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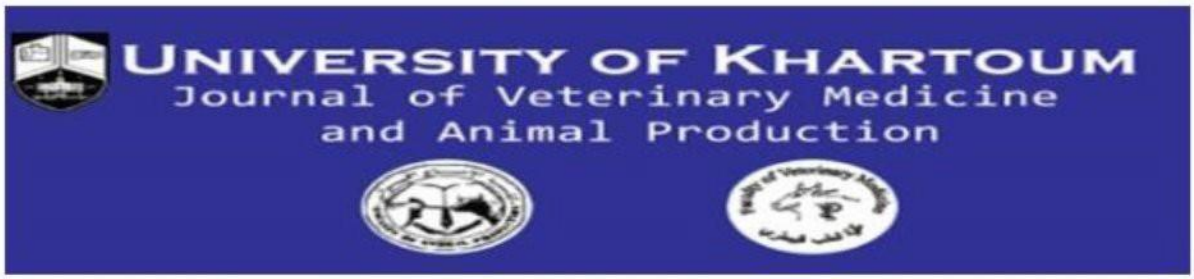

Substantiation of *Legionella pneumophila* isolates using Real Time PCR Test in Khartoum State, Sudan

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Abstract

Legionella pneumophila is considered as a causative agent of Legionnaire's disease, which affects humans causing severe fatal pneumonia, particularly in immunocompromised individuals. *Legionella* species isolates were identified using cultural, morphological and biochemical characteristics, before they were confirmed by Real Time PCR test. A total of 38 *Legionella* species isolated from water reservoirs of evaporative coolers air conditioners, distributed in Khartoum State, were subjected to Real Time PCR which gave 24 (63%), identical species to *Legionella pneumophila*.

Key word: *Legionella pneumophila, Legionnaires disease, real time PCR, evaporative coolers, air conditioners*

المستخلص

تعتبر جراثيم الفيلقة الرئوية العامل المسبب لداء المحاربين القدماء الذي يصيب البشر متسببا في التهابات رئوية حادة مميتة خاصة في الاشخاص ذوي المناعة المنتقصة. تم تعريف وتشخيص جنس جراثيم الفيلقة باستخدام التزريع و اختبارات الخواص الظاهرية والكيميائية قبل تاكيدها بأستخدام اختبار البلمرة المتسلسل الكمي اللحظي . تم إخضاع ثمان وثلاثون عزلة من جنس جراثيم الفيلقة التي عزلت من خزانات مياه مكيفات الماء المنتشرة بولاية الخرطوم لفحص البلمرة المتسلسل الكمي اللحظي. أربع وعشرون (63%) من مجمل العزلات أعطت نتائج ايجابية مطابقة لبكتريا الفيلقة الرئوية.

كلمات مفتاحية: *جراثيم الفيلقة الرئوية، داء المحاربين القدماء ، إختبار البلمرة المتسلسل الكمي اللحظي ، مكيفات الماء*

Introduction

The genus *Legionella* acquired great attention because of the increase of *Legionella* infection outbreaks throughout the world. Some studies, estimated the epidemiology of Legionnaires' disease throughout the world in a range between 0.5 and 10 % for community acquired pneumonia (Edelstein *et al*., 2005). *Legionella pneumophila* was isolated from cooling water system of one of the air conditioning plants in a large outbreak of Legionnaire's disease in Stafford district general hospital in the United Kingdom (O'Mahony*et al*., 1999). A recent study was done in the Sudan by Elsanousi and Elsanousi, (2017), who detected and isolated *Legionella pneumophila* for the first time from evaporative coolers air conditioners in Khartoum State. *Legionella* is an important pathogen in health - care units, as it causes hospital - acquired pneumonia (nosocrnial–infections), particularly in immuno

compromised patients (WHO, 1990). *Legionellaceae* are Gram – negative rods. There are more than 41 species of the genus *Legionella.* The most important one is *Legionella pneumophila* (Green wood, Richard, and John, 2002). *Legionella pneumophila* serogroup 1 is a causative agent of Legionnaires' disease and Pontiac fever which is transmitted through the inhalation or aspiration of contaminated aerosols (Reingold *et al*., 1984). Legionnaires' disease (LD) is an acute pneumonia and outbreaks of Legionnaire's disease were traced to a wide variety of environmental water sources such as cooling towers, hot tubs, showers, whirlpools, spas water systems, and public fountains (Atlas, 1999; Doleans *et al*., 2004). *Legionella pneumophila* are non-spore forming, non- capsulated rods, aerobic and are able to hydrolyse gelatin. Also are oxidase and catalase positive and produce beta lactamase. They replicate as intracellular parasites of amoebae and persist in the environment as free living microbes or in biofilms (Delgado-Viscogliosi, Solignac, and Delattre, 2009). *Legionella* species can be grown on complex media such as buffered charcoal yeast extract agar with α-ketoglutarate and iron (BCYE-α medium) at a pH of 6.9, temperature of 35 °C, 80 - 90% humidity and 2 - 5% CO_2. Antibiotics can be added to make the medium selective for *Legionella* species. (Edelstein, 1981, 1982; Smith, 1982). Culture on selective media is the standard method for detection, isolation and identification of *L. pneumophila* in clinical and environmental samples, but it can take more than 7 days. (Leoni and legnani, 2001). Quantitative Real Time PCR method has been developed for rapid detection and quantification of *Legionella* DNA in water samples and it is often used in routine monitoring (Yardou *et al*., 2007). Cooling towers and air conditioning systems have been implicated as sources of *Legionella pneumophila* (Politi *et al*., 1979). In Sudan, we commonly used evaporative coolers air conditioners where there is abundant and sufficient water sources used for the device operation and because it requires low energy, inexpensive cost of manufacturing, emit very low CO_2 and had relatively quiet operation.

The aim of this study was to sort out *Legionella pneumophila* isolated from evaporative coolers air conditioners as well as to assure and to refer to the Real Time PCR as a fast, rapid and sensitive diagnostic test used to confirm the evidence of *Legionella pneumophila* besides traditional cultural procedures.

Materials and Methods

Samples preparation

Thirty eight *Legionella* species isolates were selected randomly out of a total of 222 *Legionella* species isolated from water samples that were collected from water reservoirs of evaporative coolers air conditioners in Khartoum State and subjected to Quantitative Real Time PCR test for confirmation.

Bacterial isolation and identification

Samples were treated, cultured, purified and identified using gold standard culture procedures. Gram's stain technique and biochemical tests were performed according to Barraow and Feltham, (2003). Buffered Charcoal Yeast Extract Agar (BCYE –α) medium and Glycine Vancomycin Polymxin Cycloheximide (G V P C) medium were obtained from Oxoid Laboratories, London.

Quantitative Real Time PCR Technique DNA Extraction

This procedure was done following instructions of the manufacturer of the kit. (Sacace Biotechnologies, Italy). Pure isolated colonies suspected for *Legionella pneumophila* were emulsified in 0.5 ml of normal saline solution. A volume of 50µl of the suspension were used for DNA extraction, 50 µl of DNA-eluent solution were added. 10µl of extracted DNA were

used for amplification.

Real Time PCR Assay

For the detection of *Legionella pneumophila* in samples, Quantitative Real Time PCR assay was applied targeted a specific region within the Mip gene - The sequences of the primers and probe were as follows:

Forward primer, Mip-F1 (forward5′GCCAAGTGGTTTGCAATACAAA-3′)

Reverse primer, Mip-R1 (reverse 5′-CTCGACAGTGACTGTATCCGATTT-3′)

Probe-LPN-Mip (5′-FAM-TAATCAATGCTGGAAATGGTGTTAAACCCG-TAMRA-3′)

A quantitative Real- time PCR test kit for *Legionella pneumophila* DNA, using *Legionella pneumophila* Mip-gene, was obtained from Sacace Biotechnologies, Italy. – [The complete Real- Time PCR test kit with DNA purification kit, (TB50-50FRT)]. Quantitative standards were used to quantify *Legionella pneumophila* DNA copies. Materials and methods applied on this technique were according to manufacturer instructions.

Results Interpretation

Results obtained in Real-Time PCR were evaluated according to the manufacturer instructions and standardization.

Results

The 38 *Legionella* species isolates showed growth on BCYE - α medium and GVPC medium and revealed Gram negative bacilli, catalase, oxidase and gelatin hydrolysis tests positive. Twenty four (63%) of the isolates showed positive results for *Legionella pneumophila* using Real - Time PCR technique. Table (1) and figures (1,2and 3) summarize the Real - Time PCR results.

Table 1: Quantitative Real Time PCR results of *Legionella pneumophila* isolated from water samples obtained from evaporative air conditioning systems in Khartoum State

Sample Name	Standard Concentration	Calculated Concentration	Ct
QS1	6.23×10^{6}	6.23×10^{6}	5.98
QS2	1.70×10^{5}	1.70×10^{5}	22.12
QS3	4.00×10^{3}	4.00×10^{3}	35.03
NTC	-ve	-ve	-ve
1		9.60×10^{3}	29.47
2		3.56×10^{6}	15.67
4		9.80×10^{4}	29.45
5		1.52×10^{5}	32.78
7		1.57×10^{5}	23.98
9		1.34×10^{5}	24.75
10		5.93×10^{6}	10.93
19		6.85×10^{6}	12.19
12		8.20×10^{3}	30.14
14		1.22×10^{5}	27.89
17		8.10×10^{3}	30.31
18		9.88×10^{3}	29.65

QS: Positive standard control, NTC: Negative Standard control, Ct: Cycle threshold, - ve: negative

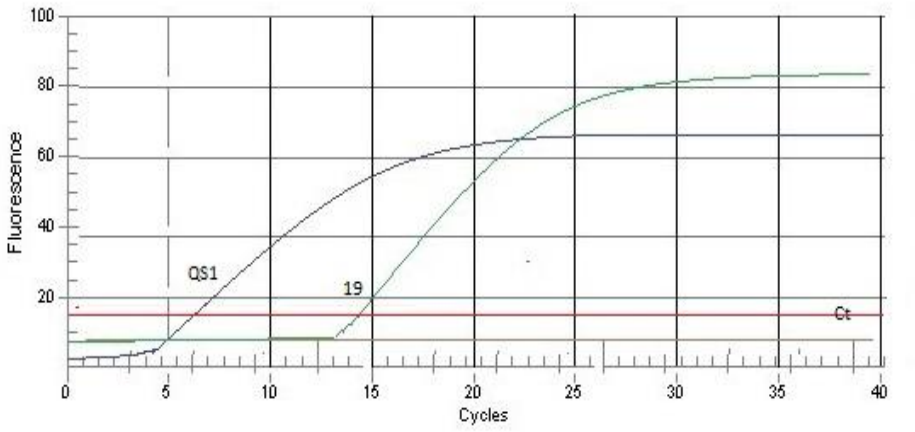

Fig.1: Amplification plot of quantitative Real Time PCR of *Legionellapneumophila* isolate obtained from water sample no. 19 of evaporative cooler air conditioning system in Khartoum State (QS1: postive standard control , Ct: Cycle threshold)

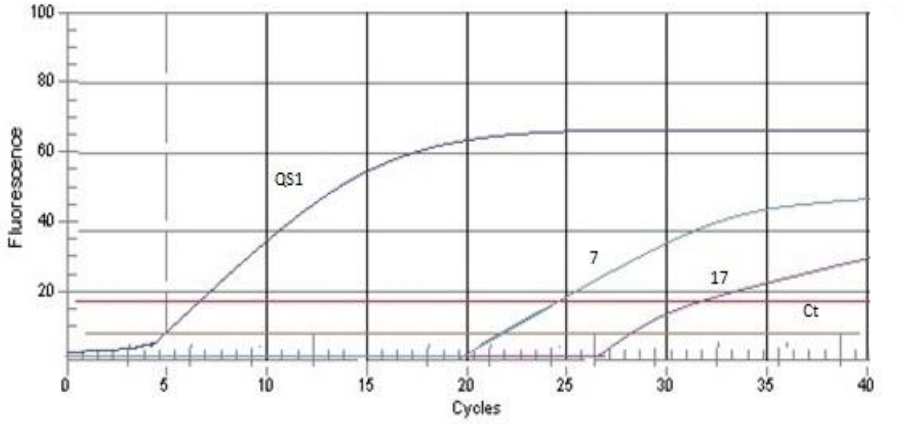

Fig. 2: Amplification plot of quantitative Real Time PCR of two *Legionellapneumophila* isolates obtained from water samples no. 7 and no. 17 of evaporative coolers air conditioning systems in Khartoum State (QS1: postive standard control, Ct: Cycle threshold)

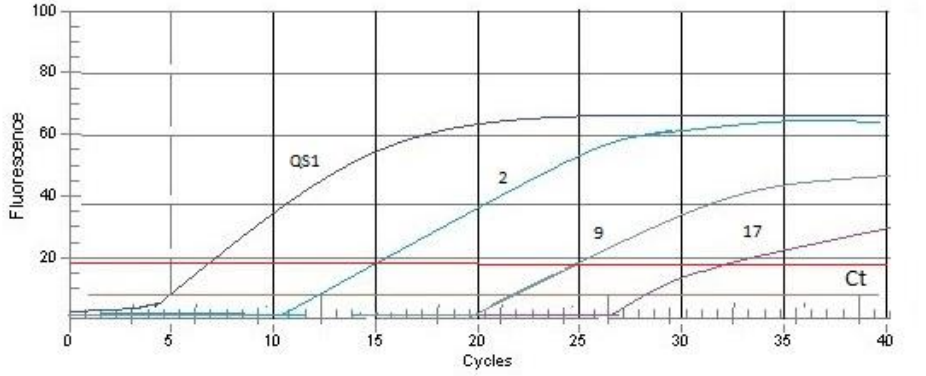

Fig. 3: Amplification plot of quantitative Real Time PCR of *Legionella pneumophila* isolates obtained from three water samples (samples no, 2,9 and 17) of evaporative coolers air conditioning systems in Khartoum State (QS1: postive standard control Ct: Cycle threshold)

Discussion A PCR – based detection, is an attractive

and sensitive technique that has been suggested as a way to overcome problems of the cultural methods, however all the PCR - methods are lacking the ability to discriminate between living and non-living (non-infectious) cells also this could include living but non culturable bacteria (Oliver, 2005).Therefore, we applied the two methods: standard culture technique and Real time PCR test.

Sixty three percent from a total of 38 *Legionella* species isolates gave positive results for *Legionella pneumophila* using Real-Time PCR technique, which is considered a rapid diagnostic method in comparison with different PCR assays. The combination strategy between gold standard culture technique and Real time PCR was developed previously by Park *et al.,* (2013) who used the technique for the identification of Vibrio species which raised the isolation ratio of all Vibrio species and provides a rapid, sensitive, and specific technique to detect Vibrio species in the environment in Korea. This assumption was confirmed by the results of this study. Also Real time PCR was used for the confirmation of *Salmonella* species isolates detected from wide variety of food and food-animal matrices by Bohaychuk *et al.,* (2007). Our study also agreed with other study done by Tan, Jiang and Yong Ng (2017) who applied the combination of culture on selective media, followed by direct colony Real Time PCR confirmation which allows faster and economical screening in Taiwan.

Conclusion

Although the standard culture methods allow the isolation and the quantification of *Legionella* from the environment but it has its limitations and requires selective media and prolonged incubation periods. Bacterial loss can occur during the concentration stages. Interference of organisms with *Legionella* spp growth may lead to wrong estimation of the real number of *Legionella* spp present in the sample. *Legionella* species may enter a viable but non-cultivable. Therefore, we suggest to apply both cultural and PCR assay.

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