ORIGINAL ARTICLE

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

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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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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

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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-ACG-GTT-GG-3′; reverse primer, 5′-CCA-TGA-GCC-TCT-CTA-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 castellanii* 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 10^4/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 C_q 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-*Chlamydiales* 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

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

Sample	*Simkania negevensis*	*Chlamydiales*	Organisms identified
Bronchoalveolar lavage	0/200	1/200	?
Nasopharyngeal aspirate[a]	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 chondrophila* (*n* = 1)

qPCR, quantitative real-time PCR.
[a]Includes 11 samples positive for respiratory syncytial virus.

independent readers under an epifluorescence microscope (Axioplan 2; Zeiss, Feldbach, Switzerland) at a magnification of ×1000, and was scored 0 if negative, 0.5 if doubtful or 1 if positive by each reader, as previously described [14]. Scores from 0 to 0.5 were considered negative, 1 to 1.5 doubtful low, 2 to 2.5 doubtful high and 3 to 4 positive. Cutoff for seropositivity was set at 1:64 as recommended [15].

Results

Simkania 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 C_q = 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 10^4/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].

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 both classical PCR and Vero cell culture approaches were used. As many as 25% of the children were positive for *Simkania* in this study [18]. Similar results were observed in a study performed in the United Kingdom using nested PCR and cell culture, in which 100 of 222 nasopharyngeal samples from children with bronchiolitis were positive by PCR [19]. Conversely, a specific qPCR could not detect any *Simkaniaceae* member when applied to 102 children with respiratory symptoms and 46 controls in Turkey [20]. Similarly, 531 respiratory samples investigated by pan-*Chlamydiales* PCR were negative for *Simkania* spp. in Finland [21]. Finally, in the United Kingdom, 847 urine samples from pregnant women were analysed for the presence of *Chlamydiales* DNA. Despite an overall *Chlamydiales* prevalence of 4.3%, including *C. trachomatis,* no *Simkaniaceae* were detected [22].

We observed the quasi-absence of human exposure to *S. negevensis* in pregnant women and young adults from Switzerland, pregnant women from England and an adult population from Israel, while previous studies had reported a seroprevalence of 46% among English pregnant women [19] and 55% to 80% among adults in Israel [7]. Both studies were performed using the same previously developed enzyme-linked immunosorbent assay (ELISA) [24]. This assay was also recently used in an Italian population and reported a similar high seroprevalence, ranging 9% to 30% [24].

These discrepancies may be related to the specificity of the PCRs and serologic tests used in previous studies. The

TABLE 2. Seroprevalence study showing results of microimmunofluorescence assay

Sex and country	Simkania		*Waddlia*		*Parachlamydia*	
	Total Ig ≥1:32	Total Ig ≥1:64	Total Ig ≥1:32	Total Ig ≥1:64	Total Ig ≥1:32	Total Ig ≥1:64
Women, Switzerland	6/132 (4.5%)	2/132 (1.5%)	59/132 (44.7%)[a]	36/132 (27.3%)	6/132 (4.5%)[a]	1/132 (0.8%)
Women, England	0/101 (0%)	UD	68/101 (67.3%)[b]	UD	12/101 (11.9%)[b]	UD
Men, Switzerland	2/105 (1.9%)	0/105 (0%)	UD	9/105 (8.6%)[c]	UD	0/105 (0%)[c]
Women, Israel	0/36 (0%)	0/36 (0%)	16/36 (44.4%)	9/36 (25%)	2/36 (5.6%)	1/36 (2.8%)
Men, Israel	0/40 (0%)	0/40 (0%)	18/40 (45%)	13/40 (32.5%)	5/40 (12.5%)	1/40 (2.5%)

Formalin-inactivated bacteria were used to test all samples except those from Swiss women, which were tested using heat-inactivated bacteria.
UD, undetermined.
[a]Derived from [1], which presents complete results including IgG and IgM analysis.
[b]Derived from [14]. Complete seroprevalence analysis of total population is available elsewhere [2].
[c]Derived from [8] and represents IgG.

previously used PCR assays might have been unreliable because of contamination with amplicons or *S. negevensis* genomic DNA [5]. In particular, nested PCR, a technique highly susceptible to contamination, has frequently been used in the past [19,25]. The molecular and serologic diagnostic tools used in these earlier studies were developed before the discovery of several *Chlamydia*-related bacteria, so their specificity towards these new members of the *Chlamydiales* order are in question. Further, the previously tested ELISA was only tested for cross-reactivity against *C. pneumoniae* [23]. Despite microimmunofluorescence being a tedious assay, it remains the reference standard for *Chlamydiales* seroprevalence studies [15]. However, some studies performed using this technique have also reported high *S. negevensis* seroprevalences, ranging from 35% to 50% in adult patients [26] and 11% to 30% in paediatric patients [27–29]. In these cases, cutoffs of 1:8 or 1:16 for IgG and of 1:10 for IgM seropositivity may have led to an overestimated prevalence. On the other hand, the lack of human exposure observed in our study correlates with the low seroprevalence reported in Japan using a microimmunofluorescence assay (4.3%) [30]. In this study, however, the seroprevalence was probably also overestimated as a result of the low cutoff of positivity used (1:8) [30], further supporting a very low human exposure to *S. negevensis.*

Similar to *S. negevensis, W. chondrophila* is an emerging *Chlamydia*-related bacterium. Several studies have implicated it in genital infections [31,32]. Indeed, both serologic evidence and molecular detection of *W. chondrophila* have been associated with miscarriages [1,2], while high antibody titres were correlated with tubal infertility [33]. Interestingly, in agreement with previous studies [8,34], we observed a high seroprevalence of *W. chondrophila* in Israel (Table 2), supporting a potential cross-reaction of the anti-*Simkania* ELISA with *W. chondrophila.* In addition, we were able to detect *W. chondrophila* from a urinary tract sample taken from a young woman, highlighting the tropism for the genitourinary tract of this emerging bacterium.

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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Conflict of Interest

None declared.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.nmni.2018.01.001.

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