Identification of differentially expressed *Legionella* genes during its intracellular growth in *Acanthamoeba*

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**ABSTRACT**

*Legionella* grows intracellularly in free-living amoebae as well as in mammalian macrophages. Until now, the overall gene expression pattern of intracellular *Legionella* in *Acanthamoeba* was not fully explained. Intracellular bacteria are capable of not only altering the gene expression of its host, but it can also regulate the expression of its own genes for survival. In this study, differentially expressed *Legionella* genes within *Acanthamoeba* during the 24 h intracellular growth period were investigated for comparative analysis. RNA sequencing analysis revealed 3,003 genes from the intracellular *Legionella*. Among them, 115 genes were upregulated and 1,676 genes were downregulated more than 2 fold compared to the free *Legionella*. Gene ontology (GO) analysis revealed the suppression of multiple genes within the intracellular *Legionella*, which were categorized under ‘ATP binding’ and ‘DNA binding’ in the molecular function domain. Gene expression of alkylhydroperoxidase, an enzyme involved in virulence and anti-oxidative stress response, was strongly enhanced 24 h post-intracellular growth. Amino acid ABC transporter substrate-binding protein that utilizes energy generation was also highly expressed. Genes associated with alkylhydroperoxidase, glucose pathway, and Dot/Icm type IV secretion system were shown to be differentially expressed. These results contribute to a better understanding of the survival strategies of intracellular *Legionella* within *Acanthamoeba*.

1. Introduction

*Legionella pneumophila* is an intracellular pathogen and the causative agent of a severe form of pneumonia known as the Legionnaires’ disease (Fields et al., 2002). *L. pneumophila* utilizes *Acanthamoeba* spp. as a host for survival and replication, but it can also thrive within human macrophage (Iovieno et al., 2010). Some species of *Legionella* cannot be cultured in vitro and requires co-cultivation within amoeba (Steinert et al., 1997). *Legionella* avoids the lysosomal degradation by forming a *Legionella*-containing vacuole (LCV), and this intracellular replication within *Acanthamoeba* could increase the virulence of *Legionella* (Gomes et al., 2018).

Several features of *Acanthamoeba* spp. makes it a suitable model organism for studying interactions between intracellular bacteria such as *L. pneumophila* and macrophages. Notably, the infection processes and the intracellular life cycle of *Legionella* within *Acanthamoeba* are similar to those demonstrated within human macrophages (Best and Abu Kwaik, 1998). These features include uptake of *Legionella* by coiling phagocytosis, formation of ribosome-studded phagosome containing *Legionella*, and inhibition of lysosomal fusion with phagosomes (Bozue and Johnson, 1996). Additionally, *L. pneumophila* uses similar genes to multiply in both hosts. The genes of the type IV secretion system required for intracellular growth in human macrophages are also required for intracellular growth in *A. castellanii* (Segal and Shuman, 1999). Pili aid in attachment of flagellated *L. pneumophila* to human macrophage and *A. polyphaga* (Stone and Abu Kwaik, 1998). The ankkyrin repeat protein B (AnkB) effector is necessary for intracellular proliferation in *Acanthamoeba* and human macrophage to form the LCV (Richards et al., 2013). Therefore, understanding how intracellular *L. pneumophila* interacts within *Acanthamoeba* could be key to unraveling the intracellular life cycle of *Legionella* and the mechanisms involved in human macrophages.

Multitudes of studies have been conducted to identify factors contributing to *L. pneumophila* pathogenicity in *Acanthamoeba*. CpxRA two-component system was reported to contribute significantly to its
vulnerence (Tanner et al., 2016). The nuclease activity of Cas2 protein in L. pneumophila was confirmed to be crucial for promoting amoebic infection (Gunderson et al., 2015). The pyroptosis-related gene, flaA, and apoptosis-related gene, vipd of Legionella were upregulated in growing A. castellanii (Mou and Leung, 2018). The Dot/Icm effector SdhA of Legionella, a virulence factor, was expressed highly in the macrophages upon Acanthamoeba infection (Gomes et al., 2018). Higher concentrations of pro-inflammatory cytokines in macrophages infected with Legionella were derived from a previous intracellular passage through Acanthamoeba (Gomes et al., 2018).

Recently, factors associated with survival and replication of L. pneumophila in Acanthamoeba are also being investigated since these are essential for its growth in human macrophage. Yet, the genome-wide expression analysis of an intracellular Legionella within Acanthamoeba remain unreported to date. To this extent, unraveling the regulation of gene expressions and their role in pathogenesis would have a significant impact on public health improvement. In this study, total transcriptional changes to genes involved in survival, replication, and virulence of L. pneumophila were investigated during its 24 h intracellular growth phase in A. castellanii through RNA sequencing analysis. This analysis demonstrated several genes involved in the survival of intracellular Legionella in Acanthamoeba.

2. Materials and methods

2.1. Cell cultures

Legionella pneumophila Philadelphia-1 strain (ATCC 33152) was cultured on BCYE (Buffered Charcoal Yeast Extract) agar plate at 37 °C with 5% CO2. Acanthamoeba castellanii Castellani (ATCC 30868) was cultured axenically in PYG (Proteose peptone-Yeast extract-Glucose) medium at 25 °C incubator.

2.2. Intracellular growth of Legionella

A. castellanii was infected with L. pneumophila as previously described (Mou and Leung, 2018). Monolayers of Acanthamoeba were incubated in T75 flask with 1 × 10^5 of Legionella at 37 °C with 5% CO2 for 1 h. After incubation, Acanthamoeba was washed with PAS (Page’s Amoeba Saline) and incubated with new PYG media containing 100 µg/ml of gentamicin for 2 h to kill extracellular Acanthamoeba. Legionella infected Acanthamoeba was washed with PAS twice and incubated with new PYG media for 24 h at 25 °C incubator. To visualize the Legionella within Acanthamoeba, Giemsa staining was used. For Giemsa stain, Legionella-infected Acanthamoeba was fixed with methanol for 5 min and stained with Giemsa solution for 10 min.

2.3. RNA isolation

A colony on BCYE agar plate was picked for free-living Legionella RNA extraction. For intracellular Legionella RNA, Legionella-infected Acanthamoeba (for 24 h) was incubated with 2 ml of dH2O for 10 min, and lysed by 20 forced passages through a 26 gauge syringe needle. The lysate was centrifuged at 150 g for 1 min for supernatant acquisition. Total RNA was purified using the RNeasy Mini kit (Qiagen, Hilden, Germany). RNA quality was assessed by Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, Netherlands), and RNA quantification was performed using ND-2000 Spectrophotometer (Thermo Inc., DE, USA).

2.4. Library preparation and sequencing

For Legionella and Acanthamoeba passaged Legionella RNAs, rRNA was removed using Ribo-Zero Magnetic kit (Epicentre, Inc., USA) from each 5 µg of total RNA. Construction of the library was performed using SMARTer Stranded RNA-Seq Kit (Clontech lab Inc., CA, USA) according to the manufacturer’s instructions. The rRNA-depleted RNAs were used for cDNA synthesis and shearing, following the manufacturer’s instruction. Indexing was performed using the Illumina indexes 1–12. The enrichment step was carried out using PCR. Subsequently, libraries were checked using the Agilent 2100 bioanalyzer (DNA High Sensitivity Kit) to evaluate the mean fragment size. Quantification was performed using the library quantification kit using a StepOne™ Real-Time PCR System (Life Technologies, Inc., USA). High-throughput sequencing was performed as paired-end 100 sequencing using HiSeq 2500 (Illumina, Inc., USA).

2.5. Data analysis

Legionella RNA sequence reads were mapped using Bowtie2 software tool in order to obtain the alignment file. Differentially expressed genes were determined based on counts from unique and multiple alignments using EdgeR within R using Bioconductor. The alignment file also was used for assembling transcripts. Quantile-Quantile normalization method was used for comparison between samples. Gene classification was based on searches done by DAVID (http://david.abcc.ncifcrf.gov/).

3. Results

3.1. Intracellular Legionella

Giemsa staining revealed intracellular L. pneumophila in A. castellanii at 24 h post infection (Figure 1B). In non-infected Acanthamoeba, Giemsa staining showed a nuclear and many mitochondras (Figure 1A). Survival and replication of Legionella within Acanthamoeba were observed until 24 h post-infection. The plasma membrane of Acanthamoeba remains intact for up to 24, however after 24 h post infection, Acanthamoeba was lysed and the culture media was contaminated with Legionella (data not shown).

3.2. Differentially expressed genes of Legionella during intracellular growth

To better understand the changes in gene expression profiles of intracellular Legionella in Acanthamoeba (L + A), RNA sequencing analysis was performed and the results were compared with those of free Legionella (L). A total of 3,003 genes from Legionella were selected, and 1,791 differentially expressed genes (DEGs) were identified. A large number of DEGs (1,676) were downregulated, and only 115 DEGs were upregulated. DEGs of intracellular Legionella were classified biological process, cellular component, and molecular function categories based on Gene Ontology (GO) analysis. GO analysis of upregulated genes was shown in Table 1. Among upregulated genes, drastic increase in the number of GO terms were not found. On the contrary, downregulated DEGs were subdivided into various GO terms under each of the GO categories (Table 2). Within the biological process category, 32 genes were involved in ‘transcription’. Analysis of the cellular component category revealed that 147 genes were involved in ‘cytoplasm’. In the molecular function category, 139 genes were involved in ‘ATP binding’, and 79 genes were involved in ‘DNA binding’.

3.3. Upregulated genes in intracellular Legionella

Genes upregulated more than 3.5 fold in intracellular Legionella during its 24 h growth phase in Acanthamoeba were listed in Table 3. Notably, lpg2350-alkylhydroperoxidase, lpg2349-alkylhydroperoxidase, and the lpg0491-amino acid ABC (ATP-binding cassette) transporter substrate binding protein were upregulated 11 fold, 5 fold, and 4 fold, respectively. The full list of 115 genes upregulated more than 2 fold was described in Supplementary Table 1. Proteins with potential relevance to lpg2350-alkylhydroperoxidase were searched by STRING database (Figure 2A). Ten proteins including AhpD (lpg2349-alkylhydroperoxidase) have been identified to be associated with lpg2350-
alkylhydroperoxidase, and their expression levels were summarized in a table (Figure 2B). A marked increase in lpg2350-alkylhydroperoxidase expression was observed during the 24 h intracellular growth. The lpg2349-alkylhydroperoxidase and lpg1815-hydrogen peroxide inducible genes activator OxyR inductions were also enhanced.

### 3.4. Glucose pathway and type IV secretion system of Legionella

To understand the intracellular survival and replication processes of *Legionella* in *Acanthamoeba*, the expression levels of genes associated with the glucose pathway and nutritional adaptation were investigated (Figure 3). STRING protein-protein association networks revealed that 12 proteins were associated with glucose metabolism in *Legionella* (Figure 3A). Of these 12, only 2 proteins lpg2486-phosphomannomutase and edd-phosphogluconate dehydratase were highly expressed while the remaining 10 were expressed at low levels following 24 h intracellular growth (Figure 3B). Expression levels of Dot/Icm type IV secretion system from *Legionella* which are required for intracellular growth and host cell lysis were summarized in Table 4. DotB and dotC were upregulated while dotD and all icm genes were downregulated during 24 h growth within *Acanthamoeba*.

### 4. Discussion

*Legionella* is an intracellular pathogen that controls the expression of various genes to survive and replicate in host cells. After entry into the macrophage, *L. pneumophila* replicated by 4 h within the LCV (Horwitz, 1983). By 18–24 h, *Legionella* is released from the LCV and remains in the cytoplasm of macrophages (Molmeret et al., 2004). Therefore, the time point 24 h post-infection with *L. pneumophila* was selected to investigate the factors associated with intracellular growth and killing of the host organism.

In this study, *L. pneumophila* within *A. castellanii* showed 1,676 differentially expressed genes (DEGs) by 24 h post-infection. In comparison to the free *L. pneumophila*, 115 DEGs were upregulated and 1,676 DEGs were downregulated. However, it is noteworthy to mention that...
culture condition, particularly temperature may have influenced the DEGs 24 h post-infection since *Legionella* were cultured at 37°C whereas *Acanthamoeba* were maintained at 25°C. Of the DEGs, *Legionella* gene with the highest upregulation following 24 h intracellular growth in *Acanthamoeba* was alkylhydroperoxidase (Table 3). Alkylhydroperoxidase is an enzyme acting to breakdown toxic peroxide compounds to alcohol and water, and the function is related to the oxidative stress response of several bacterial species (Paterson et al., 2006). In the case of *L. pneumophila*, antioxidant enzymes are of importance since the microbes become exposed to oxidative stress within the intracellular milieu of its host *A. castellanii* (LeBlanc et al., 2006). During the 24 h intracellular growth phase of *Legionella* within *Acanthamoeba*, alkylhydroperoxidases (lpg2350 and lpg2349) were upregulated, which may signify its importance for survival upon exposure to oxidative stress. Upregulation of several genes, including alkylhydroperoxide reductase are documented to be a feature reflecting *L. pneumophila* in its replicative phase (Brüggemann et al., 2006). In line with this notion, alkylhydroperoxide reductase was upregulated more than 5 fold in our study (Figure 2). The hydrogen peroxide-inducible genes activator (*OxyR*) was also upregulated nearly 2 fold in our study. Its overexpression in *L. pneumophila* has been associated with diminished growth as well as negative regulations of *icmR* and *cpxRA* in *A. castellanii* (Tanner et al., 2017). Consistent with this finding, the *icmR* expression in the *L*+*A* group deceased to 0.054 (Table 4). Interestingly, superoxide dismutase genes *sodB* and *sodC* were down-regulated in the *L*+*A* group whereas the *H₂O₂*-reducing AhpD and alkylhydroperoxidase were enriched. Previous studies have documented that catalase activity is severely lacking in *L. pneumophila*, which leaves them vulnerable to even low concentrations of *H₂O₂* (Hoffman and Pine, 1982; Hoffman et al., 1983). Based on these reports, we speculate that profuse levels of *H₂O₂* were present within

| GO category              | GO term                                                                 | Count |
|--------------------------|-------------------------------------------------------------------------|-------|
| Biological process       | GO:0006310—DNA recombination                                           | 16    |
|                         | GO:0009116—nucleotide metabolic process                                 | 8     |
|                         | GO:0030091—protein repair                                               | 6     |
|                         | GO:0043087—regulation of GTPase activity                                 | 6     |
|                         | GO:0006629—lipid metabolic process                                      | 14    |
|                         | GO:0006633—fatty acid biosynthetic process                              | 12    |
|                         | GO:0009405—pathogenesis                                                 | 10    |
|                         | GO:0019877—diaminosopimelate biosynthetic process                       | 5     |
|                         | GO:0006782—protoporphyrinogen IX biosynthetic process                   | 5     |
|                         | GO:0042619—poly-hydroxybutyrate biosynthetic process                    | 6     |
|                         | GO:0071555—cell wall organization                                       | 13    |
|                         | GO:0006099—tricarboxylic acid cycle                                     | 10    |
|                         | GO:0009073—aromatic amino acid family biosynthetic process              | 7     |
|                         | GO:0007049—cell cycle                                                  | 11    |
|                         | GO:0008299—isoprenoid biosynthetic process                              | 4     |
|                         | GO:0006281—DNA repair                                                  | 15    |
|                         | GO:0006351—transcription, DNA-templated                                 | 32    |
|                         | GO:0008652—cellular amino acid biosynthetic process                     | 9     |
|                         | GO:0009425—NAD biosynthetic process                                     | 5     |
|                         | GO:0009231—riboflavin biosynthetic process                              | 5     |
|                         | GO:0016226—iron-sulfur cluster assembly                                 | 5     |
|                         | GO:0006400—tRNA modification                                            | 5     |
| Cellular component      | GO:0042597—periplasmic space                                           | 9     |
|                         | GO:0005737—cytoplasm                                                   | 147   |
|                         | GO:0005622—intracellular                                               | 29    |
|                         | GO:0009424—bacterial-type flagellum hook                                | 4     |
| Molecular function      | GO:0003700—transcription factor activity, sequence-specific DNA binding | 24    |
|                         | GO:0008270—zinc ion binding                                            | 32    |
|                         | GO:0051536—iron-sulfur cluster binding                                 | 10    |
|                         | GO:0008113—peptide-methionine (S)-S-oxide reductase activity            | 6     |
|                         | GO:0016829—lyase activity                                              | 11    |
|                         | GO:0003684—damaged DNA binding                                         | 7     |
|                         | GO:0005524—ATP binding                                                 | 139   |
|                         | GO:0004222—metalloendopeptidase activity                               | 13    |
|                         | GO:0001555—phosphorelay sensor kinase activity                         | 12    |
|                         | GO:0008237—metallopeptidase activity                                    | 9     |
|                         | GO:0050660—flavin adenine dinucleotide binding                          | 20    |
|                         | GO:0003677—DNA binding                                                 | 79    |
|                         | GO:0003676—nucleic acid binding                                        | 20    |
|                         | GO:0033743—peptide-methionine (R)-S-oxide reductase activity            | 4     |
|                         | GO:0016987—sigma factor activity                                       | 5     |
|                         | GO:0015197—peptide transporter activity                                | 5     |
|                         | GO:0016746—transferase activity, transferring acyl groups              | 10    |
|                         | GO:0004553—hydrolase activity, hydrolyzing O-glycosyl compounds        | 6     |

Table 2. Gene ontology analysis of downregulated genes in *Legionella*.
Table 3. Genes upregulated more than 3.5 fold in *Legionella*.

| Gene symbol | Fold change | Annotation |
|-------------|-------------|------------|
| lpg2350     | 11.029      | alkylhydroperoxidase product |
| lpg2857     | 9.470       |            |
| lpg2124     | 7.240       | hypothetical protein |
| lpg1053     | 5.912       | ATP synthase F0F1 subunit epsilon |
| lpg2753     | 5.890       | 16S ribosomal RNA |
| lpg0572     | 5.165       | hypothetical protein |
| lpg2349     | 5.046       | alkylhydroperoxidase |
| lpg0689     | 4.995       | DNA binding stress protein |
| lpg1706     | 4.916       | arginine N-succinyltransferase subunit beta |
| lpg1940     | 4.882       | peptide synthetase |
| lpg0491     | 4.237       | amino acid ABC transporter substrate-binding protein |
| lpg0594     | 4.185       | hypothetical protein |
| nuoM        | 3.971       | NADH dehydrogenase I subunit M |
| lpg0797     | 3.828       | tRNA-Met |
| lpg0146     | 3.807       | transposase B, TnpA |
| mutL        | 3.715       | DNA mismatch repair protein MutL |
| lpg0166     | 3.655       | hypothetical protein |
| lpg0433     | 3.637       | hypothetical protein |
| lpg0169     | 3.554       | hypothetical protein |
| lpg0705     | 3.527       | transporter component |
| lpg0039     | 3.503       | hypothetical protein |

**Figure 2.** The STRING network of alkylhydroperoxidase and their expression levels. The gene networks of lpg2350 alkylhydroperoxidase and lpg2349 alkylhydroperoxidase of *Legionella* generated by the STRING database (A). Colored lines between the gene/protein indicate the various type of interaction evidence. The expression levels of associated genes with alkylhydroperoxidase were summarized in table (B).

**Figure 3.** Network visualization of glucose pathway in *Legionella* and their expression levels. Network by STRING showed an interaction among the genes associated with glucose metabolism in *Legionella* (A). The expression levels of twelve genes involved in the glucose pathway were present in table (B).
A. castellanii, which forced L. pneumophila to overexpress H$_2$O$_2$-reducing enzymes to cope with this environmental stress. A key component required for the intracellular growth of Legionella is glucose metabolism. Intracellular L. pneumophila metabolizes glucose through the Entner-Doudoroff (ED) pathway, and this metabolic process involves glucokinase (glk), glucose-6-phosphate dehydrogenase (zwf), 6-phosphogluconolactonase (pgl), phosphogluconate dehydratease (edd), and 2-dehydro-3-deoxy-phosphogluconate aldolase (eda) (Harada et al., 2010). DEGs analysis in this study confirmed that edd was highly expressed 24 h post-infection (Figure 3). Earlier studies have confirmed the expression of Entner-Doudoroff pathway during the replicative phase of L. pneumophila in vivo (Brüggemann et al., 2006) and consistent with this finding, upregulation of edd gene was observed in the L + A group. From these findings, it is assumed that edd gene of Legionella plays an important role in its growth within Acanthamoeba.

The intracellular life cycle of Legionella is comprised of the replicative phase and the transmissive phase (Byrne and Swanson, 1998). During the replicative phase, Dot/ICm type IV secretion system of L. pneumophila can manipulate host cellular functions to support its intracellular growth in the LCV (Goers et al., 1999). Protein translocation by the Dot/ICm complex could occur not only through host phagosomal membranes but also from one bacterial cell to another bacterial cell (Luo and Isberg, 2004). DotB and icmE are important for conjugation-mediated genetic exchange in L. pneumophila (Swanson and Hammer, 2000). Recently, a similar study investigating the differential gene expressions of L. pneumophila reported strong upregulation of dotC, dotA, icmW, and several other genes involved in stress response during the transmissive phase of L. pneumophila (Weissenmayer et al., 2011). Similar to the previous findings, our results confirmed increased dotB and dotC expressions as well several oxidative stress-related genes from Legionella 24 h post-infection in Acanthamoeba (Figure 2 and Table 4).

In this study, enrichment of genes associated with pathogenesis was not confirmed. One explanation for the lack of bacterial virulence as observed in the present study stems from the temperature used to culture L. pneumophila. Culture condition, especially temperature, is one of the factors affecting the virulence of L. pneumophila (Swanson and Hammer, 2000). Evidently, L. pneumophila cultured at 24 °C was less virulent than the L. pneumophila cultured at 37 °C (Mauchline et al., 1994). Based on this notion, the virulence-associated genes of L. pneumophila may have been induced to a lesser extent in our study since they were incubated within Acanthamoeba at 25 °C for 24 h.

In summary, we demonstrated the gene expression patterns of L. pneumophila 24 h post-infection in A. castellanii. Protection against oxidative stress is one of the most important aspects required by Legionella to remain viable within Acanthamoeba. More studies involving oxidative stress and antioxidant defense system of Legionella are necessary to understand the intracellular growth of Legionella with Acanthamoeba. In addition to this, significantly increased or decreased genes of Legionella during intracellular growth need more research. Acanthamoeba and human macrophage share similar features that permit the intracellular growth of Legionella and therefore, further investigating the genes of Legionella used for survival and replication within Acanthamoeba could provide important information to understanding its lifestyle and pathogenicity in human macrophage.

**Declarations**

**Author contribution statement**

Fu-Shi Quan: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Hyun-Hee Kong: Conceived and designed the experiments; Analyzed and interpreted the data.

Hae-Ahm Lee: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ki-Back Chu: Analyzed and interpreted the data; Wrote the paper.

Eun-Kyung Moon: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Table 4. Expression levels of Dot/ICm type IV secretion system.

| Gene symbol | Fold change | Annotation | Product |
|-------------|-------------|------------|---------|
| Region I    |             |            |         |
| icmV        | 0.062       | intracellular multiplication protein IcmV |         |
| icmW        | 0.408       | intracellular multiplication protein IcmW |         |
| icmX        | 0.262       | intracellular multiplication protein IcmX |         |
| dotA        | 0.944       | defect in organelle trafficking protein DotA |         |
| dotB        | 1.279       | ATPase     |         |
| dotC        | 2.330       | DotC       |         |
| dotD        | 0.180       | lipoprotein DotD |         |
| Region II   |             |            |         |
| icmF        | 0.171       | IcmF protein |         |
| icmG        | 0.075       | IcmG protein |         |
| icmH        | 0.054       | IcmH protein |         |
| icmI        | 0.109       | IcmI protein |         |
| icmJ        | 0.145       | IcmJ protein |         |
| icmK        | 0.180       | IcmK protein |         |
| icmL        | 0.249       | IcmL protein |         |
| icmM        | 0.160       | IcmM (DotU) |         |
| icmN        | 0.345       | IcmN protein |         |
| icmP        | 0.296       | IcmP protein |         |
| icmQ        | 0.617       | IcmQ protein |         |
| icmR        | 0.103       | hypothetical protein |         |
| icmS        | 0.075       | IcmS protein |         |
| icmT        | 0.180       | IcmT protein |         |

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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