Nov-'08	5	1 (20.0)
H	Oncology; Dermatology; Contagious diseases; Occupational Medicine; Pneumology	Nov-'08	5	0 (0.0)
W	Ophthalmology; Psychiatry	Nov-'08	2	0 (0.0)
Total N. of samples			66	22 (33.3)

Water temperature ranged from 18.9–32.6 °C, in March 2009 and in February 2008, respectively (Tab. 2). *Legionella* spp. concentrations did not correlate with temperature. *L. pneumophila* serogroup 1 was frequently recovered from Wards E and G (Tab. 2). Peak concentrations of 3.5×10^4 cfu/L were reached in Ward E in May 2008. Here, the serogroup 1 abundance was higher than in Ward G (F= 136.1; P<0.01) and the detection of high numbers of *L. pneumophila* serogroup 1 suggested the occurrence of bacterial colonisation. High concentrations of *L. pneumophila* serogroups 2–14 were found in Wards D, B (Otorhynolaryngology and Eye Diseases), F and NI. In Ward D, the highest numbers (4×10^4 cfu/L) of these serogroups were detected in April 2008; their counts were higher than in Wards A and B (F=8.88 and 6.65; P<0.05, respectively). *Legionella* serotypes 1 and 2–14 abundances were inversely related (Spearman ρ= -0.80, P<0.01). *Legionella* spp. were also recovered from Wards F and G; their numbers differed between Wards NI and A (F=144.0; P<0.01) or B (F=18.51; P<0.01).

Table 2. Quantitative and qualitative results of the search for *Legionella* spp., free-living amoebae and non-fermenting microorganisms. Water temperature values recorded in the examined samples are also recorded

Hospital buildings	Average water temperature °C	*Legionella pneumophila* serogroup 1 cfu/L	*Legionella pneumophila* serogroups 2-14 cfu/L	*Legionella* spp. cfu/L	Free-living amoebae	Not fermenting microorganisms
A	20.4				*Hartmannella* spp.	*Pseudomonas fluorescens*
			6.0E+02		*Acanthamoeba* spp.;	----
					Hartmannella spp.	----
					Hartmannella spp.	*Comamonas testosteroni*
B	32.6		3.0E+02		*Hartmannella* spp.	*Pseudomonas luteola*
					Hartmannella spp.	*Acinetobacter lwoffii; Sphingomonas spiritivorum*
			4.0E+02		*Hartmannella* spp.	Hyphomycetes
			1.7E+03		----	*Shewanella putrefaciens*
C	24.6				*Hartmannella* spp.	*Chryseobacterium indologenes*
					----	Hyphomycetes
					----	Hyphomycetes
					Hartmannella spp.	*Shewanella putrefaciens;* Hyphomycetes
D	21.4		2.0E+03		*Hartmannella* spp.	*Pseudomonas fluorescens*
			1.1E+04		----	*Pseudomonas stutzeri*
			4.0E+04		*Hartmannella* spp.	*Pseudomonas aeruginosa*
			8.0E+02		*Hartmannella* spp.	----
			3.0E+03		----	*Pseudomonas fluorescens*
E	24.5	3.5E-04			*Hartmannella* spp.	*Stenotrophomonas maltophilia*
		4.0E-03			----	*Comamonas testosteroni*
		5.5E-03			*Hartmannella* spp.	*Comamonas testosteroni*
		2.0E-04			*Hartmannella* spp.	*Pseudomonas aeruginosa*
		1.0E-04			----	*Shewanella putrefaciens*
F	18.9		1.0E+02		----	*Pseudomonas aeruginosa*
			3.0E+03		*Hartmannella* spp.	*Pseudomonas fluorescens*
			----	3.5E+03	*Hartmannella* spp.	----
			----		*Hartmannella* spp.	*Pseudomonas fluorescens*
G	18.9	1.0E-02			----	*Delfiia acidovorans*
		1.0E-02		1.0E+02	*Hartmannella* spp.	----
					----	*Pseudomonas stutzeri; Pseudomonas fluorescens*
NI	22.4		3.5-03		*Hartmannella* spp.	*Sphingomonas paucimobilis*

Cluster analysis performed on both temperature and *Legionella* spp. values reflected the different spatial distribution of *Legionella* serotypes. Four clusters were identified. The samples collected from building B, hosting *Legionella* serotypes 2–14, clustered with 99.0% similarity (S). The samples taken from building E, hosting *Legionella* serotype 1 only, grouped into a second cluster (S= 98.9%). A third larger cluster included *Legionella* serotypes 2–14 and was composed by two sub-clusters: one (S= 95.8%) grouped samples from Wards A, D and NI, while the other (S= 96.6%) consisted of Ward F samples. The fourth cluster (S= 89.7%) included samples G1, G2 and F3 and consisted of *Legionella* serotype 1 and spp.

The search for free-living amoebae (Tab. 2) recovered the not-pathogenic species *Hartmannella* spp. The pathogenic species *Acanthamoeba* spp. was recovered only from Ward A in January 2008.

Non-fermenting isolates were mostly identified as *Pseudomonas* spp. (Tab. 2), which colonized Wards D, F and G. *Ps. fluorescens* and *Ps. aeruginosa* were frequently isolated, followed by *Ps. stutzeri, Ps. luteola, Stenotrophomonas maltophilia*. Hyphomycetes were recovered from Wards B and C. The non-fermenting microflora in Ward B included *Shewanella putrefaciens*, *Acinetobacter lwoffi* and *Sphingomonas spiritivorum*. Non-fermenting microorganisms were mostly not pathogenic, excepting *Ps. aeruginosa*.

DISCUSSION AND CONCLUSIONS

Environmental surveillance of *Legionella* spp. is needed for risk assessment and prevention of hospital-acquired legionellosis [35]. In Italy, the culture method is the official method currently approved for *Legionella* spp., although its isolation from natural samples is often difficult due to multiplication inside protozoa or biofilms.

The presented study is the first on *Legionella* spp., free-living amoebae and non-fermenting Gram-negative bacteria, which could contribute to the control of the environmental persistence of these microorganisms in water systems of a Messina hospital. Italian guidelines for legionellosis prevention and control advocate no intervention, clinical surveillance or the adoption of appropriate measures for *Legionella* spp. concentrations lower than 10^2 cfu/L, equal to or below 10^5 cfu/L, and higher than 10^5 cfu/L, respectively. In the presented study, *Legionella* spp. were in the order of 10^4 cfu/L, and no cases of legionellosis were recorded; indeed, the risk of nosocomial disease was shown to be better predicted by the proportion of water-system sites positive for *Legionella* spp. than by the measured *Legionella* spp. counts [4].

The numbers of *Legionella* spp. found in the Messina hospital were similar to those found in other Italian hospitals: in the Piedmont region [14, 36], Bologna [6, 8], Modena [18] and Bari [33]. In other European countries, in Spain *L. pneumophila* was isolated from 85% of hospital water systems [37], while in Germany *Legionella* spp. was found in 68% [38], in Poland, 55–100% of hospital samples were positive for *Legionella* spp. [9], and in Greece, *Legionella* spp. was detected in 22 of 130 water samples [39]. In extra-European Countries, *L. pneumophila* was found in 63% of water systems: in Taiwanese hospitals [35]; in the USA, *Legionella* spp. was isolated from 11 of 12 hospital water systems in Texas [4]. In the presented study, qualitative analysis showed the predominance of *L. pneumophila* serogroups 2–14, while *L. pneumophila* serogroup 1 was isolated only in the in Anaesthesiology-Neurology Ward. Similar findings were reported from Poland [9] where *L. pneumophila* serogroups 2–14 accounted for 74.6% of total *Legionella* spp. In other reports [13, 33, 36, 39], *L. pneumophila* serogroup 1 represented 50% of isolates. In the current study, the lack of relationships between water temperature and *Legionella* spp. concentrations could be

explained by the survival of *L. pneumophila* in a wide range of temperatures [24, 25].

Free-living amoebae were also investigated as potential determinants for *Legionella* colonization. With the exception of January 2008, the only amoebic species found in the Messina hospital was *Hartmannella* spp., a species frequently found in hot water samples [40]. Temperature conditions and amoebic species are important for the select human-pathogenic legionellae [41]. Free-living amoebae could explain large variations in *Legionella* spp. counts. They may serve as vehicles for transmission and reservoirs of pathogens; species such as *Acanthamoeba* spp., *Hartmannella vermiformis, Naegleria* spp., are recognized as natural hosts for *Legionella* spp., enabling this pathogen to survive in hospital water systems and sanitary areas [24, 26, 40, 42, 43]. *Legionella* spp. act as facultative endoparasites, taking advantage of the nutrient-rich environment provided by protozoa [24, 26, 43]. Free-living amoebae are also responsible for opportunistic infections [35, 44].

Moderate health risks could come from non-fermenting Gram-negative bacteria. Isolates belonging to the *Acinetobacter lwoffii*, *Chryseobacterium indologenes*, *Ps. aeruginosa*, *Ps. luteola*, *Ps. fluorescens, Ps. stutzeri, Shewanella putrefaciens, Sphingomonas paucimobilis*, *Stenotrophomonas maltophilia* may be obligatory or opportunistic agents of infectious diseases. *Acinetobacter* spp., *Ps. aeruginosa*, and *Stenotrophomonas maltophilia* may cause infections by drinking the water, skin contact or aerosol inhalation [9]. *Ps. aeruginosa* is an opportunistic pathogen causing fatal hospital-acquired infections [45]. It was isolated in 12.5% of hot water samples, while *Ps. stutzeri* in 15.6% of samples [8]. Moreover, some *Pseudomonas* spp. may compete with *Legionella* for the same protozoan host [46]. *Ps. fluorescens* and *Ps. putida* may favour the environmental persistence of *L. pneumophila* serogroup 1 [47].

In overall, this first study on the water distribution system of a Messina hospital suggested potential risks to patients' health related to the detection of *Hartmannella* spp. as reservoirs for *Legionella* spp, as well as of *Ps. aeruginosa*, a Gram negative non-fermenting bacterium frequently causing nosocomial pneumonia.

In the presented study, a qualitative research (i.e. presence/absence) was performed on samples enriched for amoebae detection and consequently no statistical correlations were possible between *Legionella* spp. concentrations and the isolated free-living amoebae. Nevertheless, the recovery of *Legionella* spp. in water samples positive for *Hartmannella* spp. suggested that this non-pathogenic species may serve as a reservoir for the environmental survival of these pathogens, or as transmission vectors of pneumonia in hospitalized patients. The occurrence of *L. pneumophila*, amoebic species and *P. aeruginosa* in the examined water samples underlines the importance of hospital water surveillance through the urgent application of monitoring programmes and prevention measures suitable for ensuring water safety [7, 8, 18, 48, 49].

Although no cases of legionellosis have been notified, the obtained microbiological findings suggest some indications for the management of hospital or health care facilities that may be helpful for preventing the potential risks related to the detection of *Legionella* spp., amoebae and potentially pathogenic Gram-negative non- fermenting bacteria in water distribution systems. In a comprehensive water safety plan, the application of a global approach is recommended, which should include:

1) the appropriate maintenance of hospital water distribution systems, by mechanic cleaning-out of the tanks of possible organic matter, followed by their washing- out with disinfectants (i.e. sodium hypochlorite);
2) the maintenance of heated water at a temperature above 50 °C;
3) the application of such measures as thermal shock or hyperchlorination to decontaminate water with *Legionella* spp. concentrations over 10^4 cfu/L;
4) the systematic monitoring of the hot water distribution network, particularly in hospitals with transplant units or with immunosuppressed patients.

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