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Journal Name:	British Microbiology Research Journal
Manuscript Number:	2013_BMRJ_6433
Title of the Manuscript:	Prevalence and Molecular Diversity of <i>Legionella pneumophila</i> in Domestic Hot Water Systems of Private Houses
Type of the Article	Research Paper

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This journal's peer review policy states that **NO** manuscript should be rejected only on the basis of '**lack of Novelty**', provided the manuscript is scientifically robust and technically sound.

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PART 1: Review Comments

	Reviewer's comment	Author's comment (if agreed with reviewer, correct the manuscript and highlight that part in the manuscript. It is mandatory that authors should write his/her feedback here)
Compulsory REVISION comments	Line 46-47: RAPD is now largely obsolete, appropriate only for local outbreak investigation, does not allow interlaboratory comparisons. Please mention the current standards, including SBT and MLVA, and some associated literature. Line 72, transport of samples on dry ice, please check, as such conditions would usually kill most <i>Legionella</i> spp. Lines 214: in figure 2, patterns 10 and 12 are very different (indeed as different as could be). From the literature serogroup 2-14 isolates from different sources are not expected to be in a single genetic group. More generally, Figure 1 and Figure 2 show a different subset of isolates. Only two are common. Given the limited number (17) of isolates, all patterns should be shown. The major problem with the present investigation is that the RAPD clustering achieved is not convincing. RAPD is not appropriate to support the main conclusions of this report, and other methods need to be used, SBT or MLVA. Such methods would allow interlaboratory comparisons, international databases have been produced for this purpose.	Current standard techniques including Sequence Based Typing (SBT) and Multiple-Locus Variable number tandem repeat Analysis (MLVA) offers a powerful tool for strain typing with an excellent inter-laboratory reproducibility, and will most probably be used extensively in major reference laboratories in the near future. But on the other hand, although RAPD is now considered to be an "old tool" for DNA typing, it is still being used for characterization studies of microorganism because of its simplicity, reliability, easy adaptation and in addition, it does not require expensive materials or equipments. These characteristics are important in cost-effective studies like ours, especially that our manuscript's aim dose not focus on comparing between the reproducibility of different DNA typing techniques. "Please mention the current standards, including SBT and MLVA, and some associated literature." In the introduction part lines [44-46] "DNA-based typing methods are becoming increasingly useful for performing epidemiological surveys of <i>Legionella</i>, and the success of these epidemiological surveillance studies is related to the typing procedures applied [15-20]" the authors have already mentioned recent trends of



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		DNA- based typing methods for performing epidemiological surveys of <i>Legionella</i>, with six associated literatures two of which (ref. 16 and 19) involved SBT and MLVA typing. “collection samples were transferred to the laboratory on dry ice” few literatures have approved the transfer of <i>Legionella</i> sample on dry ice after collection as mentioned in the following reference: Zbikowska E, Walczak M, Krawiec A. Distribution of <i>Legionella pneumophila</i> bacteria and <i>Naegleria</i> and <i>Hartmannella</i> amoebae in thermal saline baths used in balneotherapy. Parasitol Res. 2013;112(1):77-83. In this manuscript there is no doubt that, one of the limitations in this study, was the small number of the total <i>L. pneumophila</i> isolates belonging to serogroup 2-14, which necessitates trying to put more effort in properly identifying the <i>L. pneumophila</i> 2-14 serogroups and observe their genetic diversity using various DNA typing methods in future work. Lines 210-214 statement was revised and highlighted in Yellow In figure one and two there was no need to show all patterns only a few representative isolates were selected from the total since as explained in the results and discussion section lines 198-205: “All selected isolates were classified into three major groups (A, B and C). Group A included all <i>L. pneumophila</i> serogroup 1 isolates. Group B
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		included <i>L. pneumophila</i> serogroup 2-14 isolates and group C contained the remaining two <i>Legionella</i> spp. isolates, as it is shown in Figure 2. Group A was further sub-divided into two sub groups (A1 and A2), the first contained <i>L. pneumophila</i> serogroup 1 isolates collected from water heaters set at a temperature ranging between 25-30°C, the second sub group contained <i>L. pneumophila</i> serogroup 1 isolates collected from water heaters set at temperature ranging between 55-60°C” As mentioned before in the reply on the previous comments Current standard techniques including Sequence Based Typing (SBT) and Multiple-Locus Variable number tandem repeat Analysis (MLVA) offers a powerful tool for strain typing with an excellent inter-laboratory reproducibility, and will most probably be used extensively in major reference laboratories in the near future. But on the other hand, although RAPD is now considered to be an “old tool” for DNA typing, it is still being used for characterization studies of <i>Legionella</i> (references shown below) because of its simplicity, reliability, easy adaptation and in addition, it does not require expensive materials or equipments. These characteristics are important in cost-effective studies like ours. Lepeuple AS, Jovic M, de Roubin MR. Molecular typing of the <i>Legionella pneumophila</i> population isolated from several locations in a contaminated water network. Water Sci Technol. 2004;50(1):281-5.
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		Perola O, Kauppinen J, Kusnetsov J, Kärkkäinen UM, Lück PC, Katila ML. Persistent <i>Legionella pneumophila</i> colonization of a hospital water supply: efficacy of control methods and a molecular epidemiological analysis. APMIS. 2005;113(1):45-53. Qasem JA, Mustafa AS, Khan ZU. <i>Legionella</i> in clinical specimens and hospital water supply facilities: molecular detection and genotyping of the isolates. Med Princ Pract. 2008;17(1):49-55.
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Minor REVISION comments	Line 22: replace "...have been identified, most notably is..." by "...have been identified. The majority of clinical cases are due to..." Line 27 "transfers", "undergoes" Line 65: be more precise about the number of independent private houses. Quote Table 1 Line 65 "till the end" Line 82 "15 minutes" Line 95, correct " bio-Me'rieux" in " bioMérieux" Line 123-127: in the band pattern comparisons, please confirm that only band presence and positions were taken into account, not relative band intensities. Were the bands automatically assigned by the software, or were they manually selected, or a combination of both? What were the band shift allowance parameters settings? Line 142, replace "... Legionella, only 17..." by "... Legionella. Seventeen ..." Line 144, replace "spp., this percentage is found to be in agreement..." by "spp. This percentage is in agreement ..." Lines 147-148: move the technical details to methods section; according to methods section, API and not only latex agglutination test contributed to identification. Line 157 and in a number of other places: missing space in "the17" Line 158, replace "...September), during ..." by "... September). During ..." Line 161, "Seven", "seven" Lines 168-169: about the sample recovered from a setting with a water heating system at maximum heating range (80-90°C). Was temperature checked (Lines 172-173 suggest that it is not the case)? What is the statistical significance, i.e. how many water systems are set to this temperature range, and is the recovery of a single positive lower than expected or higher? The temperature range associated with all 200 samples needs to be	In the band pattern comparisons, only band presence and positions were taken into account, not relative band intensities. As mentioned in line 123: "The RAPD-PCR profiles are defined only by the pattern of presence and position of bands on the gel" Although Gelcompar II software allows using both manual and automatic assigning of the band, the entire RAPD-PCR pattern's bands were automatically assigned by the software. The band shift allowance parameters settings were those of the Gelcompar II software default setting: The band position tolerance (the maximum shift allowed to consider any tow bands as matching) (position tolerance of 1%, an optimization of 1%, a change towards the end of fingerprint of 0% and minimum height and minimum surface of 0%). In lines 168-173 about the sample recovered from a setting with a water heating system at maximum heating range (80-90°C): As mentioned in the results and discussion section line 165 " most water heating systems in winter are set between 55°C to 60°C to prevent scalding, and the only isolate collected from water heating tank with temperature set at 80 – 90°C was considered statistical non significance due to the following: "When we analysed this isolate its genotypic behaviour was similar to other isolates collected from heating water tank with temperature set at 50 – 60°C, and to be sure whether this isolate can survive this elevated
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	indicated and analysed before so that suggestions such as lines 169-170 can be made based on the present data. In addition, this observation is not new, references need to be provided. Line 175, replace "in contradiction" by "in contrast" Line 178 replace "... Legionella, this may ..." by "... Legionella. This may ...". Line 181: "Several representative ..." How many? Seventeen according to Table 2. Please indicate in text. Line 226, Table 1, correct "Ligionella", Table 1 and Table 2 replace "And" by "and" in column heading Figure 1: make more self-explanatory by showing for each lane sample geographic origin and temperature range. Indicate on the figure that the Lsp lane is isolate n°9 (as in Figure 2). Line 262, correct "diversity"	temperature (80 – 90°C) a simple experiment was designed, this experiment involved inoculating this isolate on duplicate slants of CYE broth (charcoal yeast extract base) with BCYE growth supplement (ACES buffer/potassium hydroxyde, L-cysteine, ferric pyrophosphate, alpha-ketoglutarate) and MWY selective supplement (glycine, vancomycin, polymyxin B, anisomycin) and incubated at different temperatures ranging from 40–80°C for 24hr, after which the isolate was transferred to duplicate plates of CYE agar with BCYE growth supplement and MWY selective supplement and incubated at 35°C for 3–7 days to observe colony formation and it was confirmed that (NO growth was detected on plates inoculated with isolates grown for 24 hr over 60°C). (results not shown in the manuscript please advise on whether it should be added to the results and discussion section or not) Therefore it was suggested that that the temperature in the bottom of the tanks may be much lower than the set one resulted in the isolation of this strain" and as mentioned by the reviewer this is not considered to be a new observation as it has been previously reported (reference 10 and 22). The geographic origin and temperature range for each sample in figure one is already represented in Table 1, adding it to figure 1 to make it more self-explanatory will be repeating the results shown in table 1, also as explained in figure 1 legend Lsp lane referred to isolate No. 9.
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		All the minor revision corrections suggested by the reviewer were made inside the manuscript and highlighted in yellow as advised.
<u>Optional/General</u> comments	The authors have searched for the presence of <i>Legionella</i> spp in 200 samples of water collected in different residential areas in the city of Aqaba. Seventeen samples were positive, <i>Legionella pneumophila</i> represented 15 of the 17. The authors applied RAPD in order to produce a clustering of the isolates. They claim that clustering corresponds to the temperature of the water tanks. Unfortunately, although this kind of investigation is of interest, the technology applied in the investigation is not appropriate to support the main conclusions.	In this manuscript It is important to point out that, one of the limitations in this study, was the small number of the total <i>L. pneumophila</i> isolates, but despite the limited number of tested isolates, the results observed in this study should be viewed with concern and necessitates continuous monitoring through surveillance studies besides trying to put more effort in properly identifying the <i>L. pneumophila</i> serogroups and observe their genetic diversity using various DNA typing methods in future work.