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MONITORING *LEGIONELLA* SPECIES IN HOSPITAL WATER SYSTEMS. LINK WITH DISEASE AND EVALUATION OF DIFFERENT DETECTION METHODS

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Abstract: The aim of this work was to evaluate three currently available isolation methods for *Legionella* using water samples and swabs of a single pediatric hospital water system. Additionally, high risk patients were screened for the presence of *Legionella pneumophila* antigen in urine. Fifteen water samples and 11 swab samples were collected from distal sites at 18 sampling locations. The International Standard Method (PN-ISO11731-2) based on membrane filtration and direct culture of bacteria on selective media were compared with amoebic co-culture. The numbers of legionellae detected exceeded 10^2 cfu/100 ml in 50% of the samples. All the positive samples contained *L. pneumophila* SGs 2–14. Urine samples were obtained from 57 immunosuppressed children and screened for the presence of *L. pneumophila* serogroup (SG) 1 antigen by *Legionella* urinary antigen EIA. Of the 57 urine samples tested for the presence of *Legionella pneumophila* SG 1 antigen, none were positive. Our results highlight the value of combined membrane filtration and amoebic co-culture methods in detecting viable *L. pneumophila* strains. Direct plating of 0.2 ml water is a useful screening method for samples containing large bacterial amount.

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INTRODUCTION

The source of hospital-acquired Legionnaires' disease is the hospital's potable water distribution system. Environmental culturing of water systems for *Legionella* spp. and preventing Legionnaires' disease has become a focus for hospitals because they represent ideal locations for Legionnaires' disease transmission: at-risk individuals are present in large numbers; plumbing systems are frequently old and complex, favouring amplification of the organism, and water temperatures are often reduced to prevent scalding of patients [8, 16, 21, 24].

Although the magnitude of the problem is difficult to measure, reports of outbreaks continue to abound. To reduce the likelihood of Legionnaires' disease transmission in health care facilities, CDC recommends a strategy

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focusing on proper maintenance of water systems, universal testing of patients with nosocomial pneumonia with appropriate tests, and investigation of situations in which transmission has been shown to occur [7].

Legionella detection and identification methods fall into two categories: those methods that are aimed at the clinical diagnosis of the disease, and those aimed at monitoring and identifying risk in water systems [8]. With this in mind, the present work deals with the evaluation of three currently available isolation methods using water samples: i) quantitative methods based on direct membrane filtration of 500 ml water samples, and direct plating of 0.2 ml water for samples containing large bacterial amount; ii) swabs of system surfaces that allow sampling of biofilms; iii) amoebic co-culture for detection of viable but non-culturable [VBNC] *Legionella*. Additionally, high risk patients hospitalized for longer than two weeks were screened for the presence of *Legionella pneumophila* antigen in urine.

MATERIALS AND METHODS

Sampling. All samples were collected in the University Children's Hospital in Lublin. Eighteen sampling locations were selected throughout the distribution system, with a focus on patient care areas (e.g., hospital wards of haematology, Intensive Care Room, Pulmonology, balneotherapy room, operating theatre, inhalation room, as well as toilets and bathroom). Hot or cold water samples were collected from distal sites at each sampling location. The sampling sites involved faucets, air-conditioners, humidifiers, shower heads. From selected "critical" points, a total of 15 water and 11 swab samples were collected for *Legionella* spp.

The sampling protocol included the recommended method from the National Institute of Health, Poland [15]. Specimens were collected as follows: two samples were collected from individual site: one water sample (500 ml) and a swab sample. Water samples were mostly taken from the hot tap outlets. In two cases, cold water samples were collected (humidifier and sterilizer, both in the Intensive Care Room). Swab samples for the analysis of *Legionella* were used to collect potential biofilm. Swab sampling was performed by swabbing the suspected area or material and replacing the swab back into the vial. Swab samples were collected prior to any initial water flow in order to capture potential undisturbed biofilm organism.

The International Standard Method (ISO), accepted in Poland as a standard (PN-ISO11731-2) [12] based on filtration procedure and culture of bacteria on selective media was compared with amoebic co-culture procedure.

Direct membrane filtration method. The method involved sample concentration by filtration of 500 ml water through 0.45 μm cellulose membrane filters. After concentration, the concentrates of the samples were treated with acid (pH 2.2) to reduce the number of non-legionella bacteria before culture. Acid treatment was carried out by 5 min exposure to acid buffer. The buffer was then removed from the filter by washing it with Page's salt. The filter was next placed on the selective GVPC agar plate (Oxoid, Basingstoke, Hampshire, UK). The inoculated culture media were incubated at 36 ± 2°C in a humid atmosphere and read at 4-10 days. Suspected *Legionella* colonies were subcultured onto BCYE (buffered charcoal yeast extract) agar for verification. The species and/or serogroups were determined by a commercially available latex agglutination test kit (Oxoid, DR 800M). Reagents supplied in the kit allow confirmation of organisms as either *L. pneumophila* (serogroup) SG 1, *L. pneumophila* SGs 2–14, or *Legionella* species (including *L. longbeachae* SGs 1–2, *L. bozemanii* SGs 1–2, *L. dumoffii*, *L. gormanii*, *L. jordanis*, *L. micdadei* and *L. anisa*).

Direct plating. From each of the 500 ml water samples, aliquots of 0.2 ml were inoculated without concentration and without acid buffer treatment directly onto GVPC agar. The inoculum was spread with a sterile glass rod and incubated as described above.

All swabs taken were streaked directly onto GVPC agar and incubated as described above.

Amoebic co-culture. Amoebic co-culture was performed as follows. *Acanthamoeba castellanii*, strain ATCC 3034 was originally obtained from Dr W. Balamuth (Department of Zoology, University of California, USA). Amoebae free of intracellular endocytobionts were grown axenically in 300 ml Erlenmayer flasks with 100 ml of proteose peptone-yeast extract-glucose (PYG) medium, pH 6.6. The flasks were inoculated with 3-day-old amoeba culture to give an initial population of approximately 5×10^3 organisms/ml. The culture was incubated on a rotary shaker with an acentric rotation of 3 cm (120 rev/min) at 28°C. The number of cells was determined using a Büchner haemocytometer. Amoebae from the exponential phase (60–72 h) were harvested by centrifugation at 300 × g for 10 min, and washed in amoeba saline prepared after Band [1]. Aliquots of 10 ml of each tested water sample were transferred into 1 ml culture of *A. castellanii* for the final concentration of 5×10^5/ml. The samples were incubated statically for 4 days at 28°C. Every 24 h, the samples were screened for the presence of *Legionella* inside the amoebae and for bacteria released from them into the culture medium, under phase contrast microscope. After the first period of incubation, 1 ml of fresh amoebic culture from the logarithmic phase of growth (3×10^6 amoebae/ml of saline) was mixed with 100 μl of the first culture, and incubated for 4 days at 28°C, statically. The presence of amoebic intracellular pathogens as well as the presence of bacteria in the culture medium was determined under phase contrast microscope (400 ×). The appearance of bacteria in the culture medium and disappearance of amoebae indicated the presence of intracellular amoebic pathogens in the tested water sample [1].

Patients and samples. Urine samples were obtained from 57 immunosuppressed children hospitalized in University Children's Hospital for longer than two weeks at the time of the study. The patients were selected from hospital wards: Intensive Care, Pulmonology, Allergology, Laryngology and Rehabilitation. The mean age of the patients was 6.4 years (age range, 0–17 years). Patients were screened for the presence of *L. pneumophila* serogroup (SG) 1 antigen by *Legionella* urinary antigen EIA (DRG MedTek). The test was performed according to the manufacturer's instructions.

RESULTS

Fifteen water samples and eleven swab samples were evaluated for the presence of *Legionella* spp. in a comparison of the filtration method, direct plating and amoebic co-culture. The standard filtration method was compared to amoebic co-culture and direct plating of the 0.2 ml water sample (Tab. 1).

Amoebic co-culture with *Acanthamoeba castellanii* was applied to isolate the viable but non-cultivable legionellae. The sensitivity of the method with regard to its relative ability to detect legionellae from the samples indicated that amoebic co-culture was able to detect *Legionella* spp. in 12 out of 15 water samples (80%) (Tab. 1). However, the method gave only qualitative results. All but one water sample tested for the presence of *Legionella* spp. by the filtration method and amoebic co-culture gave comparable results. The legionellae growing in amoebae were not confirmed by latex agglutination.

Comparing the filtration method with the direct plating of 0.2 ml water samples, we observed concordance in 11 out of 15 cases (73.3%) (Tab. 1). In sample Nos. 1, 2, 4, 6 and 11 the quantity of *Legionella* spp. exceeded 10^3/100 ml in both methods. In sample Nos. 10, 13, 14 and 16, both techniques showed single colonies or $<10^2$/100 ml. Sample Nos. 7 and 18 were free of *Legionella*, confirmed by filtration and direct plating methods. Discrepancies between these two methods were observed in 4 cases (26.6%). In

Table 1. Comparison of *Legionella* prevalence by different detection methods.

Sample No. (Total N=18)	Sampling location	Detection method			
		Filtration	Direct plating		Amoebic co-culture
			0.2 ml water (cfu/100 ml)	Swabs	
		N=15	N=15	N=11	N=15
1	kiosk	AG	1×10^3	N. t.	positive
2	toilet	AG	1.5×10^3	N. t.	positive
3	shower head (Haematology ward)	AG	$<1 \times 10^2$	negative	positive
4	faucet (Haematology ward)	AG	1.5×10^3	negative	positive
5	ventilator (Haematology ward)	N. t.	N. t.	negative	N. t.
6	faucet (Intensive Care, Neonates)	AG	3×10^3	SC	positive
7	sterilizer* (Intensive Care, Neonates)	negative	negative	N. t.	negative
8	ventilator (Intensive Care, Neonates)	N. t.	N. t.	SC	N. t.
9	faucet (Intensive Care, Older Children)	AG	$<1 \times 10^2$	negative	positive
10	humidifier*	SC	$<1 \times 10^2$	negative	positive
11	faucet (Pulmonology, Operating theatre)	AG	1×10^3	N. t.	positive
12	faucet (Pulmonology, Inhalation ward)	AG	$<1 \times 10^2$	N. t.	positive
13	faucet (Pulmonology, bathroom)	SC	$<1 \times 10^2$	N. t.	negative
14	shower head (Pulmonology, bathroom)	SC	$<1 \times 10^2$	N. t.	positive
15	faucet (Transplantation ward, bathroom)	SC	3.5×10^3	negative	positive
16	shower head (Transplantation ward, bathroom)	SC	$<1 \times 10^2$	negative	positive
17	ventilator (Transplantation ward)	N. t.	N. t.	negative	N. t.
18	faucet (Transplantation ward)	negative	negative	negative	negative
No. of samples positive for *Legionella* (%)		8** (53.3%)	6*** (40%)	0** (0%)	12 (80%)

AG – Abundant Growth, SC – Single Colonies, N. t. – not tested, * – cold water sample, ** – SC result was assumed as negative, *** – $<1 \times 10^2$/100 ml result was assumed as negative.

the standard filtration method, three water samples (Nos. 3, 9, 12) represented abundant growth of *Legionella*, whereas after direct plating only single colonies were growing on agar medium. One water sample (No. 15) was detected as containing high *Legionella* amount (>10^3/100 ml) in direct plating. The same water sample, after filtration, showed only single colonies on agar (Tab. 1).

The direct plating of the swabs did not recover *Legionella* spp. from the sites proved positive by the methods used for water samples.

The numbers of legionellae detected exceeded 10^2 colony forming units per 100 milliliters (cfu/100 ml) in 50% of the samples. All of the positive samples contained *L. pneumophila* SGs 2–14, as detected by latex agglutination method.

Of the 57 urine samples tested for the presence of *Legionella pneumophila* SG 1 antigen, none were positive.

DISCUSSION

Culturing is generally accepted as the 'golden standard' for *Legionella* detection in the environment, but the lack of standardization of culturing methods, especially for environmental legionellae, complicates the interpretation of results [2, 21]. As a standard, Polish laboratories currently use The International Standard Method (ISO; PN-ISO11731-2), based on filtration procedure and culture of bacteria on selective media. This is a monitoring method for the isolation and enumeration of *Legionella* organisms in water intended for human use and consumption and for treated bathing waters (e.g., swimming pools). It is especially suitable for waters expected to contain low numbers of *Legionella*, as the growth of *Legionella* may be inhibited by overgrowth of other bacterial colonies on the membrane [12]. The major limitation of the method is that it can only provide information on viable, culturable *Legionella*. It also requires precautions to maintain the viability during sampling and handling as well as shipping, and takes 7–10 days to obtain confirmed results. Reduced recoveries because of antibiotics and sample treatment or inability to grow on solid media additionally strongly limit the use of these method for the detection of non-*L. pneumophila* species [4].

This project was therefore undertaken to evaluate different detection methods for monitoring *Legionella* spp. in a single hospital water system.

In our study, the standard method of membrane filtration and amoebic co-culture appeared sensitive techniques with regard to collected water samples.

Amoebae play a key role in the persistence of legionellae in the environment [23]. Under some circumstances, legionellae are able to enter a viable but non-cultivable state, remaining still virulent and able to cause human infection [2, 3, 6, 19]. The use of co-culture of water samples with amoebae has led to the isolation of *L. pneumophila* in some instances where inoculated agar plates showed only single colonies of *Legionella*. This confirmed previous findings by Sanden *et al.* who reported that incubation of environmental samples with autochthonous amoebae considerably improved the sensitivity of culture methods for legionellae [20]. The major limitation of the method, however, is the fact that it gives only qualitative results.

There is a need for a fast, reliable and quantitative method that allows preliminary screening of the water sample for legionellae. The information would be useful for selecting the appropriate culture method. In our study, we inoculated the aliquots of 0.2 ml water samples directly onto GVPC agar plates. In most cases, those samples that were highly contaminated with *Legionella* spp. (as confirmed in ISO method) were also positive in direct plating. This method seems to meet the expectation of Polish conditions. According to the reports of the Polish National Institute of Health, and on the basis of other authors' findings, about 70% of hot water systems in Poland are contaminated with legionellae at the level exceeding acceptable norms [22].

On the basis of our findings, the swab method of sample collection was not critical for determining the level of *Legionella* colonization in hospital water system. Even though swabs allow sampling of biofilms, which frequently contain legionellae [6, 23], we were able to detect only single colonies of *Legionella* in two cases. The only explanation is that the swabs were streaked directly onto GVPC agar without any pretreatment, or that the legionellae in biofilm represented VBNC state.

Most of the methods that we evaluated for the monitoring of *Legionella* spp. in a hospital potable water system were comparable in sensitivity, i.e. in their abilities to detect *Legionella* spp. However, there were some discrepancies in the quantities of *Legionella* detected. This is particularly important, given that outbreaks of Legionnaires' disease have been linked to exposure to *Legionella*, and that criteria for remedial action and disinfection have been suggested on the basis of the levels of *Legionella* spp. recovered from water samples [21]. According to the Ordinance of the Polish Ministry of Health (29 March 2007), the remedial action must be taken if *Legionella* spp. are isolated from potable water samples in quantities of >10^3 cfu/100 ml [19]. Although we have not performed the filtration method with subsequent volumes of water sample, the use of 10, 100 and 500 ml samples is recommended for accurate determination of *Legionella* number [13].

In hospital wards where immunosuppressed patients are treated, hot water systems should be totally free of *Legionella* contamination [4, 10, 18]. The results from this study indicate a high prevalence of legionellae in hospital potable water systems. Serological typing of environmental strains revealed that *L. pneumophila* serogroups 2–14 were responsible for extensive contamination of the hospital water supply system. The *Legionella* concentration at the different sites examined ranged from <10^2 to >10^3 cfu per 100 milliliters, which is an amount considered sufficient to cause one or more sporadic cases per year [24].

Even though pneumonia is common in the general pediatric population, Legionnaires' disease in otherwise healthy infants and children is extremely rare, representing just 1% of the total legionellosis cases reported [11, 17]. The available literature contains several reports of nosocomial legionellosis in children whose medical condition or treatment placed them at increased risk [5, 9, 17]. Detection and quantification of *Legionella* spp. in the hospital water distribution system is one of the cornerstones of risk assessment [17, 18].

In our setting, fifty-seven urine samples were tested to screen the role of *Legionella pneumophila* in paediatrics. The frequency of confirmed disease was 0%. *Legionella pneumophila* was not a common etiologic agent of paediatric pneumonia, even though the potable water system of the hospital was highly contaminated with the bacteria. However, it must be emphasized that the ELISA test used was able to detect the presence of *L. pneumophila* SG 1 antigen only while the environmental study showed the prevalence of other than 1 serogroups.

In conclusion, our results highlight the value of combined membrane filtration and amoebic co-culture methods in detecting viable *L. pneumophila* strains. Direct plating of 0.2 ml water is a useful screening method for samples containing large bacterial amounts. Legionnaires' disease is a sporadic disease in infants and children, but the underdeveloped immune system of children and intensive medical treatments, together with increased contact with hospital environments, should bring *Legionella* to the attention of medical personnel.

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