



Search for *Legionella pneumophila* in Domestic Water System in Benin Metropolis

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ABSTRACT: *Legionella* species, a Gram negative bacterium is the causative agent of Legionnaires' disease, a potentially fatal pneumonic syndrome of widely recognized public health importance. The aim of the study was to determine the presence of *Legionella pneumophila* in domestic water (borehole) in Benin metropolis by cultural method. One hundred and ninety-eight (198) water samples from the eight-two facilities (grouped into three: public apartments/hotels, private apartments and eateries) were cultured on BCYE made selective with the addition of legionella supplement IV and growth supplement after concentration and heat treatment at 50°C for 30 minutes and incubated at 37°C. Isolates were identified by doing gram stain, oxidase, catalase and hippurate test, final identification was done by using PCR, sequence and blast search using National Center for Biotechnology Information (NCBI). The results obtained showed that *Legionella pneumophila* or *Legionella* species was not isolated though other bacteria (such as *Burkholderia bacterium* MSMB7 (32%), *Pseudomonas antarctica* (28%), *Cupriavidus gilaridii* (14%), *Microbacterium paraoxydans* (14%), *Bacillus thuringensis* (4%), *Bacillus cereus* (2%), *Acinetobacter johnsonii* (6%)) were detected. In conclusion zero percent (0%) prevalence of *Legionella* species in the water systems investigated though other bacteria with pathogenic potential were recovered. This finding suggests that water systems in Benin metropolis may not present vehicles for the transmission of diseases associated with *Legionella* species.

DOI: <https://dx.doi.org/10.4314/jasem.v22i10.12>

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Dates: Received: 26 June 2018; Revised: 30 July 2018; Accepted: 29 August 2018

Keywords: *Legionella pneumophila*, domestic water, search, BCYE, Benin metropolis

Legionellaceae are fastidious, opportunistic Gram negative bacteria that reside in aquatic environment with wide spread distribution (Pel-Yi *et al.*, 2000). In their natural environment, Legionellaceae are intracellular parasite of free living protozoa. *Legionella* is transmitted to humans by inhalation of contaminated aerosols.

Common sources are air conditioning system, cooling towers, dental devices, river, streams, taps and showers (David *et al.*, 2007; Alli *et al.*, 2011; Maura *et al.*, 2014). Legionellosis can appear in two distinct clinical presentation: Legionnaires' disease (LD), a mild to fatal pneumonia with 3.4% fatality rate (Anonymous, 2000) and Pontiac fever, an acute self-limited influenza-like illness. Transmission between human beings has never been observed to this date (Nathalie *et al.*, 2010). Over 50% species of *Legionella* have been recognized and at least 24 of these have been associated with human infection. One species of *Legionella*, *L. pneumophila* is the etiological agent of about 90% of legionellosis case and serogroup 1 (Sg1) account for the most frequent cause of infection (Doleans *et al.*, 2004; Amemura-Maekawa *et al.*, 2010).

Domestic system are complex environment in which concentration of *Legionella* can fluctuate considerably depending upon water temperature, biocide level and presence of natural host (protozoa) for legionellae to parasitize. Domestic water in Benin are 99% from borehole which are usually 80 to 120 meters deep or more (Ikhile, 2016). Culturing the domestic water is the first step to assess the risk for *Legionella* in Benin metropolis. This approach is well adopted in the national guide-lines for European countries, France, Italy and in other regional guidelines and recommendations (Italy: Ministero della Salute, 2000; France: Ministère de l'Emploi et de la Solidarite, 2002). Legionnaires' disease has not been reported in Benin and environmental culture of domestic water for the isolation of *Legionella* has never been performed hence no documented epidemiological data to determine the presence of the bacterium. The objectives of this study was to determine the presence of *Legionella* in domestic water and air conditioner water system in Benin metropolis.

MATERIALS AND METHODS

Study area: Benin City is situated in the southern part of Edo state, Nigeria. It is the capital of Edo state. Its

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geographical coordination are 6° 20' 0" north and 5° 38' 0" east. It is estimated to have a population of 1,147,187 people (Okhakhu, 2016). Its major attractive sites includes Oba's palace, Central hospital, banks, police station, legislative building, Oba Akenzua cultural center, Okada house, museum (where some of the importance statues of ancient times are preserved). The past Benin City was known for the artisans who did excellent bronze and ivory casting.

Study facility: The study was conducted between September, 2015 and March, 2016 in eighty-two facilities grouped into three namely: private apartments, public apartments (hotels) and eateries all in Benin metropolis. Water samples were collected after obtaining verbal consent from the management of the facilities and ethical clearance from the Edo state Ministry of Health.

Sample collection: A total of one hundred and ninety-eight (198) water samples were aseptically collected from taps, showers and air conditioner (AC). Sterile swab sticks were first used to scrape the mouth of facet (shower with the shower head removed) and AC pipes and placed in 15mls sterile plastic centrifuge tube containing 3mls of water obtained from same point. A liter of water sample was collected into specimen container containing 1% sodium thiosulphate and stored at room temperature before processing. One hundred milliliter (100ml) of water from same source were stored at 2-4°C for physico-chemical analysis.

Sample processing: Water samples were processed in Central Hospital Benin Medical Microbiology laboratory for isolation of *Legionella* as described by Alli *et al.*, (2011). Briefly 100ml of each collected water sample was placed in a centrifuge tube and centrifuged at 3000g for 20minutes. The supernatant was carefully discarded leaving about 3mls of water with the sediment. The sediment was mixed (vortex) to dislodge the sediment bacteria. 1ml of the concentrated water sample was placed at 50°C in a water bath for 30min (to reduce the no of non-legionella bacteria), the heated water sample was placed on the bench for 10min in order to attain room temperature.

Culture method: Prepared concentrated sample were cultured on buffered charcoal yeast extract (BCYE) according to PHE, 2015. Briefly, an aliquot of 100µl of prepared sample was inoculated on duplicate plate of BCYE agar base made selective by adding legionella selective supplement (glycine, vancomycin, anisomycin and polymycin B sulphate) and enrich with legionella growth supplement. A set of plates

were placed in a candle jar and other without candle jar at 90% humidity incubated at 36°C. Plates were examined at periodic interval starting from the third day of incubation for the typical ground glass colony and those without growth were re-incubated and re-examined later. Plate without growth after day fourteen (14) were discarded. Each presumptive colony was first Gram stained and those that were Gram negative rods were tested for catalase, oxidase and hippurate. Subcultures were done on α-BCYE-GVP with L-cysteine and non-selective medium BCYE agar without L-cysteine and incubated as describe above.

DNA extraction of isolate: The isolated bacteria had their DNA extracted using ZR fungal/ bacterial DNA Mini Prep™. The manufacturer's DNA extraction protocol was used with elution volume of 25µl, and was kept at -20°C until PCR amplification was performed.

PCR, Sequence and Blast of isolates: DNA extract obtained from isolates had their 16S target region amplified using Dream Taq™ DNA polymerase according to manufacturer's procedure with universe primer 16S-27F 5' - AGAGTTTGATCMFGGCTCAG-3' and 16S-1492R 5' - CGGTTACCTTGTTAGGACTT- 3' in an ABI PRISM™ 3500xL Genetic Analyzer and CLC Main-Workbench 7.5.1 blast search was done using NCBI as describe by Stephen *et al.*, 1997.

Water quality analysis: Calcium, zinc, magnesium, iron and copper analysis were done adopting Akpan-Idiok *et al.*, 2012 method using Buck 210 Atomic Absorption Spectrophotometer.

Statistical analysis: All statistical analysis were done using statistical package for social science (SPSS). Values were recorded as mean and standard deviation. Comparison of mean was by one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

A search for *Legionella* species in domestic water in Benin metropolis in 82 facilities grouped into three (public apartments/hotels (18.3%), private apartments (70.7%) and eateries (11%)) was done. Out of the total facilities examined 36.6% yielded growth while 70.7% yielded no growth as presented in Table 1.

Table 1: System examined in this study

System	Number	(%) Facility with growth
Hotels	15	(18.3) 26.7
PP	58	(70.7) 41.4
Eateries	9	(11.00) 22.2
Total	82	(100) 36.6

PP = Private apartment

Figure 1 below shows microbiological positive water samples with respect to sites. One hundred and ninety-eight (198) water samples from showers, taps and air conditioners in Benin metropolis were examined, 41(20.7%) yielded bacteria growth (25 (27.2%) of the growth were from taps and 16(24.4%) from showers), while 157(79.3%) yielded no bacteria growth. No bacteria growth was observed in all the twenty-eight air conditioner water sample examined, this is in line with Alli *et al.*, 2011 study.

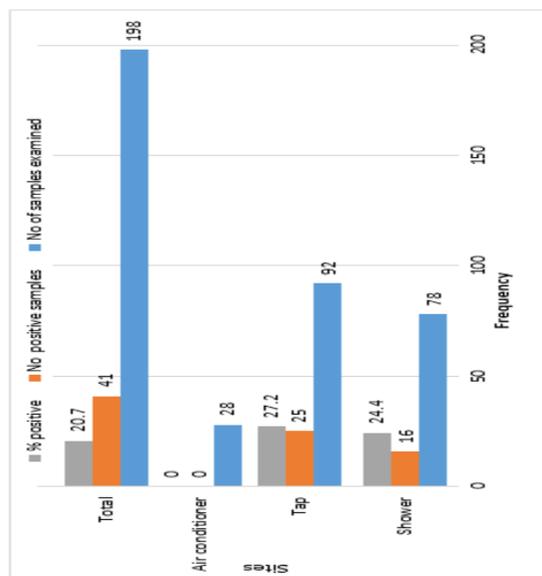


Fig 1: Microbiological positive water samples in respect to sites

Table 2 shows gram reaction and biochemical test performed on isolates. Suspected colonies were gram stained and those that were Gram negative rods were sub-cultured on BCYE (with and without growth supplement) and biochemically tested (catalase, oxidase and hippurate; a test suggestive of *Legionella*) in line with Alli *et al.*, 2011 study. All suspected isolates grew on BCYE (with and without growth supplement) and were hippurate negative. *C. gilaridii* and *P. antarctica* were both oxidase and catalase positive while *B. bacterium* was oxidase positive but catalase negative and *A. johnsonii* was oxidase negative but catalase positive. The positive control organism (*L.pneumophila*) gave typical biochemical reaction (i.e. oxidase, catalase and hippurate positive) and growth only on BCYE (with growth supplement). The Gram positive isolates were not subjected to biochemical test. The used of BCYE with antibiotic to search for *Legionella* was in line with Alli *et al.*, 2011 in which BCYE was used in detecting and isolating of legionellae from the environment although legionellae was not isolated in our study. **Table 3** shows identified bacteria from molecular level. Results shows that *Legionella* has a zero prevalence. This is in contrast to result reported by Alli *et al.*, 2011 in which *Legionella* spp was isolated from wells and streams. Other bacteria isolated are *P. antarctica* (28%), *B. bacterium* (32%), *M. paraoxydan* and *C.gilaridii* (14% each), *B. cereus* (2%), *B.thuringensis* (4%) and *A. johnsonii* (6%).

Table 2. Gram reaction and biochemical test performed on isolates

Isolates	Oxidase test	Catalase test	Hippurate test	Gram stain reaction	Growth on BCYE with L-cysteine	Growth on BCYE without L-cysteine
<i>C. gilaridii</i>	+	+	-	GNB	+ ^a	+ ^a
<i>P. antarctica</i>	+	+	-	GNB	+ ^a	+ ^a
<i>M. paraoxydans</i>	NS	NS	NS	GPB	+ ^a	+ ^a
<i>B. bacterium</i>	+	-	-	GNB	+ ^a	+ ^a
<i>B. cereus</i>	NS	NS	NS	GPB	+ ^a	+ ^a
<i>B. thuringiensis</i>	NS	NS	NS	GPB	+ ^a	+ ^a
<i>A. johnsonii</i>	-	+	-	GNB	+ ^a	+ ^a
<i>L.pneumophila</i> (positive control)	+	+	+	GNB	+ ^a	- ^v
<i>E. coli</i> (Negative control)	-	-	-	GNB	- ^v	- ^v

KEY: + = positive, - = negative, +^a = growth on BCYE, -^v = no growth on BCYE; NS = Not subjected to test, GNB = Gram negative bacilli, GPB = Gram positive bacilli

Table 3: Identified isolates and their frequency

Isolates	Frequency (%)
<i>Legionella</i> spp	0
<i>C. gilaridii</i> MSMB32	14
<i>P. antarctic</i> strain PAMC 27494	28
<i>M. paraoxydans</i> strain TH-3302	14
<i>B. bacterium</i> MSMB7	32
<i>B. cereus</i> strain EKS4-2	2
<i>B. thuringiensis</i> strain MZ-1	4
<i>A. johnsonii</i> strain JU6	6

Table 4: Mean value of Cu, Zn and DO in the study

Variables	Unit	Public apartments	Eateries	Private apartments	P-value	Significance
Cu	Ppm	0.37±0.07	0.37±0.07	0.97±0.07	0.156	NS
Zn	Ppm	0.58±0.11	0.58±0.11	0.58±0.10	0.689	NS
DO	ppm	0.06±0.01	0.06±0.01	0.06±0.02	0.398	NS

Key: Cu = Copper, Zn = Zinc, DO = Dissolved Oxygen, NS = Not significant

One question that puzzles the mind is; 'why was *Legionella* not isolated?' The main source of domestic water in the facilities examined is from the deep ground (borehole) which is 80m to 120m or more, at this depth the metals concentration (such as Cu, Zn and DO with no significant level: $p > 0.05$) (Table 4) may play important role in the survival of this bacterium (*Legionella* species) such as: (1) water percolation causes filtration of the studied organism and particles thereby accounting for the non-isolation of legionellae. (2) in this study we observed a low level DO (0.06ppm) as against a higher level reported by Wadowsky *et al.*, 1985 in which *Legionella* require 0.3-9.6ppm (DO) to survive. The low DO observed may have caused non-isolation of *Legionella* in this study. (3) the high level of copper (0.37±0.07ppm to 0.39±0.07ppm) and zinc (0.58±0.10ppm to 0.58±0.11ppm) observed in this study may have greater effect on *Legionella* as studies have shown that copper greater than 50ppb and zinc level above 200ppb or below 100ppb may inhibit or lower *Legionella* concentration (Borella *et al.*, 2004; 2005). The effect of pH on *Legionella* could not be ascertain in this study as pH of the medium was not recorded. Also observed in this study is the non-isolation of *Legionella* from the air conditioner water samples examined, this is in agreement with zero percent (0%) reported by Alli *et al.*, 2011 in Osogbo, Osun State, Nigeria, as against a study carried out in Germany in which 3.3% of the air conditioner water systems examined were positive for *Legionella* (Dermitzel *et al.*, 1992). The non-isolation of *Legionella* from air conditioner water in this study could be attributed to air conditioner being an artificial man-made that is devoid of amoeba, which is supportive of legionellae growth (Alli *et al.*, 2011). The temperature required (above 49°C or below 21°C depending on heat load, ambient temperature) by the air conditioner to effectively chill a room may not be conducive for *Legionella* to survive. The erratic power failure in Benin metropolis may also contribute to the non-isolation of the bacterium. When this occur air conditioner stops functioning causing the AC outlets to dry up and any *Legionella* present within the outlets is reduced due to the death of the bacterium. In this study, bacteria isolated were *Burkholderia bacterium* MSMB7 with 32% prevalence, *Pseudomonas antarctica* (28%), *Cupriavidus gilaridii* (14%), *Microbacterium paraoxydans* (14%), *Bacillus*

thuringensis (4%), *Bacillus cereus* (2%), *Acinetobacter johnsonii* (6%).

Conclusion: Results obtained in our study revealed a zero percent (0%) prevalence of *Legionella* in the water systems investigated, though other bacteria with pathogenic potential were isolated. This finding suggests that water systems in Benin metropolis may not provide vehicles for the transmission of diseases associated with *Legionella* species. Therefore, there is need for further study on *Legionella* in well, river and stream and a continuous assessment of borehole water in Benin metropolis and rural area in order to ascertain its presence and risk, this will facilitate the development of active prevention strategy for Legionnaire's disease.

REFERENCES

- Akpan-Idiok, AU; Ibrahim, A; Udo, IA (2012). Water quality assessment of Okpauku River for drinking and irrigation uses in Yalu, Cross-river state Nigeria. *Res. J. Environ. Sci.* 6(6):210-221.
- Alli, OAT; Ogbolu, D; Adedokun, SA; Ogundare, OE (2011). Isolation of *Legionella pneumophila* from surface and ground water in Osogbo, Nigeria. *Afr. J. Microbiol. Res.* 5(18): 2779 - 2785.
- Amemura-Maekawa, J; Kura, F; Helbig, JH; Chang, EN; Kaneko, A; Watambe, Y; Isobe, J; Nukina, M; Nakajima, H; Kawano, K; Tada, Y; Watanabe, H; the working group for *Legionella* in Japan (2010). Working group for *Legionella* in Japan. Characterization of *Legionella pneumophila* isolates from patients in Japan according to serogroups, monoclonal antibody subgroups and sequence types. *J. Med. Microbiol.* 59: 653 - 659.
- Anonymous (2000). Outbreak of Legionnaires' disease associated with an aquarium in Australia. *CDR Weekly.* 10: 161.
- Borella, P; Montagna MT; Stampi, S; Stancanelli, G; Romano-Spica, V; Triassi, M; Marchesi, I; Bargellini, A; Tato, D; Napoli, C; Zanetti, F; Leoni, E; Moro, M; Scaltriti, S; D'Alcala, GR; Santarpia, R; Bocia, S (2005). *Legionella*

- contamination in hot water of Italian hotels. *Appl. Environ. Microbiol.* 71 (10): 5805 - 5813.
- Borella, P; Montagna, MT; Romano-Spica, V; Stampi, S; Stancanelli, G; Triossi, M; Neglia, R; Marchesi, I; Fantazzi, G; Tato, D; Napoli, C; Quaranta, Q; Laurenti, P; Leoni, E; De Luca, G; Ossi, C; Moro, M; D'Alcala, GR (2004). Risk factor associated with isolation of Legionellae in domestic waters. *Emerg. Infect. Dis.* 10: 457 - 464.
- David, K; Gary, R; Gillian, ES; Babatunde, O (2007). Investigation of an outbreak of Legionnaire's disease: Hereford, UK 2003. *Resp. Med.* 101:1639-1644.
- Dermizel, A; Genenich, HH; Muller, HE (1992). *Legionella* and other bacteria in air humidifiers and cooling systems of air conditioning units- a survey. *Gesundheitswesen.* 54: 716 - 719.
- Doleans, A; Aurell, H; Reyrolle, M; Lina, G; Freney, J; Vandenesch, F; Etienne, J; Jarraud, S (2004). Clinical and environmental distributions of *Legionella* strains in France are different. *J. Clin. Microbiol.* 42(1): 458-460.
- Ikhile, CI (2016). Geomorphology and hydrology of the Benin region, Edo State, Nigeria. *IJG.* 7: 144-157.
- Maura, DJ; O'Connell, K; Stephen, JV; Jatin, HM; Dawn, K; Mitch, K; Stacy, P (2014). Widespread molecular detection of *Legionella pneumophila* serogroup 1 in cold water taps across the United States. *Environ. Sci. Technol.* 48(6): 3145-3152.
- Minister della Salute (2000). Linea guida per la prevenzione controllo della legionellosi. Istituto superiore di sanita. *Repubblica italiana.* 103.
- Ministere de l'Emploi de la solidarite (2002). Circulaire DGS/SDZA/SD5C-DHOS/E4n. 2002/243 du 4/04/2002 relative a la prevention durisque lie anx legionelles dans les etablissements de sante. France: Direction generale de la sante, Directiondel'Hospitalisation del' organisation des soins.
- Nathalie, T; Patrick, T; Nya, R; Caria, D; Victoria, N; David, NF; Francis, J; Donald, EL; Cyril, G (2010). New endemic *Legionella pneumophila* sero group 1 clones, Ontario, Canada. *Emerg. Infect. Dis.* 16(3): 447-454.
- Okhakhu, PA (2016). Assessment of the urban climate of Benin City, Nigeria. *J. Environ. Ear. Sci.* 6(1): 131-143.
- Pei-Yi ,Y; Yuseu, EL; Wel-Ru, L; Hsiu-Yu, S; Yin-Ching, C; Ren-Jy, B; Wen-Kuei, H; Yao-Shen, C; Yung-Ching, L; Feng-Yee, C; Muh-yong, Y; Ching-Chuan, L; Wen-Chien, K; Hsi-Hsun, L; Zhi-Yuan, S (2008).The high prevalence of *Legionella pneumophila* contamination in hospital potable water systems in Taiwan: implications for hospital infection control in Asia. *Int. J. Infect. Dis.* 12: 416 - 420.
- Public Health England (PHE) (2015). Identification of Legionella species. UK standards for microbiology investigation. 18(3).<https://www.gov.uk/uk-standards-for-microbiology-investigations-smi-quality-and-consistency-in-clinical-laboratories>.
- Stephen, FA; Thomas, LM; Alejandro, AS; ffer; Jinghui, Z; Zheng, Z; Webb, M; David, JL (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25: 3389 - 3402.
- Wadowsky, R M; Yee, RB (1985). Effect of non-Legionellaceae bacteria on the multiplication of *Legionella pneumophila* in potable water. *Appl. Environ. Microbiol.* 46: 1447-1449.