What is the true clinical relevance of *Simkania negevensis* and other emerging *Chlamydiales* members?

M. Vouga1,2, C. Kebbi-Beghdadi1, J. Liénard2, L. Baskin4, D. Baud1 and G. Greub2,3

1) Materno-fetal and Obstetrics Research Unit, Department “Femme-Mère-Enfant”, University Hospital, Lausanne, 2) Center for Research on Intracellular Bacteria, Institute of Microbiology, Faculty of Biology and Medicine, University of Lausanne, 3) Infectious Disease Service, University Hospital, Lausanne, Switzerland and 4) Virology Laboratory, Rambam Health Care Campus, Haifa, Israel

**Abstract**

*Waddlia chondrophila* and *Simkania negevensis* are emerging *Chlamydia*-related bacteria. Similar to the pathogenic organisms *Chlamydia pneumoniae* and *Chlamydia trachomatis*, these emerging bacteria are implicated in human genital infections and respiratory diseases. We used a screening strategy based on a newly developed *S. negevensis*–specific quantitative real-time PCR (qPCR) and a pan-*Chlamydiales* qPCR. We could not detect *S. negevensis* in 458 respiratory, genitourinary, cardiac and hepatic samples tested. One urethral swab was positive for *W. chondrophila*. We observed a low prevalence of *Chlamydiales* in respiratory samples (1/200, 0.5%), which suggests that *C. pneumoniae* is an uncommon respiratory pathogen. Furthermore, we screened 414 human serum samples from Switzerland, England and Israel and observed a low prevalence (<1%) of exposure to *S. negevensis*. Conversely, humans were commonly exposed to *W. chondrophila*, with seroprevalences ranging from 8.6% to 32.5%. *S. negevensis* is not a clinically relevant pathogen, but further research investigating the role of *W. chondrophila* is needed.

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**Corresponding author:** G. Greub, Center for Research on Intracellular Bacteria, Institute of Microbiology, Faculty of Biology and Medicine, University of Lausanne, Bugnon 48, CH-1011 Lausanne, Switzerland.

E-mail: gilbert.greub@chuv.ch

The last two authors contributed equally to this article, and both should be considered senior author.

**Introduction**

*Chlamydiales* are strict intracellular bacteria characterized by a biphasic developmental cycle. Well-known members include *Chlamydia trachomatis* and *Chlamydia pneumoniae*, which are associated with genital infections and respiratory diseases, respectively. Over the last decades, several emerging members have been isolated, such as *Waddlia chondrophila*, *Parachlamydia acanthamoebae* and *Simkania negevensis*. These may constitute a potential threat to human health: *W. chondrophila* has been documented as a potential agent of miscarriage [1,2], and *P. acanthamoebae* could be implicated in respiratory diseases [3]. *S. negevensis* was discovered in Israel in 1993 [4]. Little is known about the biology and the clinical importance of this novel bacterium, but evidence of human exposure has been reported worldwide [5], with seroprevalence increasing with age, reaching up to 70% to 80% in some Middle Eastern populations [6,7]. Several studies have shown an association of acute *S. negevensis* infection with respiratory diseases, in particular bronchiolitis and pneumonia [5]. Nevertheless, its true clinical relevance remains controversial as a result of the low prevalence of confirmed cases and the low reliability of the diagnostic tools used in most early studies. Furthermore, its ability to grow in endometrial cells suggest that *S. negevensis* could be implicated in genital infections, much like *C. trachomatis* [7]. Like other intracellular bacteria, *S. negevensis* can only be detected by molecular techniques, such as PCR, or through cell co-culture. It remains undetectable by routinely used diagnostic
methods. Its prevalence in clinical settings could thus be underestimated. Therefore, we sought to further define the clinical importance of this emerging bacterium.

**Materials and methods**

**Patients and samples**

DNA samples. We analysed 458 different clinical samples of different origins: (a) 91 nasopharyngeal swabs from children with symptoms compatible with bronchiolitis, among which 11 were positive for respiratory syncytial virus, (b) 200 bronchoalveolar lavage (BAL) samples from both adults and children who possibly had lung infections, which were negative for other common pathogens (samples originated from the internal medicine ward, emergency room, intensive care unit or pulmonary service), (c) 22 urethral samples from both men and women and (d) 135 cervicovaginal swabs. In addition, one cardiac biopsy (aortic valve) sample and nine hepatic samples were tested. The study was approved by the ethical committee of Vaud canton, Switzerland (216-15, approved 13 July 2015).

Human serum samples. We used serum samples that had been collected during previous seroprevalence studies. These samples included the following: (a) 101 samples from female patients, 36 with uneventful pregnancies, 48 with recurrent miscarriages and 17 with sporadic miscarriage from the Recurrent Miscarriage Clinic of St Mary’s Hospital (age, 25–39 years) [2]; (b) 132 patients with acute miscarriages from Lausanne University Hospital (mean age, 34 ± 6 years) [1]; and (c) 105 serum samples from asymptomatic young men at the time of army recruitment (age, 18–26 years) [8–10]. Finally, 76 serum samples taken from adult patients (mean age, 54 ± 16 years) from Rambam Health Care Campus, Haifa, Israel, were provided by Z. Kra-Oz. The gift was approved by the local ethic committee.

DNA extraction

DNA was extracted from the samples by the microbiology diagnostic laboratory of Lausanne University Hospital using the MagNA Pure 96 automated system (Roche, Rotkreuz, Switzerland) as previously described [11].

Quantitative real-time PCR

We developed a specific *Simkania negevensis* quantitative real-time PCR (qPCR) using an approach similar to the one routinely used in the molecular diagnostic laboratory of Lausanne University Hospital [11], and we followed the MIQE Guidelines [12]. Using Geneious 5.0.3 and primer3Plus software, specific primers and hydrolyzing probe (TaqMan) targeting the 16S rRNA gene of *Simkania negevensis* strain Z (ATCC VR1471) were developed. The following primers were chosen, amplifying a 125 bp fragment: forward primer, 5'–ACC-TCT-TAC-CTG-GGG-ATA-AGC-GTT-GG-3'; reverse primer, 5'–CCA-TGA-GCC-TCA-CCG-CA-3'; and probe, 5'–FAM(6-carboxyfluorescein)-GA*G-AGC-T*GG-GGT-AGC-CTG*-GTT-TCT- BHQ1(Black Hole Quencher 1)-3’. Locked nucleic acids were added in the probe, as noted by an asterisk, to ensure higher specificity. PCR reactions were performed with 0.4 μL each of primers and probe (Eurogentec, Seraing, Belgium), 10 μL iTaq Supermix with ROX (Bio-Rad, Reinach, Switzerland) and 5 μL of DNA sample in a final volume of 20 μL. The cycling conditions were 3 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. The PCR products, tested in duplicate, were detected with a StepOne instrument (Applied Biosystems, Zug, Switzerland) or QuantStudio instrument (Applied Biosystems) when 96- or 384-well plates were used, respectively. DNA-free water (PanReac; AppliChem, Darmstadt, Germany) was used as a negative PCR control. The specificity of the reaction was evaluated using DNA extracted from common respiratory and genitourinary bacteria and viruses as well as from the amoeba *Acanthamoeba castellani* and from several *Chlamydia*-related bacteria (Supplementary Table S1). Bacterial DNA was diluted at 1 ng/μL. An inhibition test was made using 4 μL of the tested species and 1 μL of the control plasmid at 10^6/μL copies to ensure the absence of inhibitors. Inhibition was considered when <50 copies were amplified.

The PCR assay’s performance was evaluated in 16 different runs and exhibited a good interrun reproducibility, with a Cq value of approximately 20.89 for 10^5 copies, high repeatability with a correlation coefficient of 0.9950 and a 95% confidence interval of 0.74 cycles between duplicates (Supplementary Fig. S1). The limit of detection was lower than 5 copies. Mean efficiency of the calibrating experiments was 98.7% ± 2.8. In clinical experiments, the previously described pan-Chlamydia PCR [13] was used as positive control.

**Microimmunofluorescence**

Microimmunofluorescence was performed by two different protocols: firstly using formalin-inactivated bacteria (Elementary Bodies (EBs) of *Simkania negevensis* strain Z, *W. chondrophila* strain WSU 86-1044 and *Parachlamydia acanthamoebae* strain Hall coccus, respectively), as described elsewhere [14], and secondly using heat-inactivated bacteria (EBs of *Simkania negevensis* strain Z, *W. chondrophila* strain WSU 86-1044 and *Parachlamydia acanthamoebae* strain BN9), as described elsewhere [2]. Serum samples were screened in duplicates for total IgH at a dilution of 1:32 and 1:64 using a goat anti-human IgH fluorescein-conjugated antibody (Fluoline H; bioMérieux, Marcy l’Étoile, France) diluted 1:400. MIFs were read blindly by two
Organisms identified by novel qPCR analysis.

Samples (n = 458) were screened using specific S. negevensis qPCR developed in this study and previously described pan-Chlamydiales qPCR [13], both based on TaqMan technology. Inhibition was excluded by an internal control routinely performed in our diagnostic laboratory. Nevertheless, six samples exhibited doubtful internal controls, a technique suspected to be less specific than formalin-inactivated bacteria [14].

In contrast, a high seroprevalence of W. chondrophila was observed, a finding which was in line with previous reports (9/105–13/40, 8.6–32.5%) (Table 2) [1,8,14]. The seroprevalence of P. acanthamoebae was low, as previously described (0/105–1/36, 0–2.8%) (Table 2) [1,8,14].

**Discussion**

Using a large screening strategy based on both this new and highly specific qPCR and the broad range pan-Chlamydiales PCR, we could not detect S. negevensis in nasopharyngeal or BAL samples of children and adults with suspected respiratory infections, as well as in genitourinary, cardiac and hepatic samples. Our results contrast with previous PCR-based studies which suggested an association with acute respiratory tract infections [18,19]. Indeed, a significant association was shown in children with bronchiolitis in a study performed in Israel in which 25% of the children were positive for *Simkia* [18].

**Results**

*S. negevensis* was detected in none of the 458 DNA samples using our newly developed qPCR (Table 1). However, using the pan-Chlamydiales PCR, we identified 15 positive *Chlamydiales* samples, all of genitourinary origin; 14 samples were also positive using a specific *C. trachomatis* PCR. The remaining one was confirmed to be positive for *W. chondrophila* using the specific *W. chondrophila* PCR [16]. In addition, 17 samples were considered doubtful (one well out of two positive). After performing a second test for these 17 samples, only one of them, from a BAL sample, was considered positive (three out of four wells positive; mean Cq = 39). Unfortunately, further identification of the corresponding family-level lineage could not be achieved because of the lack of remaining material for subsequent analysis. Inhibition was excluded by an internal control routinely performed in our diagnostic laboratory. Nevertheless, six samples exhibited doubtful internal controls and were therefore retested using 4 μL of the tested species and 1 μL of the control plasmid at 10⁴ μL. No inhibition was observed. Our PCR assay appears to be specific for *S. negevensis* at the species level, as demonstrated by the absence of amplification of four DNA samples isolated from ticks and assigned to the Simkaniaceae family by sequencing of the 16S rRNA gene region amplified with the pan-Chlamydiales PCR (data not shown) [17].

Congruent with molecular data, we observed an extremely low seroprevalence of *S. negevensis* (2/414, <1%) using our microimmunofluorescence protocol (Table 2). Interestingly, the two positive serum samples were identified using heat-inactivated bacteria, a technique suspected to be less specific than formalin-inactivated bacteria [14].

**TABLE 1.** Organisms identified by novel qPCR analysis. Samples (n = 458) were screened using specific *S. negevensis* qPCR developed in this study and previously described pan-Chlamydiales qPCR [13], both based on TaqMan technology. Inhibition was excluded by an internal control routinely performed in our diagnostic laboratory. Nevertheless, six samples exhibited doubtful internal controls, a technique suspected to be less specific than formalin-inactivated bacteria [14].

| Sample                  | Simkania negevensis | Chlamydiales | Organisms identified |
|-------------------------|---------------------|--------------|---------------------|
| Bronchoalveolar lavage  | 0/200               | 1/200        | ?                   |
| Nasopharyngeal aspirate | 0/91                | 0/91         |                     |
| Cardiac biopsy          | 0/1                 | 0/1          |                     |
| Hepatic biopsy          | 0/9                 | 0/9          |                     |
| Cervicovaginal swab     | 0/135               | 10/135       | *Chlamydia trachomatis* (n = 10) |
| Urethral swab           | 0/22                | 5/22         | *C. trachomatis* (n = 4), Waddlia chondroba (n = 1) |

qPCR, quantitative real-time PCR. *Includes 11 samples positive for respiratory syncytial virus.*
We found only one sample positive for a Chlamydiales bacterium (BAL sample) (1/200, 0.5%) and a complete absence of members of this order in nasopharyngeal aspirates (0/91); in particular, no C. pneumoniae DNA was detected. This low prevalence correlates with several other European studies describing a prevalence of C. pneumoniae infection of <2% [21,35,36]. The molecular detection rate of this recognized pathogen in respiratory samples does not significantly differ from detection rate of Parachlamydiaceae [37–39]. Nevertheless, C. pneumoniae remains a well-established agent of respiratory diseases, sometimes causing outbreaks [40,41].

In conclusion, we found strong evidence for low human exposure to S. negevensis and confirmed that it is not an important human pathogen. We also observed a low prevalence of C. pneumoniae infection. This work further supports common human exposure to W. chondrophila and encourages research investigating the role of this emerging pathogen.

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