TITLE: The Effect of Hydrogen Peroxide on Reducing the Colonization of *Legionella spp.* in a Hospital Water Network

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ABSTRACT

Objectives: To evaluate the effectiveness of hydrogen peroxide (HP) use in the hospital water network disinfection to control *Legionella spp.* colonization.

Methods: Following the detection of high levels of Legionella contamination in a 136-bed general hospital water network, an HP treatment of the hot water (25 mg/L) was adopted. During a period of 34 months, the effectiveness of HP on Legionella colonization was assessed. Legionella was isolated in accordance with ISO-11731 and identification was carried out by sequencing of the *mip* gene.

Results: Before HP treatment *L.pneumophila* sg 2-15 was isolated in all sites with a mean count of 9950±8279 CFU/L. After one month of HP-treatment, we observed the disappearance of *L. pneumophila* 2-15, however other Legionella species never cultured before appeared; Legionella *pneumophila* 1 was isolated in 1 out of 4 sampling sites (2,000 CFU/L) and other non-*pneumophila* species in all sites (mean load 3,000 ±2887 CFU/L).

Starting from September 2013, HP-treatment was modified adding food-grade polyphosphates and in the following months we observed a progressive reduction of the mean load of all species ($p<0.05$), until to a substantial disappearing of Legionella colonization.

Conclusion: Hydrogen peroxide demonstrated a good efficacy in controlling Legionella. Although in the initial phases of treatment it seemed unable to eliminate all the species, by keeping HP levels to 25 mg/L and adding food-grade polyphosphates, a progressive and complete control of colonisation was obtained.

INTRODUCTION

Legionella spp. is a human Gram-negative aerobic opportunistic intracellular bacterium, responsible for a severe pneumonia called Legionnaires' disease [1]. It is a waterborne pathogen frequently associated with nosocomial infections, particularly among immunodepressed patients like acquired immune-deficiency syndrome (AIDS) affected, transplanted patients, and those undergoing aggressive chemotherapy [2].

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The case fatality rate of LD associated with outbreaks is lower than that of sporadic cases, generally around 8–15%, but it can be higher, above all in the case of hospital-acquired infections, AIDS patients, transplant patients, and those undergoing aggressive chemotherapy [3].

The genus Legionella includes 57 species (including subspecies) and about 70 serogroups, not all associated with human disease. The species most frequently detected in diagnosed cases [4] is Legionella *pneumophila* (L.*p.*) consisting of 16 serogroups; L.*p.* serogroup 1, responsible of the first identified outbreak, occurred in 1976 in Philadelphia (USA), is the cause of 95% of infections in Europe and 85% in the world [5, 6].

In Italy, 1,497 cases were reported in 2014 and only 62 (4.1%) of them were hospital-acquired infections, although among the latter the highest case fatality rate was registered, 30.8% compared to 10.1% of community infections. The species of Legionella implicated in all cases was *Legionella pneumophila* [7].

Legionella colonization control in hospital hot water networks is an important patients' safety related target but, at the same time, it represents a challenge to find a cost effective solution.

Several concerns are related to Legionella risk control; the complexity of the water system structure in huge buildings with the possibility, in some branches, to have low water flow and a higher likelihood of the development of biofilm. Old pipeline systems frequently show high corrosion levels that provide a favorable substrate for bacterial growth and the need to operate with sanitation interventions. Hot water systems, which usually operate at 40-50ºC provide the ideal growth conditions for Legionella, whose optimal temperature for in-vitro growth is 36°C (limits 15–43°C), with a generation time of 99 minutes under optimal conditions [8-10].

The second important issue is represented by intrinsic bacterial features. Legionella is a resistant and adaptable microorganism, able to survive in a wide range of natural and artificial environments [11], including adverse environmental conditions in quiescence, to recover activity and pathogenicity afterwards. Similarly, Legionella demonstrate resistance to chemical agents at concentrations usually applied for water disinfection.

The first comprehensive review of disinfection methodologies was published in 1990 [12] and the most recent in 2011 [13], no evidence-based recommendation can yet be made for any of the potentially applicable treatments to the hospital water network so that the CDC Guideline for Preventing Health-Care-Associated Pneumonia suggests to validate the decontamination procedures by collecting specimens for culture at 2-week intervals for 3 months after treatment to ensure the institution's safety practices [14-16].

Several chemical products, mostly chlorine based, have been employed as water disinfection systems; all of them are useful to control Legionella mean count, but no one is able to completely eradicate the colonization. Bacterial cell shelter in biofilm and free living protozoa, especially cysts, can protect intracellular bacteria against disinfectant [17-21]. In particular, amoebae play a role in re-emergence of *L. pneumophila* in water sources after disinfection, since the bacteria can be protected by the trophozoites and/or the cysts forms of amoebae [22].

As demonstrated by several studies, complete eradication of Legionella from hospital water network system seems impossible to achieve despite long-time disinfection [23-25], and in high risk area is necessary to install point-of-use filters to prevent hospital infections [26].

This evidence highlight the need to evaluate new disinfectant products to obtain better results in disinfection efficacy and cost-effectiveness.

Recently, in some hospital Hydrogen Peroxide (HP) was applied for hot water disinfection. Silver stabilized hydrogen peroxide has a history of use in the control of Legionella in water systems, but only few experimental studies have tested HP alone (water system, cleaning baths for dental and medical instruments), that is now regaining a role of central interest.

Continuous treatment of water distribution systems using HP, generally in combination with silver salts, is now allowed in many countries. In France the Ministère de la Santé Publique considers this formulation in the guidelines for treatment of *Legionella pneumophila* [27].

HP is a strong oxidizing agent that oxidize the microorganisms' enzymatic system, releasing free ~~of~~ oxygen atoms (nascent oxygen). Its production occurs naturally in the body as part of our defense against antigens.

HP as a water disinfectant offers various advantages. It is a strong oxidizer, bactericidal at 3% solution (D value E.coli: 0,57 min), sterilant at 6% in 6 hours, more powerful than chlorine dioxide and more stable at high temperatures and pH compared to chlorine-based disinfectants. Furthermore, it's non-toxic to human and environment~~al~~, taste free and without mutagenicity and carcinogenicity activity [28].

HP still presents numerous advantages from both economic and operative point of view, in fact operating cost are highly lower, compared with traditional water disinfection systems, so as investment costs and expenditure for equipment. HP solution is readily available and presents a very long storability (maximum loss of concentration 3% per year). Moreover, it has a low corrosive effect, while pipeline corrosion is a frequent and no-negligible problem with chlorine-based disinfection systems.

HP-based disinfection has recently been applied, with satisfactory results in reduction of microbial contamination in dental unit water; recent studies have demonstrated the efficacy of HP treatment versus several microorganisms, including Legionella [29-31].

RESULTS

Before the start of the HP treatment, high Legionella concentrations were detected in all the examined water point. Concentrations ranged from 3,000 to 20,800 CFU/L, with a mean value of 9950±8279 CFU/L in flushed water samples, proving a significant colonization of the entire hospital building water network (sampling on July the 3rd 2013). The bacterial species identified belonged to *Legionella pneumophila* 2-15 (Figure 1).

This potentially critical situation led, in July 2013, to apply a HP-based disinfection strategy that started with a shock treatment of the water network with a solution 10X of HP (10 mg/L) and ~~of~~ silver

ions (10 µg/L) for 12h, followed by a continuous treatment with 10 mg/L of HP and 10 µg of silver ions.

On July the 29th 2013 the first sampling was performed after the beginning of the treatment. Culture analysis demonstrated the absence of Legionella *pneumophila* 2-15 in all the four sampling site. However, Legionella species never cultured before appeared: Legionella *pneumophila* 1 was isolated in 1 out of 4 sampling sites (2,000 CFU/L) and other non-*pneumophila* species in all the sites (mean load 3,000 ±2887 CFU/L).

On samples resulted positive by PCR and where non-*pneumophila* Legionella species were identified, the sequence of the *mip* gene showed the presence of *Legionella longbeachae* serogroup 1 (strain NSW150).

These results, considered not satisfactory, led to the change of the disinfectant product; from September 2013, HP and silver ions were replaced by a formulation of HP only, in higher concentration, 25 mg/L, with the addition of polyphosphates as film-forming product.

This disinfection strategy obtained the first tangible results after six month of treatment.

The efficacy of the new disinfection was observed starting from January 2014; a progressive reduction of non-*pneumophila* Legionella species loads, to less than 500 CFU/L and the complete disappearance of L. *pneumophila* 1 were observed.

In July 2014, a reduction in HP concentration from 25 to 10 mg/L, probably due to a failure of the dispensing HP device, caused an increase in Legionella concentrations and the reappearing of more than 3000 CFU/L of L. *pneumophila* 2-15. However, the mean bacteria load remained lower compared to the initial situation (800±1524 CFU/L for *L.pneumophila* 2-15 and 220±178 CFU/L for non-pneumophila Legionella).

Restored and kept the HP concentration to 25 mg/L, in the following control (November 2014), all collected samples were negative for Legionella, except for the site on the fourth floor in Obstetrics and Gynecology.

In the following months, five different sampling were performed; in February 2015, in May 2015, in October 2015, in February 2016 and in June 2016. The controls showed the absence of Legionella contamination in all sites, with the exclusion of two instances, in May 2015 (600 CFU/L of L.p. 2-15) and June 2016 (400 and 5800 CFU/L of species non-*pneumophila*.) when Legionella was detected again in the washbasin on the fourth floor (Fig. 2, Fig. 3). The higher levels of contamination in this point may be due to its location, the most distal in the water network from the dispensing HP device, and to its infrequent use and consequently water stagnation in the terminal portion.

Statistical analysis demonstrates as abatement in Legionella loads after HP treatment is significant. Application of Nemenyi test evidenced significant p-values in comparison to the initial situation starting from sampling of November 2014 (p-value < 0.05). [Table 1]

The application of the new disinfectant formulation based on only HP at 25 mg/L and the addiction of filming product demonstrated a positive effect on corrosion reduction ~~of~~; the detection of iron concentrations dissolved in water, used as a proxy of pipelines corrosion, evidenced a reduction of its levels (data not shown). Actually, even the interventions for water system maintenance decreased.

DISCUSSION

This is one of the few studies performed as yet in hospital settings, although previous experiences reported in literature used HP either with a formulation with silver salts or with acetic and peracetic acid, either in different concentration.

In An Israeli study, performed on a 50-bed ward of a great hospital, a formulation of HP and silver salts, during the 24 months of application, demonstrated a significant reduction of Legionella contamination, from mean count values of 200-14.000CFU/L to the total absence [32].

Other two experimentations, performed in Italian long-term-care facilities, didn't yield a total abatement of the contamination, although a reduction in Legionella mean count of about 2 log was attained [33,34].

Remarkable results are obtained in a recent study by Modena-Reggio Emilia University (Italy), with a treatment based on an association of HP with acetic and peracetic acid, performed on a highly-contaminated building. Initially, Legionella counts were rapidly reduced, remaining stably at lower levels in the first seven months, until complete disappearing of bacteria in more than 90% of samples during the subsequent eighth month. During the treatment, another interesting evidence was the *L. pneumophila* (serogroups 1, 6, 9) progressive substitution, with environmental species of Legionella, such as *L. jamestowniensis* [35].

The results of this 36-month field study performed at the Villafranca general hospital demonstrated a good efficacy of HP treatment in controlling Legionella. In particular, the new dosage of HP 25 mg/L with the addition of polyphosphates emerges as a valid alternative to the more frequent association with silver salts.

Overall, our data suggest that the effectiveness of HP is evident in long period. In the early stages of treatment some Legionella species, other than *L. pneumophila*, probably resist to disinfectant and subsequently replace *L. pneumophila*; these species are less pathogenic and have almost never shown to be associated to human disease. *Legionella longbeachae*, although infection is unusual, was identified as responsible of 43 cases between 2005 and 2012 by European Surveillance System (Tessy) and for a cluster of six cases in amateur gardener in 2013 in Scotland [36]. These findings demonstrate the importance of an accurate environmental surveillance to monitor the presence of all Legionella species including non-pneumophila ones.

This characteristic trend, with a first stage of *L. pneumophila* reduction followed by its substitution by less pathogenic Legionella, and a long-term efficacy in reduction of all Legionella species, was highlighted by Marchesi *et al.* [35], applying HP disinfection associated with peracetic acid.

Such as other water disinfection methods, it was demonstrated that keeping an appropriate and uninterrupted concentration of HP is very important, since new increase of Legionella loads were observed during the study period due to a lower dosage of disinfectant. Through our data, we can assert that 25 mg/L HP levels ensure a good control of Legionella colonization and its almost

complete disappearing in the long period, qualifying this method as a reliable new option to water treatment.

This study is the first that evaluated the application of HP as the only disinfectant in the Legionella colonization control, demonstrating good performances of HP-based disinfection. This product could represent a valid alternative to chlorine-based disinfectants, with at least comparable results in efficacy and interesting advantages in costs reduction as direct costs for installation of dosing device and disinfectant and indirect costs with reduction of pipelines corrosion and maintenance fees.

MATERIALS AND METHODS

Setting

The Villamarina general hospital of Piombino (Leghorn, Italy), is a 136-beds hospital (120 ordinary and 16 DH) with a catchment area of about 60,000 inhabitants. Built in early '90s as an extension of a previous structure, it has been active since 1992.

The hospital architectural structure is a monoblock with a central plate on 3 levels (-2, -1, ground floor) and three vertical towers, one in five floors and the other two in four floors.

The reference specialties are: Cardiology, General Medicine, Oncology, General Surgery, Urology, Orthopedics, Ophthalmology, Obstetrics and Gynecology, Pediatrics.

In July 2013, within the water safety plan (WSP) implementation program, the hot water system disinfection, and a systematic monitoring program with sampling of final points of use of water network began.

Water disinfection

Considering the water network characteristics, with old and galvanized steel-made pipelines, the need to minimize the risk of corrosion and related maintenance costs led to discard chlorine dioxide disinfection and select HP as disinfection product.

The first performed action was a shock disinfection with a solution of HP and silver ions for 12 h. Subsequently, a continuous disinfection system, based on a formulation of 10 mg/L HP and 10 mg/L silver ions, was applied.

In September 2013, the HP-silver ions disinfection was replaced by the use of HP alone at 25 mg/L. Furthermore, polyphosphates were added to disinfectant as film-forming product to reduce pipeline corrosion. This formulation was employed from September 2013 to June 2016, with the only exception of July 2014, when HP concentration went down to 10 mg/L, probably due to a dosing device malfunction.

Sample collection and Detection of *Legionella* spp.

Between July 2013 and June 2016, 59 hot water samples were collected. Four different target points, one for each floor, were selected considering their representativeness of the hospital water network: a emergency room bathroom (basement), an intensive care room (ground floor), an Oncology Day Hospital room (first floor) and the hand basin of the Obstetrics and Ginecology delivery room (fourth floor). Since July 2014, a fifth sampling point was added at the circulation circuit of the hot water network.

Water samples were assayed for Legionella and tested for the following physical-chemical parameters: temperature (°C), pH (pH units), conductivity (µS/cm), turbidity (NTU), iron ions (µg/L Fe).

At the same time, the disinfectant concentration in water was regularly measured at the point of use to have an accurate control of HP levels into the water supply. The HP concentration was determined by the colorimetric test Merckoquant® test strips.

During the first sampling, to detect basal levels of Legionella colonization, two water samples were collected for each sampling point, one instantly and one after 5 minutes of flushing. In following

sampling, only flushed samples, more representative of overall water network contamination levels, were collected.

The isolation of *Legionella* spp. in hot water samples was performed in accordance with standards procedure [37-38]. One liter of water was filtrated through a membrane having a porosity of 0.2 m diameter (Millipore, Billerica, MA). After filtration, the membrane was immersed in 10 ml of the same water and subjected to a treatment of sonication for 5 minutes, allowing the detachment of cells from the membrane and their suspension in water. Suspension was subjected to a thermal inactivation treatment at 50°C for 30 minutes with the aim to select *Legionella* spp., inactivating all microbial species not resistant to high temperature. Afterwards 0.1 ml of the suspension was seeded on BCYE agar plates, incubated at 37°C for 7-10 days within jars in which a modified atmosphere (2.5% CO_2) Finally, Legionella colonies grown on BCYE were subjected to species and serogroup identification analysis using a multi-purpose latex agglutination test (Legionella Latex Test, Oxoid Ltd, Basingstoke, Hampshire, UK). Identification of Legionella species was carried out by sequencing of the *mip* gene (558 bp) [39].

DNA template was prepared by resuspending the colony in 500 µL of sterile water and incubated at 99°C for 10 minutes. For each Polymerase Chain Reaction (PCR) 50 µL of mix were prepared with 31,25 µL of water; 5 µL of 10X PCR Buffer (15 mM MgCl2), 1 µL of dNTPs mix (10 µM); 1,25 µL of mip595R 5'-CATATGCAAGACCTGAGGGAAC (20 mM); 1,25 µL of mip58F 5'-GCTGCAACCGATGCCAC (20 mM); 0,25 µL of HotStarTaq DNA Polymerase (5U/µL); and 10 µL of extract (HotStarTaq DNA Polymerase, Qiagen, United States). PCR reaction steps were as follows: initial denaturation at 95°C for 15 minutes; denaturation at 94°C for 1 minute; annealing at 55°C for 1 minute; extention at 72°C for 1 minute; termination at 72°C for 10 minutes. Denaturation to extention steps were repeated 35 times. 10 µL of the amplified PCR mixture was loaded to a 1% agarose gel with ethidium bromide. A 1,5 Kb ladder was used to compare amplified PCR product. After electrophoretic run, applied at 110 V for 30 minutes, *mip* gene amplification results were visualized in UV transilluminator.

Amplified *mip* gene was sequenced in outsourcing (GATC, Biotech, Germany) and sequence alignment was performed by BioEdit Version 7.0.0. Sequences identification was obtained by Basic Local Alignment Search Tool (BLAST) Database.

Statistical Analysis

Data are shown as means, standard deviations and medians. Normality of distribution was assessed using the Kolmogorov–Smirnov test, the variable was not gaussian. Nemenyi test for paired data was performed to compare the values of different times. Post-hoc power tests were conducted to estimate the sample size, 1-beta values of significant data were > 0.8, assuring an appropriate sample size. The statistical analysis was carried out using the IBM SPSS software package, version 17.0.1.

Table 1: Statistical comparison between Legionella loads detected in every date of sampling (p-value).

p-values	T0 3 Jul 2013	T1 29 Jul 2013	T2 25 Oct 2013	T3 29 Jan 2014	T4 7 Jul 2014	T5 11 Nov 2014	T6 2 Feb 2015	T7 25 May 2015	T8 26 Oct 2015	T9 15 Feb 2016	T10 27 Jun 2016
T0 - 3 Jul 2013	1	0,670	0,790	0,136	0,241	**0,003***	**0,003***	**0,012***	**0,003***	**0,003***	**0,033***
T1 - 29 Jul 2013	0,670	1	0,873	0,286	0,456	**0,011***	**0,011***	**0,038***	**0,011***	**0,011***	0,088
T2 - 25 Oct 2013	0,790	0,873	1	0,220	0,365	**0,007***	**0,007***	**0,025***	**0,007***	**0,007***	0,062
T3 - 29 Jan 2014	0,136	0,286	0,220	1	0,749	0,136	0,136	0,311	0,136	0,136	0,522
T4 - 7 Jul 2014	0,241	0,456	0,365	0,749	1	0,070	0,070	0,183	0,070	0,070	0,337
T5 - 11 Nov 2014	**0,003**	**0,011**	**0,007**	0,136	0,070	1	1,000	0,631	1,000	1,000	0,394
T6 - 2 Feb 2015	**0,003**	**0,011**	**0,007**	0,136	0,070	1,000	1	0,631	1,000	1,000	0,394
T7 - 25 May 2015	**0,012**	**0,038**	**0,025**	0,311	0,183	0,631	0,631	1	0,631	0,631	0,709
T8 - 26 Oct 2015	**0,003**	**0,011**	**0,007**	0,136	0,070	1,000	1,000	0,631	1	1,000	0,394
T9 - 15 Feb 2016	**0,003**	**0,011**	**0,007**	0,136	0,070	1,000	1,000	0,631	1,000	1	0,394
T10 - 27 Jun 2016	**0,033**	0,088	0,062	0,522	0,337	0,394	0,394	0,709	0,394	0,394	0

(*) Statistical significance

Figure 1: *Legionella pneumophila* 2-15 loads (CFU/L) detected in Villamarina general hospital before the start of disinfection program with hydrogen peroxide (sampling of 07th July 2013); for comparison are reported bacterial load in instantaneous samples and in fluxed ones.

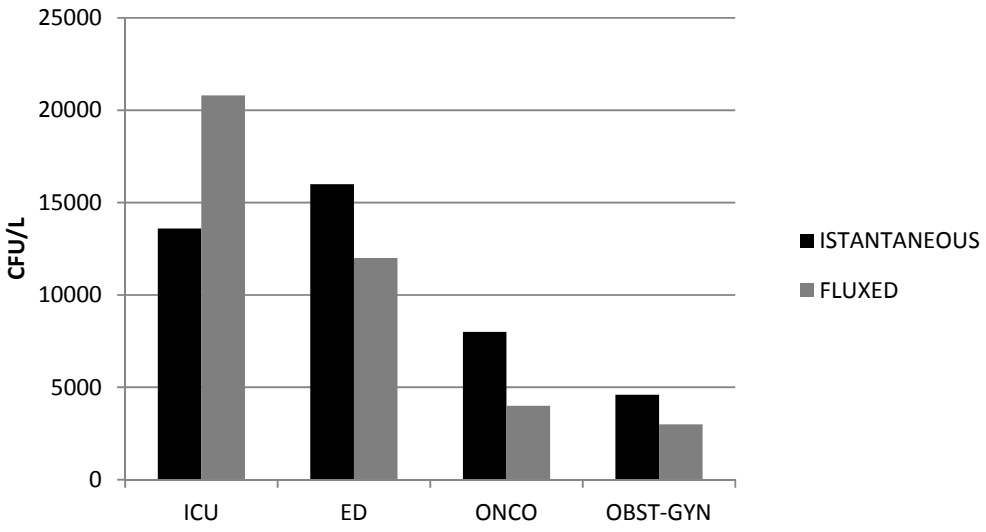

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Figure 2: Different species of Legionella loads (*L. pneumophila* 1, *L. pneumophila* 2-15, *Legionella* spp) (CFU/L) detected in the four different sampling sites in the hospital (Intensive Care Unit, Emergency Department, Oncology, Obstetrics and Gynecology) during the period from July 2013 to June 2016.

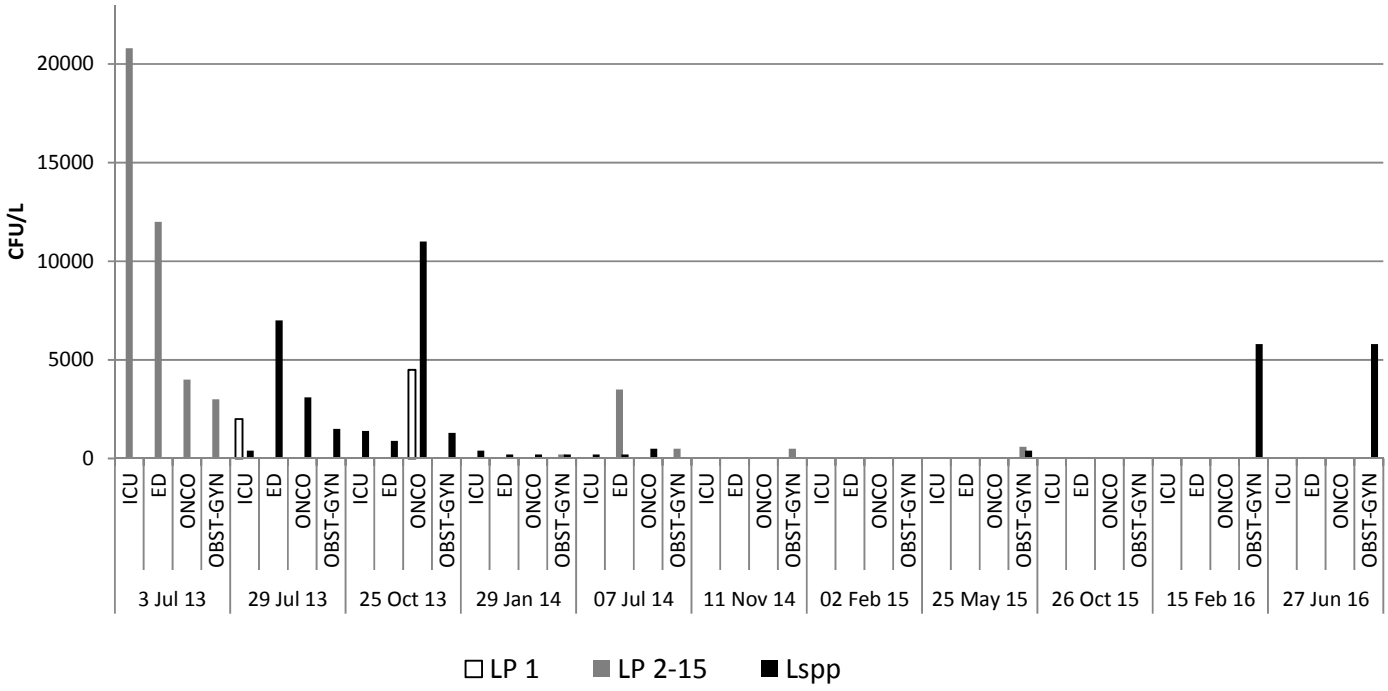
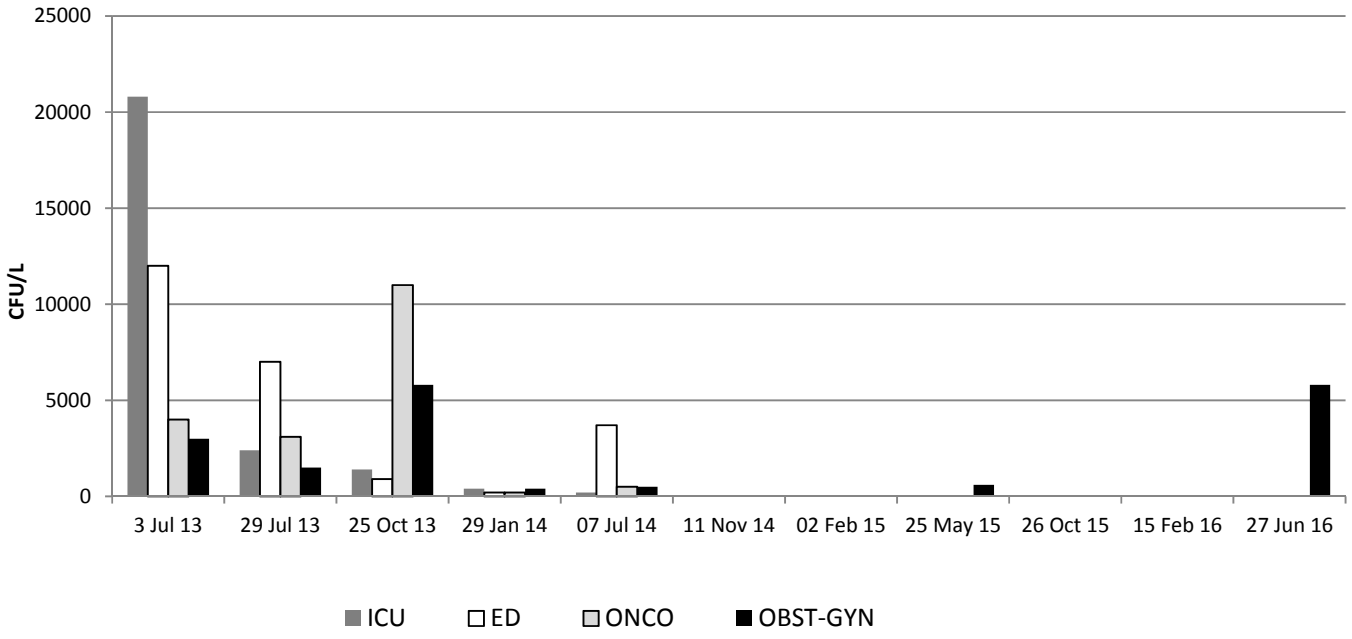


Figure 3: Legionella loads (CFU/L) detected in each of the four sampling sites in the hospital (Intensive Care, Emergency Department, Oncology, Obstetrics and Gynecology) during the period from July 2013 to June 2016.



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