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Full Length Research Paper

Isolation and serotyping of *Legionella pneumophila* from Goreangab Dam and hostel shower heads in Namibia

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This is the first report of *Legionella pneumophila* serotypes in Namibia. Using modified buffered charcoal extract agar selective medium, *L. pneumophila* was isolated from Goreangab Dam water and University of Namibia (UNAM) student hostel shower heads. Latex agglutination tests showed that *L. pneumophila* serotypes 1 - 15 were present in Goreangab Dam water and serotypes 2 - 15 were present in blocks A and B of the UNAM old hostel shower heads. Further studies are needed to help understand *L. pneumophila* ecology and risk of human infection in Namibia.

Key words: Namibia, *Legionella pneumophila*, isolation, serotypes, dam water, shower heads.

INTRODUCTION

Legionella pneumophila (Family Legionellaceae) is a Gram negative, broad host range, and facultative intracellular bacterium that causes a respiratory infection called legionellosis or legionnaires' disease (LD) (Purcell and Shuman, 1998). Legionellosis is the most severe form of infection, which includes pneumonia, and the fatality rate can be 50 - 80% in immunocompromised patients ((Murdoch et al., 1996; Yu, 2000). Immunosuppression, chronic lung disease, alcoholism, cigarette smoking, advanced age, and general anaesthesia are risk factors for LD (Murdoch et al., 1996; Over the past few years, the incidence of legionellosis has increased with numerous outbreaks being reported (Delgado-Viscogliosi et al., 2005). One of the worst outbreaks occurred from November, 2003 to January, 2004 in the industrial region of Lens, Northern France, where 86 cases of LD were found, resulting in 15 deaths (Miquel et al., 2004).

Legionella was first isolated and described into a new genus and species in 1977 following an epidemic of acute pneumonia among veterans of the American Legion in Philadelphia, United States of America (McDade et al., 1977; Brenner et al., 1979). By 2005, there were 47 *Legionella* species (Delgado-Viscogliosi et al., 2005) and about 64 serogroups (Cloud et al., 2000) documented in the literature. *L. pneumophila* accounts for about 90% of the cases of LD, and about 85% are due to serogroup 1 (Helbig at al., 2002; Yu et al., 2002). Other *Legionella* species are rarely pathogenic in humans, the most common being *L. longbeachae* (causes 4% cases of LD) and *Legionella bozemanii* (causes 2.4% cases of LD) except in Australia and New Zealand where *Legionella longbeachae* reportedly caused 30% of LD (Yu et al., 2002).

Legionella normally inhabit freshwater or wet soil but the main reservoirs are man-made aquatic environments, particularly warm water systems (Borella et al., 2005). In freshwater, legionellae survive as intracellular parasites of free-living protozoans, which are their natural hosts (Borella et al., 2005). In plumbing water systems, legionellae are found in biofilms that provide shelter, nutrients and support for their survival and multiplication outside host cells (Borella et al., 2005). These peculiar characteristics of growth are responsible for their frequent contamination of artificial water systems. They also reduce biocide efficacy, making it difficult to eradicate them from contaminated water systems.

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Legionella infection occurs mainly by inhalation of aerosols generated from water sources such as distribution systems and cooling towers (Steinert et al., 2002). Transmission to humans has reportedly occurred only through mechanical means, such as air-conditioning units, showerheads, and sprinklers (Piao et al., 2006). Outbreaks of LD have been traced to a wide variety of environmental water sources and human-made systems such as cooling towers, hot tubs, showerheads, whirlpools, heated water spas, and public fountains (Wery et al., 2008; Delgado-Viscogliosi et al., 2005). These outbreaks take place in homes, offices, hotels, hospitals, and cruise ships, among other locations (Delgado-Viscogliosi et al., 2005). Different distributions of *Legionella* species and serogroups have been observed depending on the water type (Borella et al., 2005). Various *Legionella* species were detected in groundwater and potable water, whereas *L. pneumophila* was found to be adapted to warm water systems, where it grows and multiplies most efficiently (Borella et al., 2005).

Thus, the relative distribution of different *Legionella* species and serogroups may have a substantial impact on public health as the majority of LD is caused by *L. pneumophila* serogroup 1 (Helbig et al., 2002). Surveying and monitoring of legionellae in the environment are needed to prevent and control legionellosis, and *Legionella* concentrations in environmental sites may be used as a predictive risk factor (Shelton et al., 1993). Although there have been no reported LD outbreaks in Namibia or around Goreangab dam, the risk of sporadic and outbreak infections still looms. This paper presents results of an inaugural effort to isolate and serotype *L. pneumophila* found in Goreangab Dam water and University of Namibia (UNAM) student hostel shower heads. Knowledge of *L. pneumophila* serotypes and their levels of contamination in various environmental sites will help raise public health awareness, preparedness, prevention, and management of any future outbreaks of LD in Namibia.

MATERIALS AND METHODS

Media

The basal medium for the isolation of *Legionella* species was a modification of the buffered charcoal yeast extract agar (BCYEA) selective medium as reported by Feeley et al. (1979). Yeast extract (10 g), agar (17 g), and activated charcoal (10 g) were made up in 1 L of sterile distilled water. A supplement of L-cysteine (0.40 g) and ferric chloride (0.25 g) dissolved in 10 ml of sterile distilled water was sterilized by filtering using 0.45 µm filter membrane (Schleicher and Schuell microscience GmbH, Germany) and later added to the sterile medium prepared above. The pH was adjusted to 6.9 by adding 1 M KOH solution at 45°C. Since *Legionella* spp. require L-cysteine to grow, a set of modified BCYEA plates without L-cysteine were also prepared.

Sample collection

Water samples were randomly collected from Goreangab Dam situated west of Windhoek city (Figure 1). Water samples (500 ml) were collected in sterile 500 ml Schott bottles (Schott, Germany) containing 0.5 ml of 1.8 (w/v) solution of sodium thiosulfate. In total 8 samples were collected; 4 at the edges of the Dam and the other 4 from distal sites in the middle of the dam. Sterile swabs were used to collect biofilms on the interior surfaces of the shower heads in the old hostels of the UNAM at Pioneers Park, Windhoek. All samples were from Block A and B of the old hostels; 4 samples were from the first floor while the other 4 samples were from the second floor. These swabs were re-suspended and mixed in 10 ml of water from the shower. Samples were collected on 4 occasions; on the 21st of every month between June and September, 2009.

Culturing of water suspension

Due to the presence of plankton, 150 ml of Goreangab Dam water was diluted 2-fold with sterile distilled water to avoid clogging on filter membranes during filtration. Diluted water samples (300 ml) were filtered through 0.45 µm pore size membrane filters (Schleicher and Schuell microscience GmbH, German). Intact membranes were plated on the modified BCYEA as previously reported by Feeley et al. (1979). The suspensions from shower heads were cultured on modified BCYEA by the spread plate technique with an inoculum of 1 ml. Plates were inoculated in duplicate on modified BCYEA with and without L-cysteine supplementation. Plates were incubated at 35°C for 7 days. Colony forming units per ml were recorded.

Legionella spp. identification

Grayish-white colonies that grew on modified BCYEA-L-cysteine supplemented medium were presumptively identified as *Legionella* spp. pure cultures to be used in further examinations were sub-cultured on modified BCYEA containing L-cysteine. A battery of tests was done to confirm the presence of *L. pneumophila*. Gram staining was performed, and all colonies that were not gram negative bacilli were discarded. Colonies that showed to be catalase positive and non-spore formers were tested for glucose fermentation (Thacker et al., 1988). The latter was performed using methyl red voges proskauer (MR-VP) broth (Scharau Chemie, SA, Barcelona, Spain) as explained in Lammert (2007). Positive glucose fermentation colonies were considered to be those of *Legionella* spp. The latex agglutination test was used to confirm the presence of *L. pneumophila* serogroups. The agglutination test was carried out using the agglutination serogroup 1 - 15 kit according to the manufacturer's instructions (Oxoid). Negative and positive controls were included. A positive reaction with *L. pneumophila* latex 1 was revealed by agglutination of latex particles in 1 min while a positive reaction with *L. pneumophila* latex 2 - 15 was revealed within 4 min.

RESULTS

L. pneumophila colonies were isolated from Goreangab Dam water and UNAM shower heads (Figure 2). Legionellae were Gram negative bacilli, catalase positive, non-spore formers, and glucose fermenters. The number of colony forming units per ml (95% CI of mean ± s.e.) of *L. pneumophila* are in given in Figure 3. The mean number of colony forming units for Goreangab dam, UNAM hostels A1, A2, B1, and B2 were 12.0 ± 1.85, 6.0 ± 1.43, 13.4 ± 2.99, 3.6 ± 0.75 and 3.0± 1.14, respectively. *L. pneumophila* serotypes 1-15 were found in Goreangab

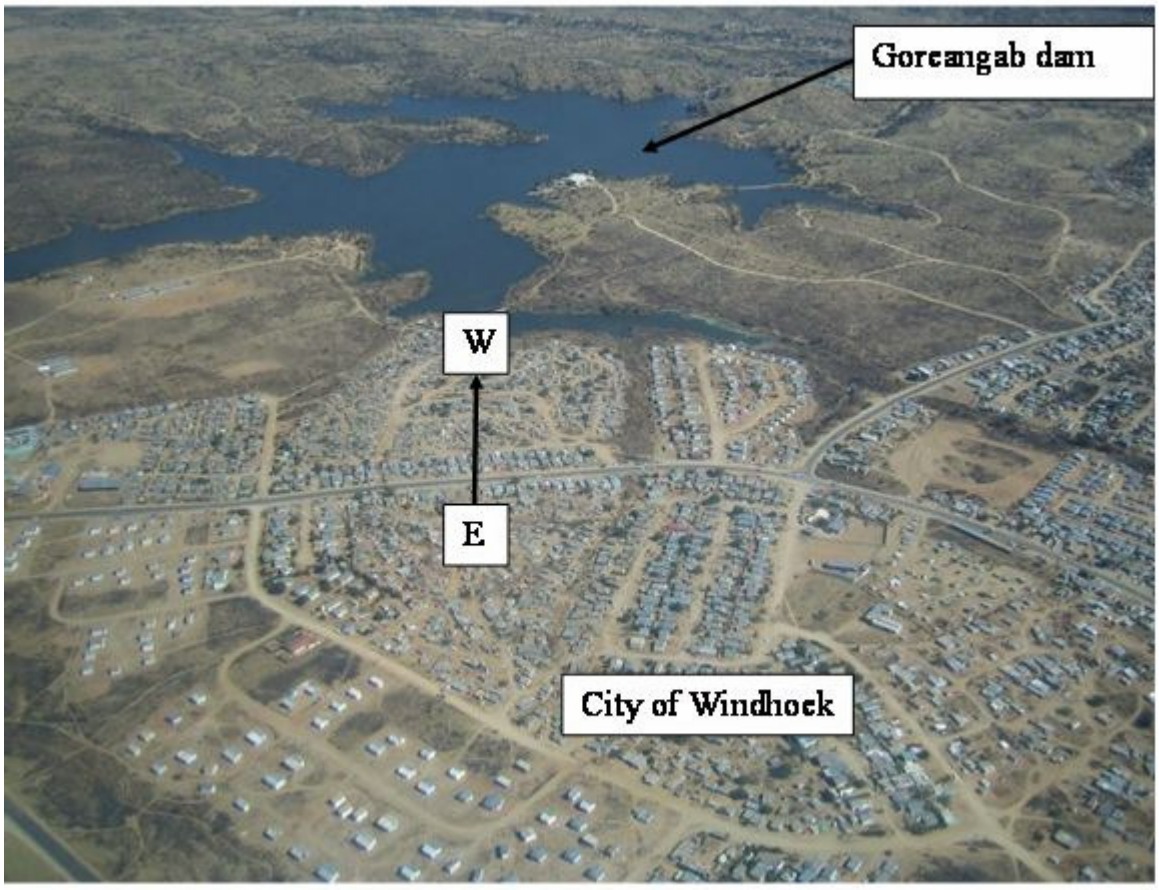

Figure 1. Aerial view of the Goreangab Dam water mass located west of Windhoek city (W, West; E, East).

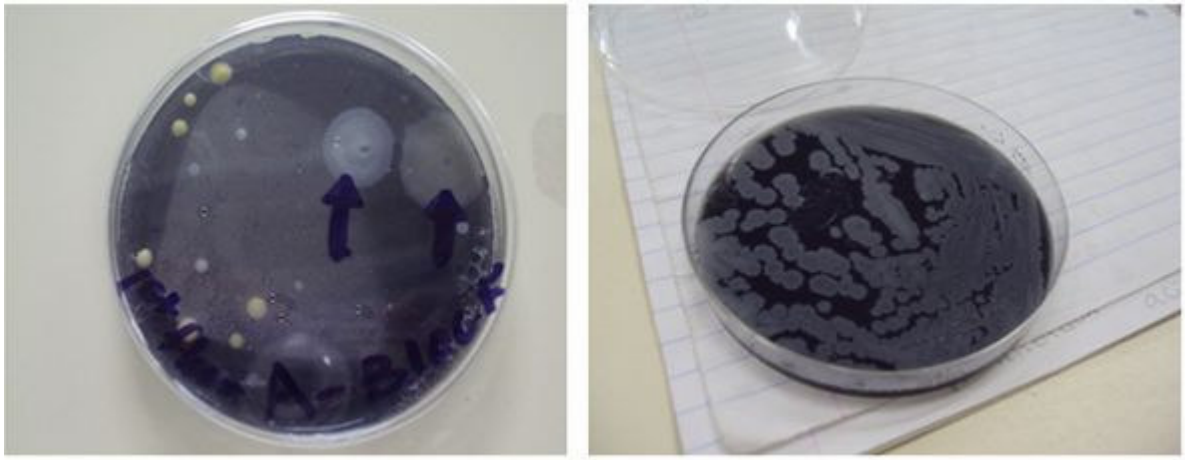

Figure 2. Left: grey-white colonies suspected to be *Legionella* spp. Right: a pure culture of *Legionella* spp. used in further tests to confirm identification of *L. pneumophila*.

Dam water but only serotypes 2 - 15 were present in UNAM shower heads. The prevalence of *L. pneumophila* was 75% (Goreangab Dam water samples), 87% (block A1 and A2 hostel shower heads), 100% (block B1 hostel shower heads), and 75% (block B2 hostel shower heads). Figure 4 shows positive and negative agglutination

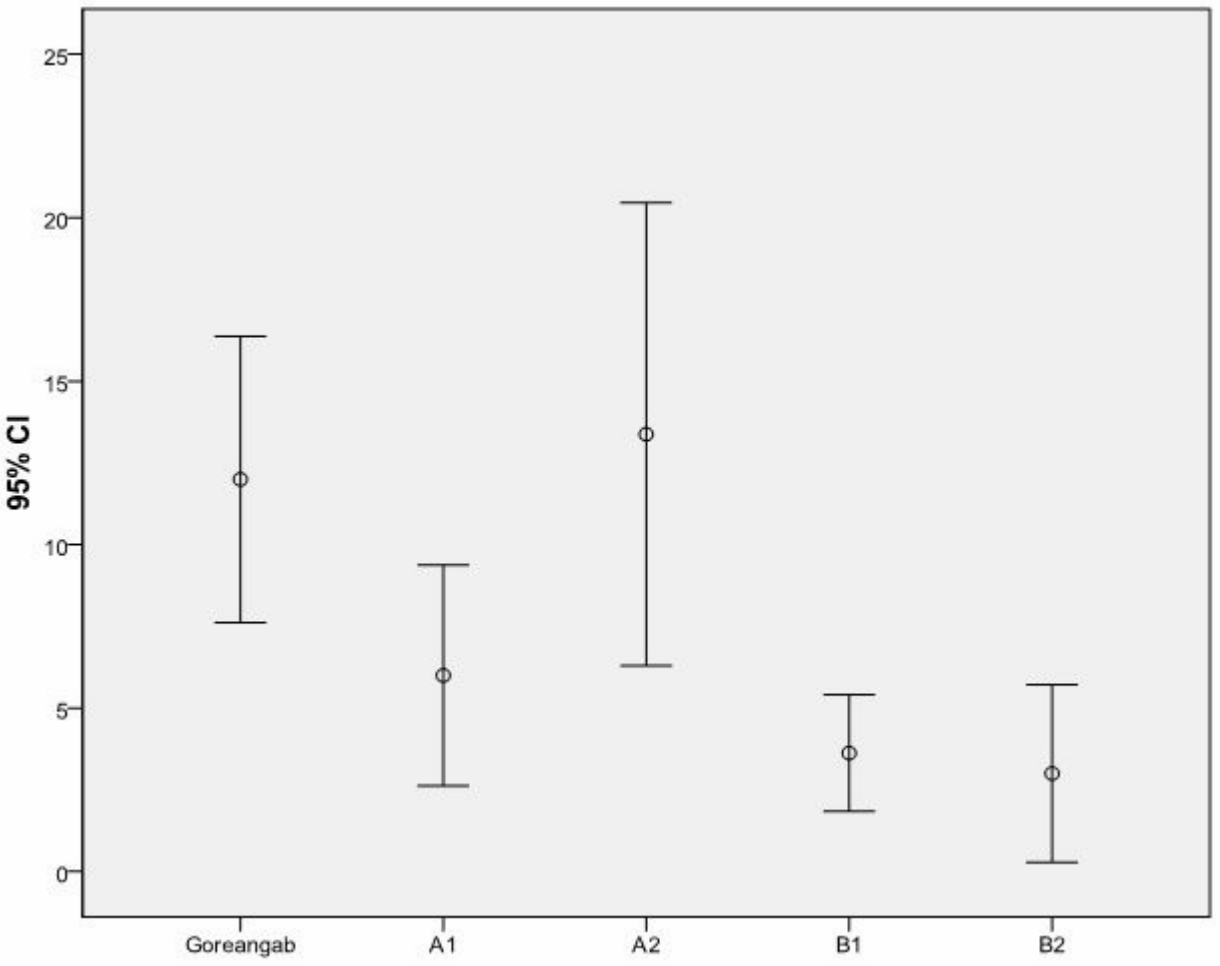

Figure 3. The number of colony forming units per ml (95% CI of mean ± S.E.) of *L. pneumophila* (mean of 16 samples). Sampled UNAM hostel blocks are indicated as A1, A2, B1, and B2.

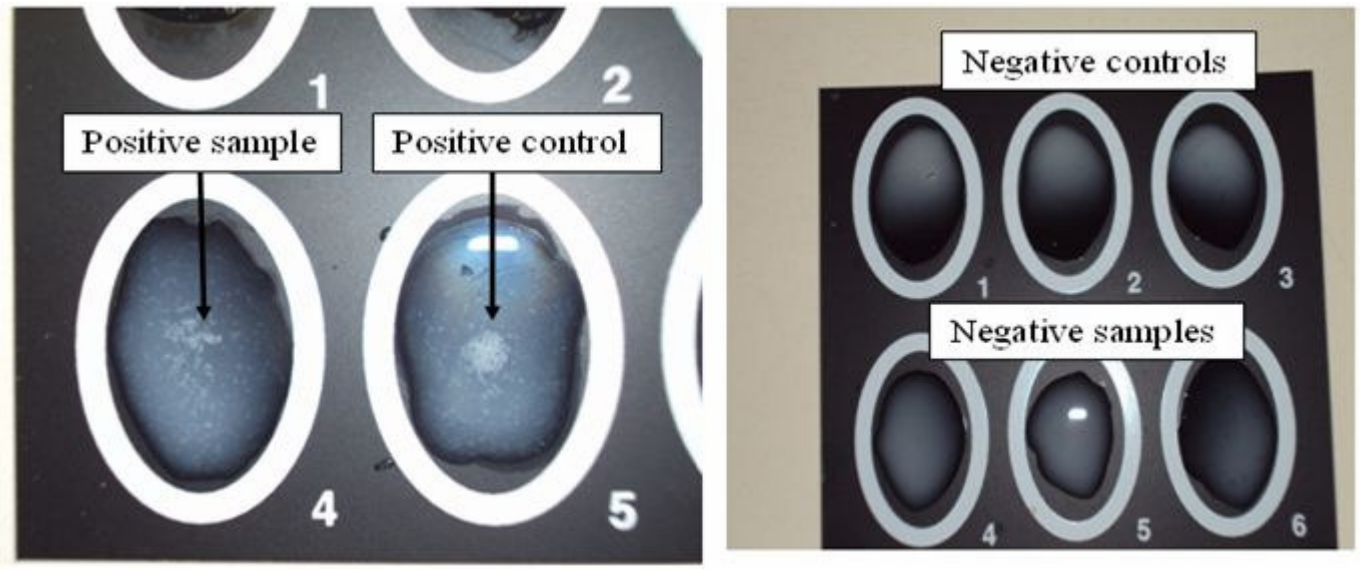

Figure 4. Agglutination test results: left (positive agglutination; 4 = positive sample from Goreangab Dam water; 5 = positive control), and right (negative agglutination; 1, 2, 3 = negative control; 4, 5, 6 = negative samples from hostel shower heads).

tests of *L. pneumophila* serotypes.

DISCUSSION

Legionella spp. have an absolute growth requirement for L-cysteine and without this amino acid they were unable to grow. Colonies with typical morphology on selective media were sub-cultured onto BCYEA without L-cysteine (Reinthaler et al., 1993). Those isolates that grew on BCYEA but failed to grow on this media were presumptively identified as *Legionella* spp. Pathogens that are catalase-positive, such as *Mycobacterium tuberculosis*, *L. pneumophila*, and *Campylobacter jejuni*, make catalase in order to deactivate the peroxide radicals, thus allowing them to survive unharmed within the host (Srinivasa et al., 2003). The life cycle of *L. pneumophila* has been studied and no spores were reported (Suzina et al., 2004; Faulkner and Garduno, 2002). Harada et al. (2010) have studied glucose metabolism in *L. pneumophila* using a combination of the Entner-Doudoroff (ED) pathway, genetic, and biochemical approaches. The bacterium utilized exogenous glucose for synthesis of acid-insoluble cell components, but manifested no discernible increase in growth rate.

To our knowledge, this is the first empirical report of *L. pneumophila* serotypes in Goreangab Dam and a UNAM student hostel. *Legionella* species are ubiquitous in freshwater environments but can create problems for public health (Azara et al., 2006). Goreangab Dam serves as the biggest raw-water reservoir for the city of Windhoek. It is possible that the origin of *L. pneumophila* in the dam was mainly due to faecal contamination. *L. pneumophila* serotype 1 is the pre-eminent pathogen of sporadic and community-acquired LD. The presence of a human pathogenic *L. pneumophila* serotype 1 in this important source of municipal water should be of public health significance. We recommend that water treatment procedures should include specific measures to disinfect for *L. pneumophila* serotype 1. Most public institutions in Windhoek use partially-treated water from Goreangab Dam to water grass lawns through sprinklers. This is the same water that contains pathogenic *L. pneumophila* serotype 1. It is therefore important that health risks are assessed in lieu of human contact with aerosols from such sprinklers.

Namibia is the driest country south of the Sahara desert; hence, most Windhoek residents are attracted to the water at Goreangab Dam during their leisure. These social visitors to Goreangab are potentially at risk to *L. pneumophila* infection either through physical contact with contaminated water or through infective aerosols. Elsewhere, *L. pneumophila* had been shown to be transmitted through contact with infective aerosols carried over distances of 900 m (Addis et al., 1989; Bhopal et al., 1991). Proximity of homes to sources of infection was found to be a risk factor for non-outbreak LD (Bhopal et al., 1991). Distance and risk relation were compatible with aerosol movement in an urban environment, and air turbulences aided aerosol deposition (Bhopal et al., 1991). Imperato (1981) observed that legionellosis was transmitted by inhalation of organisms aerosolized from environmental sources, mainly water and soil. Further, Bolin et al. (1985) explained that aerosols from shower heads were implicated as a means of transmission of *L. pneumophila* from potable water to the patient, hence, showering was a risk factor for nosocomial LD. Roig and Rello (2003) stated that the precise mode of transmission was controversial but it was clear that aspiration of colonized water and inhalation of aerosols were involved in the acquisition of LD. Given such evidence, we can only speculate that UNAM students that use showers, visitors to Goreangab Dam, and residents within 900 m radius from the dam may be at risk of sporadic or non-outbreak *L. pneumophila* infection.

Legionellosis can easily be confused with symptoms of swine flu (Imperato, 1981). Twenty years ago, the World Health Organization observed that the morbidity and mortality due to sporadic and epidemic legionellosis was under-reported in most health statistics (World Health Organization, 1990). We believe that this scenario still exists in many countries especially in Africa. In Namibia, there is no routine surveillance and reporting of *L. pneumophila* infection. The current report of *L. pneumophila* contamination of Goreangab Dam and student hostels is a reminder that health authorities ought to include *L. pneumophila* on its routine surveillance list. In England and Wales, the passive surveillance system based on reports from laboratories triggers an active surveillance system that helps to locate common sources of infection (World Health Organization, 1990). The frequency of sporadic cases is an important component of community-acquired pneumonias (World Health Organization, 1990). Namibia has a generalized HIV/AIDS epidemic with about 230,000 out of a total population of about 2 million people living with HIV/AIDS (Chinsembu, 2009). Given that legionellosis is frequent in immune-suppressed, aged, cancer and radiation treatment patients (Imperato, 1981; World Health Organization, 1990), we recommend that targeted behavioural control messages be directed at these sectors of population in order to warn them against the possible risks of *L. pneumophila* infection from environmental sources. The number of CFUs obtained in this preliminary study is low but further studies should be carried out to ascertain the levels of *L. pneumophila* contamination of potential environmental sources that may serve as reservoirs for infection.

Previous studies found that Legionellae frequently inhabit cooling water systems (Kurtz et al., 1982), hot water systems (Wadowsky et al., 1982), shower baths and heads (Dennis et al., 1984), tap water (Yee and Wadowsky, 1982; Reinthaler et al., 1993), and many other drinking water outlets (Tison and Seidler, 1983; Hsu

et al., 1984; Marrie et al., 1992). *Legionella* species are recognized as environmental inhabitants of natural water such as in lakes, ponds, and streams, and man-made environments such as hot water tanks, air-conditioning and plumbing systems of hospitals and hotels (Hsu et al., 1984). Knowledge of such sources of *L. pneumophila* infection will be important in public health campaigns and future *L. pneumophila* management programs. Although, less pathogenic *L. pneumophila* serotypes 2 - 15 were detected in student hostel shower heads, there is a likelihood of cross-contamination with the more pathogenic *L. pneumophila* serotype 1 found in Goreangab Dam. However, the presence of *L. pneumophila* in shower heads should be reason enough for University of Namibia hostel authorities to embark upon a targeted *L. pneumophila* disinfection program using biocides. *L. pneumophila* often multiplies in many species of protozoa, and this host-parasite interaction is central to the pathogenesis and ecology of this species (Kwaik et al., 1998).

The success of eradication measures directed at water distribution systems will be dependent upon data obtained from the ecology of the organism within the habitat of the water distribution system (Stout et al., 1985). Whenever high levels of *Legionella* are detected in hot water systems, disinfection of water is critical for controlling outbreaks of legionellosis (Delgado-Viscogliosi et al., 2005). Disinfections are carried out by the use of oxidizing biocides such as chlorine (Delgado-Viscogliosi et al., 2005). However, such disinfections can be rendered useless by the intricate lifecycle of *L. pneumophila*, a facultative intracellular bacterium that can invade and replicate inside amoebae in the environment (Greub and Raoult, 2003). Free-living amoebae are the evolutionary crib for *L. pneumophila*; they serve as a reservoir for *L. pneumophila* as well as provide protection from environmental stresses such as chlorination (Greub and Raoult, 2003). Further studies are needed to understand the ecology and risk of LD. Monitoring and surveillance of *L. pneumophila* in various environmental sites will help shed light on possible sources of human infection.

Conclusion

Using modified BCYEA as a selective medium, *L. pneumophila* was isolated from Goreangab Dam water and UNAM student hostel shower heads. Latex agglutination tests showed that *L. pneumophila* serotypes 1 - 15 were present in Goreangab Dam and serotypes 2 - 15 were found in blocks A and B of the UNAM old hostel showerheads.

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