

## Prevalence study of *Simkania negevensis* in cooling towers in Spain

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### ABSTRACT

*Simkania negevensis* is an obligate intracellular bacterium grouped into the order Chlamydiales. This new amoeba-resistant intracellular bacterium might represent a novel etiologic agent of bronchiolitis and community-acquired pneumonia and occurs in aquatic habitats such as drinking water and reclaimed wastewater. Another amoeba-related bacterium, *Legionella pneumophila*, is an etiologic agent of pneumonia transmitted by environmental aerosols or contaminated water/air cooling systems. These transmission pathways are important in the natural history of *Legionellae* infections and possibly other intracellular microorganisms such as *Parachlamydiaceae*; thus, understanding the feasibility of *Simkania* infection by these routes is relevant. In the present work, we investigated the prevalence of this newly identified pathogenic bacterium in cooling towers by quantitative PCR (qPCR) and its possible relationship with *Legionella pneumophila* co-infection. Our results show *Simkania* detection in 2 of 70 cooling towers analyzed. To our knowledge, this report is the first describing *Simkania negevensis* detection in this category of environmental water samples.

**Key words** | cooling towers, environmental water samples, real time PCR, *Simkania negevensis*

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### INTRODUCTION

*Simkania negevensis* is an obligate, intracellular, gram-negative bacterium that shares several characteristics with the Chlamydiae, especially those related to its growth cycle (Kahane *et al.* 1993, 1995, 2002). To this point, only one strain has been recognized, Z<sup>T</sup> (ATCC VR 1471<sup>T</sup>), initially called “Z”. Taxonomic studies have grouped this strain into a new bacterial family, *Simkaniaceae*, in the order Chlamydiales, based on its intracellular bimorphic parasitic growth in cultured cells and on rDNA sequence comparisons (Everett *et al.* 1999a, b; Kahane *et al.* 1999). Its full-length 16S and 23S rDNA sequences are 80% and 87%, respectively, identical to

those from members of the *Chlamydiaceae* family (Everett *et al.* 1999a, b).

*S. negevensis* has also been considered as the etiological agent for several respiratory tract infections in humans, including bronchiolitis in infants (Kahane *et al.* 1998) and community-acquired pneumonia and acute exacerbation of chronic obstructive pulmonary disease in both adults and children (Lieberman *et al.* 1997, 2002; Petrich *et al.* 2002; Greenberg *et al.* 2003; Fasoli *et al.* 2008). It is important to note that the determination of the prevalence of *Parachlamydia* and *Chlamydia*-like organisms has recently been the

subject of several studies, in part as a consequence of its association with lung infection (Casson *et al.* 2008; Corsaro *et al.* 2009; Greub 2009).

Serological evidence of past exposure to *S. negevensis* has been well documented, indicating that natural infection with this pathogenic microorganism seems to be highly prevalent worldwide (Friedman *et al.* 1999, 2003; Kumar *et al.* 2005; Yamaguchi *et al.* 2005; Baud *et al.* 2009). Recently, it has been suggested that *S. negevensis* could be a ubiquitous microorganism present in most aquatic habitats such as drinking water and reclaimed wastewater because of its relative resistance to chlorination (Kahane *et al.* 2004, 2007) and its ability to replicate and survive over long periods of time in amoebic cysts (Kahane *et al.* 2001). Moreover, Friedman *et al.* (2006) reported that *Simkania* typically found in domestic water supplies was the same organism isolated from patients with lower respiratory tract infection and suggested that water itself may possibly have been the source of these *Simkania* infections.

Similar transmission of Legionellae has been previously described (Fallon & Rowbotham 1990). In fact, amoebae are thought to play an important role in the natural history of infections with Legionellae and may play a similar role in other intracellular microorganisms such as the Parachlamydiaceae (Harb *et al.* 2000; Swanson & Hammer 2000; Greub & Raoult 2002). However, in the case of Legionnaire's disease, transmission by environmental aerosols or contaminated water/air conditioning systems, such as cooling towers, is also possible (Stout & Yu 1997; Pagnier *et al.* 2009).

To date, no studies are available from Spain or elsewhere in Europe specifically addressing the epidemiology of *Simkania negevensis*. For this reason, the aim of the present work was to investigate the potential existence of this bacterium in cooling water towers to determine if these sources may be environmental reservoirs.

## MATERIALS AND METHODS

### Water sampling

Water samples from 70 cooling towers in 17 Spanish cities were collected during a two-week period. From each cooling tower, 2 L of water were taken in sterile plastic containers,

supplemented with 2 ml of sodium thiosulfate (3%). Selection of sampling points was carried out with the main criterion of locating a practicable area on the returning line. As a consequence, water was usually sampled in the tower basin below the filling material.

### Water concentration and DNA extraction

One liter of sample was concentrated by membrane filtration using a nylon or cellulose membrane (0.45 µm porous diameter; Millipore, MA, USA). Cells were resuspended in 10 ml of sterile saline solution and vigorously vortexed for 60 s. Two milliliters of the resulting suspension were concentrated to 200 µl by centrifugation (10000 × g, 5 min) and DNA was extracted with the Omega Biotek Tissue DNA Kit (D3396-02, Norcross, GA, USA) according to the manufacturer's instructions.

### Microbiological analysis

*Legionella pneumophila* levels were analyzed by microbiological culture according to the ISO11731:1998(E) standard (Water quality – Detection and enumeration of *Legionella*). Briefly, 0.2-ml aliquots of the original and concentrated samples (heat treated, acid treated, and untreated) were spread on GVPC agar plates (Oxoid Ltd., Basingstoke, Hampshire, UK). The plates were incubated at 36°C in a humidified environment for at least 10 d and examined on days 3, 5, and 10 with a plate microscope. For each plate, at least three colonies containing possible *Legionella* were subcultured on BCYE (with cysteine) and CYE (cysteine-free) media (Oxoid Ltd.) for 3 d. Colonies grown on BCYE were subsequently identified by an agglutination test (*Legionella* latex test; Oxoid Ltd.). The detection limit was defined according to the Spanish quality technical regulations for cooling towers and corresponded to 100 CFU/L.

*Simkania negevensis* levels were analyzed by quantitative PCR (qPCR). The specific primers and probes used for DNA amplification were those described by Johnsen *et al.* (2005). Quantification was performed using real-time PCR with the LightCycler 1.5 PCR system (Roche Applied Science, Mannheim, Germany). The reaction mixture, 20 µl in total, consisted of 10 µl of Fast Start SYBR Green (Roche Applied Science) and 10 µl of sample, giving a final concentration of

0.5  $\mu\text{mol/L}$  of reverse (5'-GAG CTC CGG AAT TTC ACA TCT G-3') and forward primers (5'-AAA GGT AAC GAA TAA TTG CCT-3'). The experimental LightCycler protocol was one 10-min step at 95°C for Taq polymerase activation, 45 cycles of PCR amplification (95°C for 15 s, 55°C for 30 s for annealing, and 72°C for 30 s for elongation), and a final melting temperature ramp from 65°C to 95°C at 0.1°C per second.

A positive control of *Simkania* DNA (kindly provided by Dr Friedman from the Department of Virology and Genetic Development of the Faculty of Health Sciences, University of Negev, Israel) was used as a standard for DNA quantification. Serial logarithmic dilutions were carried out from  $10^1$  to  $10^4$  cells/reaction and used to construct a calibration curve. All measurements were performed in duplicate, and the efficiency of the amplification was 1.92. Samples were considered to be *negative* when there was no amplification or when the *Tm* (melting temperature) was outside of the range established in previous amplifications of the negative control (PCR-grade double-distilled water; Eppendorf, Hamburg, Germany) and the positive control (*S. negevensis* strain). Samples were considered to be *positive* when at least one of the replicates showed a *Tm* that fell within the positive control curve. Finally, samples were considered to be positive but not quantifiable when the amplification yielded a *Tm* inside the positive range but with a *Ct* over 40. The theoretical quantification limit was 500 cells/L calculated by the dilution method assuming a direct equivalence between the target copy and the cell. The qPCR products from positive samples were run on a 2% agarose gel using E-gel Base system equipment (Invitrogen, USA), following the manufacturer's instructions, and confirmed by DNA sequencing (Secugen, Madrid, Spain) after purification with the Cycle Pure Kit (Omega Bio-Tek, Norcross, GA, USA). DNA sequences were analyzed using the basic local alignment search tool (BLAST) (Altschul et al. 1997) at the NCBI server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Different researchers completed the *Legionella pneumophila* and *Simkania negevensis* analyses, and the results were not known until the end of the experiment.

## RESULTS

From the 70 cooling tower samples processed, two were positive for *Simkania negevensis* (2.85%) and four for *Legionella*

*pneumophila* (5.70%). For *Simkania negevensis*, only one sample showed a quantifiable level of  $6.7 \times 10^3$  cells/L; the other one was positive but not quantifiable, as defined above ( $<500$  cells/L). The sequencing results of the positive controls displayed a 100% identity to the corresponding 16S rRNA from *Simkania negevensis* Z strain (accession number NR 029194). The sequences of the PCR products obtained from the two positives cooling tower samples showed 99% (E value  $5e-54$ ) and 100% (E value  $5e-50$ ) identity with the target reference.

In the case of *Legionella pneumophila*, the results were  $1.0 \times 10^2$ ,  $2.0 \times 10^2$ ,  $3.0 \times 10^2$ , and  $3.6 \times 10^2$  CFU/L, respectively. None of the samples was simultaneously positive for both microorganisms. In both positive cases of *Simkania negevensis*, the samples were collected from different industries and cities.

## DISCUSSION

Because of the potential similarity between infection pathways for *Legionella* and *Simkania*, we decided to evaluate the presence of this *Chlamydia*-like pathogen in water from cooling towers. The prevalence of *Simkania negevensis* in our samples was very low (2.85%). Although *Simkania negevensis* is reported to be ubiquitous in water systems (Kahane et al. 2004, 2007), it may be that the presence of this pathogenic bacterium in the analyzed samples was below our detection limit.

As expected, because of the intensive control of cooling towers by Spanish public health authorities, the prevalence of *Legionella pneumophila* was also low (5.70%). This outcome is likely the result of good management programs focused on continuous water treatment using biocides, such as chlorine. For this reason, in cooling towers with appropriate water treatment programs, the colonization prevalence of these pathogenic microorganisms is usually low.

We also attempted to determine whether a relationship exists between *S. negevensis* and *L. pneumophila* presence because of the connection of legionellosis outbreaks from water samples of this type and the two pathogen's relationship with respiratory tract infections (Stout & Yu 1997; Swanson & Hammer 2000). This hypothesis could not be addressed because of the small percentage of either type of

bacteria measured in the cooling towers. Systems that are under tight control may not usually represent a significant sanitary risk for the development of pulmonary infectious diseases caused by these microorganisms. Thus, the risk of contagion from *Simkania* by other infection routes and its effect on public health as a potential opportunistic pathogen remain unclear.

In conclusion, efficient management treatments to avoid colonization of cooling towers by *Legionella* seem to have positively affected microbial water quality and may have reduced the presence of another emergent pathogenic microorganism, *Simkania negevensis*, to a non-detectable level. We did, however, detect *Simkania* DNA in 2 of 70 cooling towers analyzed, the first report of *Simkania* detection in this type of environmental water sample in Spain.

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