Review

SIMKANIA NEGEVENSIS, AN INSIGHT INTO THE BIOLOGY AND CLINICAL IMPORTANCE OF A NOVEL MEMBER OF THE CHLAMYDIALES ORDER

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Simkania negevensis is a Chlamydia-related bacterium discovered in 1993 and represents the founding member of the Simkaniaceae family within the Chlamydiales order. As other Chlamydiales, it is an obligate intracellular bacterium characterized by a biphasic developmental cycle. Its similarities with the pathogenic Chlamydia trachomatis and Chlamydia pneumoniae make it an interesting bacterium. So far, little is known about its biology, but S. negevensis harbors various microbiological characteristics of interest including a strong recruitment of the ER to the Simkania-containing vacuole and the presence of an intron in the 23S rRNA encoding gene. Evidence of human exposition has been reported worldwide. However, there is a lack of robust clinical studies evaluating its implication in human diseases; current data suggest an association with pneumonia and bronchiolitis making S. negevensis a potential emerging pathogen. Owing to its fastidious growth requirements, the clinical relevance of S. negevensis is probably underestimated. In this review, we summarize the current knowledge on S. negevensis and explore future research challenges.
INTRODUCTION

Several members of the Chlamydiales order are of high clinical importance. For example, *Chlamydia trachomatis* is currently the second most common sexually transmitted disease after human papillomavirus (HPV) (Forhan et al., 2009). This infection can cause pelvic inflammatory disease (PID) (Bartlett et al., 2013) and lead to infertility as well as obstetrical complications such as miscarriage and preterm birth (Baud et al., 2011a, 2008; Gimenes et al., 2014; Pellati et al., 2008). Similarly, *Chlamydia pneumoniae* commonly causes bronchitis and pneumonia in children and young adults (Asner et al., 2014; Blasi et al., 2009; Principi and Esposito, 2001) and *Chlamydia psittaci* is an important zoonotic agent associated with atypical pneumonia as well as systemic infections (Knittler and Sachse, 2015; Schachter, 1986). In the past decades, various Chlamydia-related bacteria have been discovered, notably *Simkania negevensis (S. negevensis)*. These bacteria have been classified in the Chlamydiales order, as they exhibit a biphasic developmental cycle with infectious Elementary Bodies (EBs) and replicative Reticulate Bodies (RBs) similar to what is known for *Chlamydia* spp.. Growing evidence suggests a pathogenic role for many of these Chlamydia-related bacteria mainly in respiratory diseases and in obstetrical complications (Baud et al., 2014, 2008; Corsaro and Greub, 2006; Greub, 2009; Greub et al., 2003a; Greub and Raoult, 2002a; Lamoth and Greub, 2010). Due to the lack of commercial diagnosis methods and clinical guidelines, these strict intracellular bacteria are, however, not routinely screened in the clinic. It is therefore essential to better understand the biology and to clearly define their clinical importance as they may represent important human pathogens.
In this review, we summarize the current knowledge on *S. negevensis* and explore future research challenges.
**HISTORY**

*S. negevensis* was discovered in 1993 as a cell culture contaminant. In their first report, Simona Kahane *et al.* describe the micro-organism “Z” as an intracellular bacterium, which exhibited a two-phase replicative cycle similar to *Chlamydia* spp.. However, it was not recognized by *Chlamydia*-specific PCR primers, nor by *Chlamydia*-specific antibodies (Kahane et al., 1993). The analysis of the 16S rRNA encoding gene showed an 83% homology with *Chlamydia* spp. and 73% with *Rickettsia* (Kahane et al., 1995); the authors concluded to the discovery of a new *Chlamydia*-like bacterium. To better characterize the relationship of the microorganism with members of the *Chlamydiaceae* family, the 16S/23S rRNA intergenic spacer and Domain I of the rRNA encoding gene of these species were analyzed (Everett and Andersen, 1997). This work led to the description of the *Simkaniaceae* family within the *Chlamydiales* order with the microorganism *Simkania* «Z» as a founding member. It was renamed, afterwards, *Simkania negevensis* in honor of Simona Kahane from the Ben-Gurion University of Negev, Israel (Everett et al., 1999a).

*S. negevensis* was the first *Chlamydia*-like organism described. Its discovery gave an insight of the wide ecological diversity of the *Chlamydiales* order, and opened new research fields. Indeed, in the past decade, various *Chlamydia*-like organisms have been discovered (Corsaro and Greub, 2006; Corsaro and Venditti, 2009; Horn, 2008; Lienard et al., 2011b), notably using amoebal co-culture techniques (Jacquier et al., 2013). In 1999, based on the percentage of the 16S and 23S rRNA encoding genes sequences similarities (>80 and <90%), Everett *et al.*, proposed, in addition to the *Simkaniaceae* family, two novel families within the *Chlamydiales* order: the *Waddliaceae* and the
Parachlamydiaceae (Everett et al., 1999a). These “Everett” cut-offs have been validated by the International Subcommittee of Taxonomy (Greub, 2010) and have been recently extended by Pillonel et al. to use 6 additional taxonomically informative genes (Pillonel et al., 2015). Currently, the order is divided in nine family-level lineages, namely the Chlamydiaceae, Clavichlamydiaceae, Criblamydiaceae, Parachlamydiaceae, Parilichlamydiaceae, Piscichlamydiaceae, Rhabdochlamydiaceae, Simkaniaceae and Waddliaceae (Bavoil et al., 2013; Stride et al., 2013). Such division might however only represent a small portion of the ecological diversity of this order as suggested by Lagkouvardos et al., who estimate that more than 180 potential family-level lineages likely exist (Lagkouvardos et al., 2014).

MICROBIOLOGY

Simkaniaceae diversity

The Simkania genus belongs to the Simkaniaceae family, within the Chlamydiales order and consists of a single species, S. negevensis. Simkaniaceae comprise 3 genera: Simkania, Fritschea and Syngnamydia; the latter two genera are reported as Candidatus strains, since they have not been yet recovered by culture. Fritschea were first identified in 2003 as endosymbionts of arthropods and are subdivided in 2 Candidatus species: F. bemisiae and F. eriococci (Everett et al., 2005; Thao et al., 2003; Xue et al., 2012). Syngnamydia, which also includes 2 Candidatus species (S. venezia and S. salmonis), were recently described as pathogens in fishes, causing epitheliocystis, a disease characterized by large cysts in the gills (Fehr et al., 2013; Nylund et al., 2014). Additionally, seven DNA sequences recovered in environmental samples were recently
described as putative members of the *Simkaniaceae* family based on their 16S rRNA and 23S rRNA encoding genes sequences. The first sequence (EF177461) was recovered in gastrodermal cells of *Xenoturbella*, a primitive marine worm (Israelsson, 2007). Three other sequences (FJ976094, FJ976095 and AF448723.3) were identified in environmental water samples and grouped as the cvE9 cluster (cvE38, cvE41 and cvE9) (Corsaro et al., 2002; Corsaro and Venditti, 2009). The last three were recovered from unicellular eukaryotes present in the ocean crust and from citrus plants suffering from the Yellow dragon disease (*Huanglongbing*), a mortal disease in citrus, caused by vector transmitted Gram-negative bacteria (Sagaram et al., 2009; Santelli et al., 2008).

**Simkania negevensis**: genome and evolution

All *Chlamydiales* derived from a *Chlamydiae/Planctomycetes/Verrucomicrobia* common ancestor (Budd and Devos, 2012). Three evolutionary clusters appear to exist: (i) the *Chlamydiaceae/Clavichlamydiaceae* cluster, which harbors the smallest genomes and seems to have branched the earliest, (ii) the *Parachlamydiaceae/Waddliaceae/Criblamydiaceae* cluster and (iii) the *Simkaniaceae/Rhabdochlamydiaceae* cluster (Lagkouvardos et al., 2014). Indeed, *Simkaniaceae* share an 86-87% homology in the 16S rRNA encoding gene sequence with *Rhabdochlamydiaceae* (Kostanjsek et al., 2004) and only 82% homology with *Chlamydiaceae* (Kahane et al., 1995). Comparative analysis of the full genome sequences showed more than 400 conserved core genes preserved in all *Chlamydiales* members (Bertelli et al., 2010; Collingro et al., 2011; Pillonel et al., 2015). Among them, 20 are
highly informative taxonomically and should be preferentially used to assign new strains at species level (Pillonel et al., 2015).

*S. negevensis* genome is characterized by a 2-3 fold larger size compared to the *Chlamydiaceae* members, but comparable to the genome size of *Waddliaceae* and *Parachlamydiaceae* members (Collingro et al., 2011), as shown in table 1. This suggests a large biosynthetic ability, allowing adaptation to various environments. The analysis of *S. negevensis* genome revealed the presence of a type I intron localized in the 23S rRNA gene (Everett et al., 1999b). Type I introns are self-splicing motile introns, which have so far only been described in eukaryotic cells. To date, *S. negevensis* and *Coxiella burnetii* are the only bacterial species, which possess such genetic structure (Nesbø and Doolittle, 2003). This molecular pattern is also present in the amoeba *Acanthamoeba castellanii* as well as in the algal chloroplasts (Everett et al., 1999b) and therefore may have been acquired from horizontal gene transfer. This discovery, along with the high proportion of eukaryotic-like genes in chlamydial genomes, questions the possible relationship between *Chlamydiales* and chloroplasts and their possible implication in the eukaryiogenesis (Brinkman et al., 2002). The function of this intron remains unclear, but evidence showed that the intron is not spliced from the 23S rRNA and might delay growth by reducing ribosomal function (Everett et al., 1999b). It is not known whether this intron was also present, at first, in other *Chlamydiales* growing in amoebae and was subsequently eliminated to improve replication or whether it was horizontally acquired after the divergence of *S. negevensis* from other *Chlamydiales*.

*S. negevensis* possess a 132kb plasmid, which carries a type IV secretion system (T4SS) encoded by a *tra* operon. A similar *tra* operon is also present in the genome of
Protochlamydia amoebophila (Greub et al., 2004a) and of Parachlamydia acanthamoebae (Greub et al., 2009), two members of the Parachlamydiaceae family. These operons exhibit a striking similarity with the tra genes carried by the conjugative F-plasmid of Escherichia coli. Thus, it is suspected that these chlamydial operons encode a conjugative DNA transfer system. In addition, the S. negevensis tra operon also exhibits genetic similarities to the T4SS conjugative system encoded in Rickettsia belli, where conjugation has been demonstrated (Ogata et al., 2006), reinforcing the putative role of the S. negevensis T4SS in DNA transfer. Independent of the presence of the T4SS, the identification of a plasmid in the S. negevensis genome is of utmost interest, as it might play a significant role in the pathogenesis of S. negevensis infection, as suggested for the 7'500kb Chlamydia spp. plasmid. Indeed, it has been shown both in vivo (Kari et al., 2011; O’Connell et al., 2007) and in vitro (Porcella et al., 2015) that infections with a plasmid deficient strain resulted in attenuated infections exhibiting a lower inflammatory response. An adaptive immune response was however elicited enabling protection against re-infection (Kari et al., 2011; O’Connell et al., 2007; Porcella et al., 2015). The mechanisms by which the chlamydial plasmid modulates the pathogenesis are still unclear, but they are probably related to: (i) direct effect of proteins encoded by the plasmid (Liu et al., 2014, p. 201; Li et al., 2008) and (ii) regulation of the expression of other virulence factors encoded by the chromosome (Carlson et al., 2008; Song et al., 2013). Interestingly, the S. negevensis plasmid encodes various proteins of interest, among which one copy of the Macrophage Infectivity Potentiator (Mip) (Collingro et al., 2011). This lipoprotein is highly similar to the Mip protein of Legionella pneumophila. In Chlamydia, it has been shown to be essential for an optimal intracellular infection and to
induce the production of pro-inflammatory cytokines (Bas et al., 2008; Lundemose et al., 1993a, 1993b).

Biochemical characteristics

Cell wall and surface proteins. The composition of the outer membrane is of significant importance for the pathogenesis of Chlamydiales. In the EB state, membrane proteins are implicated in the entry within the host and serve as major antigens for the immune response. In the RB state, membrane proteins may be part of the secretion systems used to corrupt the host cell whereas others may serve as porins to uptake nutrients from the host cytoplasm. A comparison of the structure of the membrane of various members of the Chlamydiales order is shown in table 1.

The Chlamydial Outer Membrane Complex (COMC) of the Chlamydiaceae is mainly composed by the MOMP (ompA) protein and by 2 cystein-rich proteins, OmcA and OmcB (Caldwell et al., 1981). MOMP (ompA) represents 60% of the surface proteins, at least in purified EBs and serves as a porin (Bavoil et al., 1984). Aistleitner et al. have recently studied the structure of S. negevensis outer membrane (Aistleitner et al., 2014).

Using a computerized proteomic approach, 65 OMPs were identified as major components of the S. negevensis EB’s membrane. Among them 37 were MOMP-like proteins, which resemble the MOMP (ompA) of Chlamydiaceae. Out of the 37 MOMP-like proteins, 30 contained beta-sheet conformations, suggesting a role as porins. Two of them demonstrated high similarity to MOMP proteins of veterinary Chlamydia species (Collingro et al., 2011). S. negevensis outer membrane lacks the cysteine-rich proteins (OmcA and OmcB) present in other Chlamydiales. These cysteine-rich proteins are
sensitive to the redox state of the environment and they might provide some rigidity and resistance to osmotic pressure through the formation of disulfide bridges. Some authors suggested that they may replace the peptidoglycan layer present in the periplasm of other Gram-negative bacteria (Everett and Hatch, 1995; Sun et al., 2007). No homologues of OmcA and OmcB were identified in *S. negevensis* genome and none of the identified MOMP-like proteins could serve this function as their content in cysteine was extremely low (Aistleitner et al., 2014). Such differences may result in a more flexible envelope and a lack of surface proteins regulated by the redox state. Such regulations are associated with modifications of the function of several surface proteins in *Chlamydiaceae* through reduction and reoxidation of the cysteine residues and could play a role in the switch from the EB to the RB state. For example, the permeability of MOMP is increased once reduced (Bavoil et al., 1984) and the type III secretion system (T3SS) in *Chlamydia* is also described as influenced by the redox state (Betts-Hampikian and Fields, 2011).

Studying the morphology of EBs and RBs particles is difficult and has been the subject of various works. As an example, crescent bodies and star bodies were reported as infectious EB-like particles of *Parachlamydiaceae* and *Criblamydiaceae*, respectively (Lienard et al., 2011b; Rusconi et al., 2013; Thomas et al., 2006). Current knowledge suggests that these observations are largely influenced by the buffer and the fixatives used and therefore do not allow taxonomic discrimination, even if they correspond to some structural differences in the cell wall of the different bacterial families (Pilhofer et al., 2014; Rusconi et al., 2013). Noteworthy, *S. negevensis* EBs were described as highly pleomorphic particles, especially once located in the cytoplasm (Pilhofer et al., 2014).
Such observation reinforces the hypothesis of a relative high cell wall flexibility due to the lack of cysteine-rich surface proteins in *S. negevensis*.

The inner membrane of most bacteria is protected by a cell wall composed of peptidoglycan. This structure is important for membrane stability as well as cell division. Until recently, it was thought that *Chlamydiales* were lacking peptidoglycan and their partial sensitivity to cell wall inhibitors such as penicillin was therefore not understood and considered as a paradox called the *Chlamydiales* anomaly. However, the presence of peptidoglycan was recently demonstrated in some members of the *Chlamydiales*, namely *C. trachomatis*, in which they were present as a ring structure at the division septum (Liechti et al., 2014) and *P. amoebophila*, which synthesizes sacculi that contain a modified form of peptidoglycan (Pilhofer et al., 2013). Additionally, a recent work identified two peptidoglycan-remodeling enzymes (the AmiA amidase and the NlpD endopeptidase) implicated in coordinated cell wall constriction during division. Such enzymes were shown to recognize peptidoglycan-like peptides in *Chlamydiales* (Frandi et al., 2014). Most of this work was done on *Waddlia chondrophila*, a *Chlamydiales* member that is susceptible to high dose of penicillin and also to fosfomycin, a drug active on MurA (Frandi et al., 2014; Jacquier et al., 2014). The MurA enzyme is implicated in peptidoglycan biosynthesis, suggesting the presence of peptidoglycan-like structures in at least 3 family-level lineages of the *Chlamydiales* order, namely the *Chlamydiaceae*, *Waddliaceae* and *Parachlamydiaceae* (Frandi et al., 2014; Jacquier et al., 2015, 2014). In their first report, Kahane *et al.* described *S. negevensis* as being resistant to 10µg/ml of penicillin, as opposed to what was observed for *C. trachomatis*. It was, however,
sensitive to cycloserine, an inhibitor of the peptidoglycan synthesis, similarly to other
*Chlamydiaceae*. In addition, activity of the AmiA amidase and the NlpD endopeptidase
was, recently, documented in *S. negevensis*, suggesting the presence of peptidoglycan-
like structures in *S. negevensis* (Frandi et al., 2014), despite failure to isolate
peptidoglycan containing sacculi in *S. negevensis* (Liechti et al., 2014). Their detection
might be prevented by a restricted time-frame synthesis (Jacquier et al., 2015) or might
require an additional method, similar to the one recently described by Packlam et al.,
which allowed the isolation of peptidoglycan fragments in *C. trachomatis* (Packiam et al.,
2015).

**Secretion systems.** Intracellular bacteria parasitize their host’s metabolic pathways to
their own advantage. In parallel, they must inhibit the trigger of the host’s immune
system. One strategy consists in secreting various proteins into the host cytoplasm or
environment through secretion systems (Greub, 2013). *S. negevensis* encodes a Type III
secretion system (T3SS) (Collingro et al., 2011). Hsia *et al.* first suggested the existence
of a type III secretion system in *Chlamydia* (Hsia *et al.*, 1997), which was likely present
in the *Chlamydiales* common ancestor given its presence in every genome sequenced so
Stephens *et al.*, 1998), as summarized in table 1. The T3SS appears as a needle inserted in
the inner and outer membranes of the bacterium, which protrudes into the cytoplasm of
the cell through the inclusion membrane (Pilhofer *et al.*, 2013). Although, the structure of
the T3SS is highly conserved among *Chlamydiales*, effectors secreted through this
system largely vary among families and genera (Collingro *et al.*, 2011). Interestingly, *S.*
negevensis does not possess genes encoding for major chaperones of the T3SS, namely the SctF, CdsF, CT666 and the Specific Chlamydiales Chaperon 3 (SCC3), which are conserved in Waddliaceae, Parachlamydiaceae and Chlamydiaceae (Collingro et al., 2011).

As mentioned above, S. negevensis plasmid encodes a T4SS. In addition to its likely role in plasmid propagation and DNA exchange, it may also allow secretion of proteins and may represent an important virulence factor.

**Metabolism.** Strict intracellular bacteria are often characterized by their important lack of metabolic pathways and the necessity to parasitize their hosts for nutrients and energy. However, genomic studies have revealed extended metabolic capabilities of S. negevensis compared to Chlamydiaceae (Collingro et al., 2011; Omsland et al., 2014). This extended metabolic capacity has also been observed for other Chlamydia-related bacteria exhibiting large genomes (Bertelli et al., 2010; Collingro et al., 2011; Greub et al., 2009; Horn et al., 2004), as shown in Table 1. Among major differences, we note their ability to directly use glucose for the glycolysis pathway due to the presence of a glucokinase, making them independent from the host cell glucose-6-phosphate (Bertelli et al., 2010; Collingro et al., 2011; Greub et al., 2009; Omsland et al., 2014). Additionally, they possess a complete citric acid cycle as opposed to Chlamydiaceae, which lack important enzymes and need a constant exchange with the host cell to produce energy by oxidation of acetyl-CoA (Omsland et al., 2014). In S. negevensis, entry in the citrate cycle can occur: (a) through acetyl-CoA produced from pyruvate following glycolysis and (b) through the transformation of asparagine in fumarate (Collingro et al., 2011; Omsland et
Moreover, *Waddliaceae, Parachlamydiaceae* and *Simkaniaceae* are able to synthetize NAD+ from nicotinamide (Bertelli et al., 2010; Omsland et al., 2014) and *S. negevensis* can, additionally, use asparagine as a source of NAD+ (Collingro et al., 2011; Knab et al., 2011; Omsland et al., 2014). Finally, an *in vitro* experiment by Kahane *et al.* suggests that *S. negevensis* is able to grow in a glucose free medium; indeed the number of infectious forming units was similar using a Roswell Park Memorial Institute (RPMI) media depleted in glucose and supplemented with 1% glucose (Kahane et al., 1999). Taken together, these findings suggest that *S. negevensis* is less dependent from the host cell in term of energy production compared to other *Chlamydiaceae* (Omsland et al., 2014).

Similarly to *Chlamydiaceae*, *S. negevensis* is unable to synthesize nucleotides *de novo* and relies on nucleotides transporters for their uptake. Four isoforms of nucleotides transporters have been identified in *S. negevensis*: SnSTT1, an ADP/ATP antiporter; SnSTT2, a guanine/ATP/H+ symporter; SnSTT3 a global nucleotide triphosphate antiporter, also able to transport deoxy-CTP (dCTP). The function of the last transporter, SnSTT4, is unknown (Knab et al., 2011). The ability to transport dCTP is unique among prokaryotes and might represent a selective metabolic advantage for *S. negevensis*, favoring its relative growth in environments rich in cytosine.

Regarding amino-acid biosynthesis, *S. negevensis* is able to synthesize tyrosine, phenylalanine, proline, alanine, aspartate and glutamate (Collingro et al., 2011; Omsland et al., 2014). In addition, *S. negevensis* possesses a complete tryptophan operon (*trp* operon) (Collingro et al., 2011; Omsland et al., 2014). Some pathogenic *Chlamydiaceae*, notably the genitourinary strains of *C. trachomatis*, are partially capable of synthesizing this metabolite but enzymes required in this pathway are completely absent in other
Chlamydia-related bacteria (Carlson et al., 2006; Omsland et al., 2014). It appears that tryptophan plays an important role in the pathogenesis of Chlamydia spp. infections. Tryptophan is, indeed, an important regulator of the chlamydial development cycle (Bonner et al., 2014). In case of tryptophan depletion, the expression pattern changes and forces the bacteria into a persistent form (Baud et al., 2008; Brunham and Rey-Ladino, 2005; Ibana et al., 2011). Tryptophan depletion can be induced in humans by IFN-γ production (Taylor and Feng, 1991). Thus, S. negevensis might be less sensitive do IFN-γ than Chlamydiaceae, which could play a role in the pathogenesis.

Culture characteristics

Cell permissiveness and growth cycle. S. negevensis is able to grow and survive in vitro in mammalian cells such as Vero cells (Kahane et al., 1993), epithelial cells from the respiratory and genitourinary tract, endothelial cells (Kahane et al., 2007a) as well as macrophages (Kahane et al., 2008). Growth is also described in arthropods (both in vitro and in vivo) (Croxatto et al., 2014; Sixt et al., 2012), and amoebae (Kahane et al., 2001). Similarly to other Chlamydiales, S. negevensis growth cycle is characterized by the presence of two developmental stages: one large replicative form (Reticulate Body, RB) and one small compact form, which resembles to electron dense (Elementary Body, EB) form of Chlamydiaceae. These two forms can be selectively fractionated on density gradients (Kahane et al., 1999, 1993). The high density of EBs is likely due to the presence of condensed DNA as shown by the presence of filamentous material on electron microscopy tomography (EMT). Even though gene expression might be
downregulated through DNA condensation, EB particles are likely to be metabolically active as suggested by the presence of ribosomes (Pilhofer et al., 2014).

The growth cycle of *S. negevensis* is characterized by a production phase lasting approximately 3 days, during which particles are produced in an exponential way. During that time, the cytoplasm of the host cells shows increasing dark perinuclear patches. This is followed by a plateau phase, during which no or few particles are produced, but instead the particles previously produced are stored in multiple large cytoplasmic vacuoles of the host cells and cytopathic changes are observed. Based on culture observations, *S. negevensis* does not seem to induce cell lysis, or at least not to the same extent as what is observed for other members of the *Chlamydiales* order, such as *W. chondrophila* (Kebbi-Beghdadi et al., 2011). In comparison, *C. trachomatis* particles are released between 30 to 68 hours post infection depending on the strain (Miyairi et al., 2006), *W. chondrophila* particles are released 48-72 hours upon cell lysis (Goy et al., 2008) and *P. acanthamoebae* induces lysis of infected amoebae after 72-96 hours depending on the initial Multiplicity Of Infection (MOI) and incubation temperature (Greub et al., 2003b; Greub and Raoult, 2002b). However, despite the apparent absence of cell lysis, some particles seem to be released in the extracellular environment, as suggested by the presence of *S. negevensis* particles in the supernatant of infected insect cell cultures capable of inducing a second round of infection (Sixt et al., 2012). Recently, Hybiske et al. showed that *C. trachomatis* may exit from the host cell through the extrusion of a membrane-bound compartment (Hybiske and Stephens, 2007). This extrusion depended on actin polymerization, as well as on various proteins implicated in the remodeling of
the cytoskeleton. A similar mechanism might be implicated in the extrusion of *S. negevensis*.

Interestingly, it has been proposed that *S. negevensis* RBs might be infectious, an aspect that has not been observed for other *Chlamydiales* so far. Indeed, Kahane *et al.*, observed similar growth kinetics of *S. negevensis* purified EBs and RBs (Kahane *et al.*, 2002). However, this has not been confirmed yet and further investigations are needed before making any definite conclusions.

**The Simkania containing vacuole and its interaction with cellular organelles.** *S. negevensis* replicates in a single intracellular vacuole hereafter called the Simkania Containing Vacuole (SCV). Interestingly, the SCV is closely associated with the endoplasmic reticulum (ER) of the host cell, being almost completely surrounded by the typical ribosomes-covered ER membrane (Mehlitz *et al.*, 2014; Pilhofer *et al.*, 2014). Such ER recruitment was observed in infected epithelial cells, macrophages-derived cells and amoebae, emphasizing the strong conservation of the phenotype. Intracellular traffic of *S. negevensis* clearly differs from *P. acanthamoebae*, which survives in the late endosome (Greub *et al.*, 2005) and from *C. trachomatis*, which intercepts Golgi-derived vesicles and causes Golgi apparatus fragmentation (Heuer *et al.*, 2009). Recruitment of the ER also occurs upon infection with other members of the *Chlamydiales* order, such as *C. trachomatis* and *W. chondrophila*, but to a lesser extent than what is observed following *S. negevensis* infection (reviewed in Derré, 2015). The mechanisms by which *S. negevensis* induces this tight association remain to be elucidated, but might rely on specific inclusion proteins or type III effector proteins. Indeed, *Chlamydia* spp. use their
IncD protein to recruit the host ER-CERT protein to acquire ceramide (Derré et al., 2011). A recent work suggested that early retrograde transport is required to achieve an optimal *S. negevensis* infection, which seems to be implicated both in the SCV maturation, as well as in lipids uptake (Herweg et al., 2015). This was shown by the enrichment, at the SCV membrane, of Clathrin and COP1 related proteins, typical of the early endosomes and Trans-Golgi Network (TGN). This hypothesis is further supported by the reduced bacterial replication observed in the presence of specific retrograde transport inhibitors. In addition, ceramide vacuolar uptake was modified by these inhibitors, suggesting that *S. negevensis* has developed a mechanism to directly obtain ceramides from the Golgi.

The tight association observed between the SCV and the ER raises the question of activation of the ER-stress, which plays a significant role in limiting viral replication. Interestingly, *S. negevensis* is capable of inhibiting the ER-stress response despite a strong initial activation, and thus promoting host survival and indirectly promoting *S. negevensis* replication (Mehlitz et al., 2014).

In addition to the strong ER association, some recruitment of mitochondria at the SCV was observed in mammal’s cells, similarly to what is observed for *W. chondrophila* (Croxatto and Greub, 2010) and *C. psittaci* (Mehlitz et al., 2014). However, the phenotype of mitochondria association to the replicative vacuole is much stronger for *W. chondrophila*, which recruits mitochondria as early as 3 hours post-infection using a redundant mechanism based on both microtubules and actin microfilaments (Croxatto and Greub, 2010). Mitochondria might represent an additional source of lipids, as well as ATP (Croxatto and Greub, 2010). It is still unclear whether *S. negevensis* has developed a
specific mechanism to recruit the mitochondria, or if their association to the SCV is related to their natural association with the ER.
EPIDEMIOLOGY

**Prevalence**

Scarce data on the prevalence of *Simkania* are available in the literature, most of them provided by old studies. Caution should be taken when interpreting these results. The seroprevalence appears high in the Middle East (Israel and Jordanian), ranging from 55-65% among healthy adults and reaching 80% in Bedouins (Al-Younes and Paldanius, 2014; Friedman et al., 1999). Serological evidence of past infection was documented in 46% of pregnant women in Cornwall, UK (Friedman et al., 2006) and in 41% of Danish blood donors (Johnsen et al., 2005). In contrast, an overall prevalence of only 23.5% was observed among children and adults in Brooklyn, USA (Kumar et al., 2005) and only 4.5% of the population tested in Japan were seropositive, a low prevalence observed despite the low cut-off titer of 1:8 used in the study (Yamaguchi et al., 2005). The large discrepancy between these results could be explained by geographical differences in terms of exposure and risk factors. However, the heterogeneity of diagnostic tools used between the studies makes comparisons difficult. Indeed, the prevalence observed in the Danish and Jordanian population was obtained using an immunofluorescence assay with a cut-off titer of 1:16 and 1:8, respectively, which probably overestimated the prevalence (Al-Younes and Paldanius, 2014; Johnsen et al., 2005). Nevertheless, the relatively high prevalence in Israel, England and USA was observed using the same ELISA assay based on purified EBs (Friedman et al., 2006, 1999; Kumar et al., 2005). Authors tested a 1/100 diluted serum and considered an OD (optical density) higher than 0.5 as positive. In conclusion, serological evidence of human exposure to *Simkania* infection was
documented worldwide and prevalence seems to increase with increasing age (Friedman et al., 2006; Johnsen et al., 2005; Kumar et al., 2005).

**Transmission and reservoir**

*S. negevensis* was discovered as a cell culture contaminant. However, the natural host of *Simkaniaceae* remains unknown. As mentioned above, *S. negevensis* is able to grow *in vitro* in a wide range of hosts (i.e. mammalian cells, arthropods, amoebae) (Kahane et al., 2008, 2007a, 2001; Sixt et al., 2012). Evidence of *Simkaniaceae* was documented *in vivo* in ticks (Croxatto et al., 2014; Pilloux et al., 2015) and in granulomatous lesions in reptiles (Soldati et al., 2004). *Fritschea* were discovered in arthropods (Everett et al., 2005) and *Syngdamydia* in fish (Fehr et al., 2013; Nylund et al., 2014). Additionally, various *Simkania*-related DNA sequences were amplified from marine environments (Israelsson, 2007; Santelli et al., 2008) and in one case in vegetal cells (Sagaram et al., 2009). This wide host range is quite impressive and contrasts with the narrow host range of *Chlamydia trachomatis* (human-specific) and *Chlamydia felis* (cat-specific).

Various hypotheses on the reservoir of *Simkaniaceae* and their mode of transmission to humans have been proposed. Firstly, contaminated water might be a source of human infection, either as a free-living organism or through infected amoebae. Indeed, a report showed that *S. negevensis* infectivity *in vitro* was still preserved after a 7 days incubation of free particles in distilled water and was even increased in cases of co-infection with amoebae (Kahane et al., 2004). In comparison, the infectivity of *C. trachomatis*, considered as a sexually transmitted disease, was completely abolished after 5 hours of
incubation in the same conditions (Kahane et al., 2004). Moreover, co-culture of
macrophages with *S. negevensis*-infected amoebae resulted in the rapid death of the
amoebae and infection of the macrophages (Kahane et al., 2008). Amoebae could
therefore serve as reservoir and contribute to the selection of virulence traits (Greub and
Raoult, 2004; Lamoth and Greub, 2010). This is in agreement with the identification of
*Simkania* and amoebae’s antigens in drinking water and in reclaimed wastewater used to
irrigate crop in Israel (Donati et al., 2015; Kahane et al., 2004). Similarly, *Simkania* DNA
was amplified from environmental water in Western Europe (Corsaro et al., 2002;
Corsaro and Venditti, 2009) and, recently, in Italian swimming pools (Donati et al., 2015;
Pérez et al., 2012, 2011). Transmission to humans through water is also suggested by a
prospective study, in which it was possible to identify the presence of *Simkania* in the
corresponding drinking water of children suffering from a *Simkania*-associated
pneumonia in as much as 76.5% of cases. In this study, results were only considered as
positive when congruent results were obtained by at least two different approaches
(Nested PCR, culture in Vero cells and/or Membrane Enzyme Immuno-Assay (MEIA))
(Kahane et al., 2007b). Nevertheless, these results should be treated with caution as (i)
only 34 children were included in the study and (ii) nested PCR contamination cannot be
excluded due to the very high proportions of positive results observed in the study.
Finally, an oral transmission of *S. negevensis* is likely since (i) a significant IgA response
to *Simkania* was observed in patients with gastro-intestinal symptoms (Donati et al.,
2013) and (ii) *S. negevensis* may replicate *in vitro* within gastro-intestinal cell lines
(Kahane et al., 2007a).
Arthropods, such as ticks, might represent another source of infection. Vector transmission is quite common for other intracellular bacteria such as *Rickettsia* (Walker and Ismail, 2008). Moreover, *Simkania*-associated DNA was amplified from Swiss *Ixodes ricinus* ticks, though with a lower prevalence and amount than *Rhabdochlamydiaceae* (Croxatto et al., 2014; Pilloux et al., 2015).

Additional hypotheses of transmission include direct human to human transmission through aerosols or droplets due to the association with respiratory diseases (see pathogenic potential, hereafter), as well as sexual contacts or contacts with animals, which were described for other *Chlamydiales* (Baud et al., 2007; Baud and Greub, 2011; Gottlieb et al., 2013; Knittler and Sachse, 2015; Schachter, 1986). Sexual contact seems unlikely due to the high prevalence in children and the absence of cross-prevalence with *C. trachomatis* (Friedman et al., 1999). Finally, a zoonotic transmission has so far not been documented, but cannot be excluded.
PATHOGENIC POTENTIAL

In vitro

Several *in vitro* aspects of *S. negevensis* infection suggest an important pathogenic potential. First, *S. negevensis* is able to grow and survive within macrophage-type cell line U937 (Kahane et al., 2008). Moreover, uninfected epithelial cells can be actively infected after co-culture with infected macrophages (Kahane et al., 2008). Interestingly, *S. negevensis* is resistant *in vitro* to LL-37 cathelicidin human peptide present in phagocyte cells, but is highly sensitive to 5 other animal cathelicidins (Donati et al., 2011). Moreover, Karunakaran *et al.* demonstrated that *S. negevensis* prevents TNF-α-induced apoptosis in host cells through the inhibition of the release of mitochondrial caspase-activating proteins (Karunakaran et al., 2011). This is similar to what is known for the pathogenic *C. trachomatis* and *C. pneumoniae* (Fan et al., 1998). However, the phenotype seems to be stronger in *S. negevensis* as it could be observed even at a very low MOI (MOI=0.5) (Fan et al., 1998; Karunakaran et al., 2011). This anti-apoptotic capability represents an important virulence factor and is not observed in all *Chlamydiales*. For example, *Parachlamydiaceae* appear to instead induce apoptosis in macrophages (Greub et al., 2003c) and in insect cell lines (Sixt et al., 2012). The ability to inhibit apoptosis and the absence of cell lysis might allow *S. negevensis* to persist within its host and establish a persistent latent infection. Such infection might then be reactivated and might represent an important pathogenic mechanism.

Two types of *S. negevensis* infection have been described: (i) an active infection with production of new bacterial particles and observation of cytopathic effects in the host, and (ii) a persistent infection without any cytopathic effects (Kahane et al., 2007a). In
persistent infection, mostly RBs of atypical morphology were visualized within the infected cells. Persistence of infectious particles within the host was demonstrated by inoculation of uninfected Vero cells with persistently infected cells, which produced an active infection. Infection was however less efficient compared to inoculation with actively infected cells.

In Chlamydia spp., persistence may be induced by iron depletion (Raulston, 1997), IFN-γ (Beatty et al., 1994; Rottenberg et al., 2002) and penicillin treatment (Matsumoto and Manire, 1970). With S. negevensis, a latent infection can be observed either spontaneously in specific cell lines, namely intestinal or genitourinary lines, or could be induced by iron depletion. Reactivation was then possible after serial trypsin treatment (Kahane et al., 2007a). Interestingly, reactivation was also observed when persistently infected cells were cultured with macrophages-derived cells. The mechanism, by which reactivation is induced, is still unclear, but might rely on recognition of the infection by the macrophages and production of cytokines, which could promote the release of infectious S. negevensis particles (Kahane et al., 2008). In addition, an inflammatory response was observed in both types of infections, mostly through the production of IL-8 and IL-6 (Kahane et al., 2007a).

Lessons from the clinic

Since the discovery of S. negevensis, only few studies have been conducted to evaluate the implication of Simkania spp. in human diseases (see list of all studies in supplementary materials). An implication in respiratory diseases, particularly in bronchiolitis and pneumonia in children and young adults, is likely, as shown in tables 2
and 3, respectively, which summarize the findings of major clinical studies. Thus, a significant association between Simkania and acute bronchiolitis was shown in children from Israel (Kahane et al., 1998). The authors used both a PCR and a cell culture approach to detect the organism in nasopharyngeal samples. A total of 239 patients and 78 controls were included over a period of two years. Of the 116 patients, from whom a possible etiologic agent was isolated, 60 were positive for Simkania by at least one of the techniques used. This was the second most common agent identified after respiratory syncitial virus (RSV) and statistically different from controls. Clinical findings were similar to patients infected by RSV. Similar results were also obtained in a study performed on English children with bronchiolitis. In 26% of the patients, evidence of a Simkania infection was provided by both PCR and culture. This rate reached 45% when considering only samples positive by PCR. However no controls were included in this study (Friedman et al., 2006).

Various works showed evidence of an acute infection with Simkania in cases of pneumonia in children and young adults (Donati et al., 2011; Fasoli et al., 2008; Heiskanen-Kosma et al., 2008; Kahane et al., 2007b; Lieberman et al., 1997; Lienard et al., 2011a; Nascimento-Carvalho et al., 2009). Acute infection was indicated either by a rise in paired sera of IgG, IgA or IgM defined as a 4 fold increase of antibody titers observed by micro-immunofluorescence (Fasoli et al., 2008; Heiskanen-Kosma et al., 2008; Nascimento-Carvalho et al., 2009) or a rise of 0.5 OD in the ELISA signal (Lieberman et al., 1997), or by elevated titers of IgM or IgA or direct identification of Simkania through PCR, culture or antigen detection (Kahane et al., 2007b). Globally, a low prevalence of acute infection was observed (2-10%), except in the study performed...
by Kahane et al., in which *Simkania* was identified in almost all samples, including controls. Evidence of co-infections with other putative pathogens was found in 40 to 60% of the cases; the most commonly associated pathogens were *Mycoplasma pneumoniae*, RSV, *Streptococcus pneumoniae* and *Chlamydia pneumoniae* (Fasoli et al., 2008; Lieberman et al., 1997; Nascimento-Carvalho et al., 2009). Noteworthy, the association of *Simkania* infection with the occurrence of bronchiolitis or pneumonia was not confirmed in a study performed on children, in Brooklyn, USA (Kumar et al., 2005). An association between *Simkania* infection and other respiratory diseases was also studied. However, no association was found with asthma (Korppi et al., 2006; Kumar et al., 2005), nor with exacerbation of Chronic Obstructive Pulmonary Disease (COPD) (Lieberman et al., 2002), chronic cough (Johnsen et al., 2005) or acute lung rejection (Husain et al., 2007). Results are difficult to interpret owing to low statistical power of these studies. However, a significant correlation with serological evidence of an acute infection was shown in unspecified respiratory tract infection among adults, in England (Friedman et al., 2006) and a higher prevalence of *Simkania* was documented in patients with a lung transplant as compared to other immunocompromised patients or immunocompetent patients (Husain et al., 2007).

Caution should be taken when interpreting the results since only few studies included a control group. In addition, the same group from the Ben-Gurion University of the Negev, in Israel, discovered *S. negevensis* and performed most of the studies suggesting a pathogenic role. The specificity of the diagnostic tools might have not been optimal and contamination could have occurred within the laboratory. Indeed, the prevalence
observed in the studies performed by this group (26% in Israel (Kahane et al., 1998), 63.6% in Inuit patients (Greenberg et al., 2003) and 80 to 97.5% in lung transplants (Husain et al., 2007)) are strikingly high, even in the control groups (Kahane et al., 2007b; Kumar et al., 2005).

Moreover, recent studies performed by other groups have failed to confirm such high prevalence. Indeed, using a specific quantitative PCR, Niemi et al. could not identify any positive case of Simkania infection in 97 respiratory samples taken from adult patients with a suspected respiratory tract infection (Niemi et al., 2011). Similarly, only two samples from Swiss children with bronchiolitis were identified as positive for a Simkaniaaceae infection by a validated pan-Chlamydiales quantitative PCR (Lienard et al., 2011a) and only one case of a co-infection with Simkania and C. psittaci was identified in a large German study investigating community acquired pneumonia (Dumke et al., 2015). This discrepancy between the results could either be due to differences in the reliability of the diagnostic techniques used or to geographical variations of the prevalence of the infection.
Various techniques have been described to diagnose a *S. negevensis* infection, reviewed by Corsaro *et al.* (Corsaro and Greub, 2006). Most of them were developed in the early 2000’s. They may now lack specificity due to the recent discovery of novel *Chlamydiales* species.

**Direct identification of *Simkania* in clinical samples**

**PCR.** PCR is currently the technique of choice to diagnose *C. trachomatis* or *C. pneumoniae* infections. Diagnostic real-time PCRs have also been developed and are routinely used in our laboratory to detect *W. chondrophila, P. acanthamoebae* and *P. neagleriophila* in clinical samples (Casson *et al.*, 2008a, 2008b; Goy *et al.*, 2009). By analogy, this technique should be preferentially used to detect *Simkania* spp.. Most of the primers and probes developed earlier, which are listed in Table 4, are based on the detection of amplicons on agarose gel, a technique with a lower sensitivity and specificity than real-time PCR, at risk of contamination by amplicons. Thus, we currently recommend the use of a pan-*Chlamydiales* PCR (Lienard *et al.*, 2011a) followed by sequencing to detect putative cases of infection.

**Culture.** Isolation of organisms through culture is crucial to prove the presence of viable bacteria in clinical samples and to provide a reference strain for a precise downstream characterization, as well as to provide antigens for serological assays. *Simkania* spp. may be difficult to recover from clinical samples through culture, as it requires cell culture, which is at risk of contamination by other bacteria present in the flora of non-sterile
samples such as sputa, nasopharyngeal swabs or vaginal swabs. Owing to these culture
difficulties, the sensitivity of culture seems to be lower than PCR (Corsaro and Greub,
2006; Kahane et al., 1998).
Culture in Vero cells is currently the method of choice to isolate Simkania spp. in clinical
samples (Corsaro and Greub, 2006), although amoebal co-culture might represent an
ideal alternative for respiratory samples, given the non-susceptibility of amoebae to
Mycoplasma and to most bacteria present in the physiological oropharynx flora (Greub et
al., 2004b; Jacquier et al., 2013). Testing the cells used for cell culture for the presence of
a possible contamination should be routinely done; this screening should be able to detect
Mycoplasma spp. as well as Chlamydiales, including S. negevensis, since the later was
first isolated as a cell culture contaminant. We recommend microscopy examination of
cell lines and a Pan-Chlamydiales PCR (Lienard et al., 2011a), when contamination is
suspected.

Others. Immunohistochemistry based on rabbit or mouse anti-S. negevensis antibodies
may be used to visualize this bacterium in lesions when biopsies are available. By
analogy with other Chlamydiales, Gram staining should be avoided since the results are
inconsistent, as EBs result generally Gram-positive, whereas RBs result Gram-negative
(Lamoth and Greub, 2010).

Serological studies
Micro-immunofluorescence (MIF) is currently the technique of choice to detect anti-C.
pneumoniae specific antibodies (Dowell et al., 2001). By analogy, this technique should
also be applied to detect anti-*Simkania* spp. specific antibodies. Current recommendations for *Chlamydia* and *Chlamydia*-related bacteria propose a cut-off titer of \( \geq 1:64 \) for IgG to confirm a past infection and a cut-off of \( \geq 1:32 \) for IgM for an acute infection (Corsaro and Greub, 2006). Acute infections may also be documented based on seroconversion defined as an increase in IgG titer from 0 to \( \geq 1:64 \) in paired sera or a \( \geq 4 \)-fold rise in the IgG titer between acute- and convalescent-phase sera (Corsaro and Greub, 2006). Such recommendations should also be applied to detect a *Simkania* infection in order to enable inter-laboratories comparison of obtained results. Alternatively, an ELISA assay developed in our laboratory to detect *W. chondrophila* (Lienard et al., 2014) could be adapted for *Simkania* infections. Such ELISA might be a good alternative, as the results are not biased by subjective analysis. Nevertheless, we do not recommend using the assay developed by Lieberman *et al.* (Lieberman et al., 1997) since it has not been properly validated and may overestimate true seroprevalence.
TREATMENT AND PREVENTION

Antibiotic susceptibility

So far, *Simkania* infections have been treated empirically with macrolides, similarly to other *Chlamydia* infections. Pneumonia was indeed successfully treated with a regimen of erythromycin (Lieberman et al., 1997). However, *in vitro* experiments evaluating antibiotic susceptibility are lacking. Long-term *in vitro* treatment (4 months) with either rifampicin or azithromycin was shown to eliminate infectivity; however, DNA was still detectable and reactivation of the infection was possible with trypsin treatment after exposure to rifampicin (Kahane et al., 2007a). A treatment by doxycycline might alternatively be proposed for *Simkania* spp. infections based on the fact that it is one of the recommended treatment of *Chlamydia* and *Chlamydia*-related infections (de Barsy et al., 2014; Goy and Greub, 2009; Hammerschlag and Kohlhoff, 2012; Vouga et al., 2015).

Similarly to *P. acanthamoebae* and *N. hartmannellae* (de Barsy et al., 2014; Vouga et al., 2015), *S. negevensis* is resistant to quinolones (Casson and Greub, 2006). This resistance is probably due to a mutation in two quinolones resistance-determining regions (QRDR) as shown by genomic analysis, namely in the QRDR of GyrA and ParC, respectively (Casson and Greub, 2006; de Barsy et al., 2014). Studies reported a resistant phenotype to β-lactams derivatives (Kahane et al., 1993). However, high concentrations of β-lactams induce the development of aberrant bodies in *W. chondrophila* (Jacquier et al., 2014), despite the fact that it is traditionally considered resistant to β-lactams (de Barsy et al., 2014). The recent discovery of peptidoglycan among *Chlamydiales* may explain this finding (Jacquier et al., 2015). A similar phenotype might be observed upon exposure of
S. negevensis to high dose of β-lactams and might induce a partial bacteriostatic effect despite apparent resistance at lower concentrations.

FUTURE CHALLENGES AND CONCLUSIONS

Simkania negevensis, an emerging pathogen?

So far the pathogenic role of S. negevensis is difficult to hammer out, due to the lack of standardized studies. Although a high serological prevalence was often observed, direct detection of the pathogen or evidence of acute infections was rare. These findings are similar to what is observed for C. pneumoniae, whose pathogenic role is nevertheless commonly accepted. Recent studies reported a prevalence of C. pneumoniae infection of <2% in patients suffering from community acquired pneumonia (Dumke et al., 2015; Pletz et al., 2011; Senn et al., 2011; Wellinghausen et al., 2006). In addition, the correlation between serology and direct identification of C. pneumoniae through PCR is not good (Wellinghausen et al., 2006) and might be explained by a delay (2-3 weeks) in the apparition of IgM (Kuo et al., 1995). Thus, recent studies evaluating the role of S. negevensis infection in pneumonia using MIF might have missed some of the cases. Therefore, an implication of Simkania in respiratory diseases, especially in children and young adults, cannot be ruled out and requires further investigations. Despite the questionable reliability of some of the older studies that did not include controls, cases of Simkania infection were identified even in recent studies, either as the only putative pathogen or with other organisms (Dumke et al., 2015; Fasoli et al., 2008; Heiskanen-Kosma et al., 2008; Nascimento-Carvalho et al., 2009). The identification of a few patients with either a specific Simkania-associated pneumonia or bronchiolitis suggests
that it might be effectively a true pathogen and might be problematic in endemic regions. Table 5 summarizes the clinical findings of these patients. Physicians practicing in such regions, especially in Middle-east, should be aware of the existence of Simkania. In addition, infection might be promoted in cases of reduction of host defenses, either by a co-infection or by reduction of the mucociliary function. No information on the prevalence of Simkania infection in hospitalized patient is available, but, similarly to C. pneumoniae (Steinhoff et al., 1996), the prevalence might be higher due to increased proportion of immunocompromised patients.

Since other members of the Chlamydiales order including C. trachomatis and W. chondrophila have been associated with miscarriages (Baud et al., 2011b, 2007), a possible association of S. negevensis with adverse pregnancy outcomes should be investigated. In addition, pregnancy represents an immunotolerant state where S. negevensis could find an opportunity to reactivate (Alijotas-Reig et al., 2014; Kropf et al., 2007).

**Simkaniaceae, toward a better comprehension of the Chlamydiales**

S. negevensis harbors specificities not present in other Chlamydiales, notably, (i) the existence of a conjugative plasmid, which could promote genetic variations in this strict intracellular bacterium and (ii) a type-I intron suggesting a common evolutionary history with amoebae and/or plant plastids. In addition, the distinct features of S. negevensis membrane and the specificities of its metabolic pathways could help understanding the biology and evolution of this fascinating order.
In conclusion, *S. negevensis* represents a *Chlamydia*-related bacterium of high interest as it provides new insights into the biology and evolution of the *Chlamydiales* order. An implication in human diseases, particularly in respiratory tract infections in children and young adults, is suspected. Clinicians should thus consider *S. negevensis* in the differential diagnosis of respiratory tract infections and should search this pathogen using a pan-*Chlamydiales* PCR (Lienard et al., 2011a) or using another specific molecular test, that will be available in the near future. We recommend an antibiotic treatment with macrolides based on previous empirical observations, but doxycycline might represent a good alternative.
SEARCH STRATEGY AND SELECTION CRITERIA

We searched PubMed for articles published from January 1st, 1990 to September 30th, 2015 with the term “Simkania*” to identify both “Simkania” and “Simkaniaceae” and identified 81 published articles. We did not search for articles older than this date range as the first report describing Simkaniaceae was published in May 1993. We also reviewed relevant references cited in these articles, as well as articles referring to the “microorganism Z”, which was the first name of S. negevensis. In addition, we searched the NCBI nucleotide database with the terms “Simkania” and “Simkaniaceae” to recover potential related sequences.
SUMMARY POINTS

1. *Simkania negevensis* is a novel *Chlamydia*-related bacterium and the founding member of the *Simkaniaceae* family within the *Chlamydiales* order.

2. *S. negevensis* is able to replicate within a wide range of hosts including amoebae, arthropods, mammalian cells and reptiles.

3. Potential virulence factors include the presence of a conjugative plasmid, as well as a type 3 and a type 4 secretion systems. Pathogenesis may include the ability to induce persistent latent infections.

4. *S. negevensis* possess a type I intron in the 23S rRNA encoding gene suggesting a common evolutionary history with amoebae and/or plant plastids.

5. Evidence of human exposition has been described worldwide. Transmission may occur through contaminated water.

6. *S. negevensis* might represent an emerging pathogen associated with bronchiolitis and pneumonia in children and young adults.

7. Current diagnosis methods comprise PCR, culture with Vero cells or amoebae and serological assays.

8. Macrolides are the treatment of choice. Quinolones should be avoided, as *S. negevensis* is naturally resistant. Caution should be made when using penicillin as it may induce a persistent stage.

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DECLARATION OF INTEREST

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Statement

The authors report no declarations of interest.
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Table 1: General features of *Simkania negevensis* compared to other *Chlamydiales* members

Table 1 highlights various biological similarities and differences of *S. negevensis* and other *Chlamydiales*.

Abbreviations: CPAF, Chlamydia Protease-like Activity Factor; MOMP, Major Outer Membrane Protein; n.a., not applicable; nt, Nucleotides; PG, Peptidoglycan; Pmp, Polymorphic membrane protein; T3SS, Type 3 Secretion System; T4SS, Type 4 Secretion System

1 (Collingro et al., 2011)
2 (Carlson et al., 2008; Stephens et al., 1998)
3 (Bertelli et al., 2010)
4 (Horn et al., 2004)
5 (Bertelli et al., 2010; Carlson et al., 2008; Collingro et al., 2011; Horn et al., 2004; Stephens et al., 1998)
6 (Birkelund et al., 2009; Frandi et al., 2014; Jacquier et al., 2014; Kebbi-Beghdadi et al., 2015; Liechti et al., 2014; Pilhofer et al., 2014, 2013)
7 (Collingro et al., 2011; Rusconi and Greub, 2013)
8 (Bertelli et al., 2010; Carlson et al., 2006; Collingro et al., 2011; Horn et al., 2004; Knab et al., 2011; Omsland et al., 2014)
9 Two of them are considered as homologous of OmpA
10 Strain 2032/99 does not have a plasmid
11 Putative Pmp encoded in the genome, but not isolated at the membrane
Putative Pom encoded in the genome, but not isolated at the membrane

One homologue encoded in the genome, but not isolated at the membrane
| Table 1: General features of *Simkania negevensis* compared to other Chlamydiales members |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                  | *S. negevensis* | *C. trachomatis* D/UW-3/CX | *W. chondroplia* WSU B6-1044 | *P. acanthamoebae* UV-7 |
| Genetic features<sup>3</sup>      |                 |                 |                 |                 |
| Chromosome size (nt)             | 2.96337         | 1.042519        | 2.141577        | 3.072383        |
| Plasmid size (nt)                | 132.038         | 7.493           | 15.593<sup>10</sup> | no              |
| rRNA encoding genes              | 16S             | 2               | 2               | 3               |
|                                | 23S             | 2               | 2               | 3               |
| Outer membrane structure<sup>3</sup> |                 |                 |                 |                 |
| Major membrane protein          | MOMP-like       | MOMP            | MOMP-like       | Hypothetical protein (puv) |
| Cysteine-rich proteins (membrane stabilization) | absent | OmCA/OmcB | OmCA/OmcB/woc | Puv<sub>27500</sub>/Puv<sub>07550</sub><sup>12</sup> |
| Porins                          |                 |                 |                 |                 |
| MOMP (ompA)                      | MOMP-like       | MOMP            | MOMP-like       | Hypothetical protein (puv) |
|                                | 2               | 1               | no              | no              |
| MOMP like                       |                 |                 |                 |                 |
|                                | 35<sup>9</sup>  | n.a.            | 11              | 1<sup>11</sup>  |
| Pmp (auto transporter/cell adhesion) | yes (PmpB)     | yes             | yes             | ?<sup>11</sup> |
| PG-like structure               | not detected    | yes             | yes             | ?               |
| NlpD (PG remodeling enzyme)     | yes             | yes             | yes             | yes             |
| AmiA (PG remodeling enzyme)     | yes             | yes             | yes             | yes             |
| Host pathogens interactions<sup>7</sup> |                 |                 |                 |                 |
| CPAF (CMH expression, apoptosis, immune response) | no            | yes             | yes             | yes             |
| Catalase                        | no              | no              | yes             | yes             |
| T3SS                            | yes             | yes             | yes             | yes             |
| T4SS                            | yes             | no              | no              | yes             |
| Metabolic pathways<sup>8</sup>  |                 |                 |                 |                 |
| Glucokinase                     | yes             | no              | yes             | yes             |
| Citric acid cycle               | complete        | incomplete      | complete        | complete        |
| Nucleotides transporters        | 4               | 2               | 5               | 5               |
| Tryptophan operon               | complete        | incomplete      | absent           | absent           |
Table 2: Prevalence of an acute *Simkania* infection in patients suffering from bronchiolitis

Abbreviations: PCR, Polymerase Chain Reaction; NS, not significant

<table>
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<th>Country, patients</th>
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<th>Patients (%)</th>
<th>Controls (%)</th>
<th>p values</th>
<th>Co-infections (%)</th>
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<td>Israel, 239</td>
<td>PCR</td>
<td>60/239 (25)</td>
<td>0/78 (0)</td>
<td>&lt;0.001</td>
<td>22/60 (36)</td>
<td>Kahane, 1998</td>
</tr>
<tr>
<td></td>
<td>Immunoperoxidase</td>
<td>14/92 (15)</td>
<td>1/78 (1)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inuit Canada, 22</td>
<td>PCR</td>
<td>14/22 (63)</td>
<td></td>
<td></td>
<td>12/14 (85)</td>
<td>Greenberg, 2003</td>
</tr>
<tr>
<td>USA, 66</td>
<td>PCR</td>
<td>9/55 (16)</td>
<td>2/28 (7)</td>
<td>NS</td>
<td></td>
<td>Kumar, 2005</td>
</tr>
<tr>
<td>UK, 222</td>
<td>Nested PCR</td>
<td>100/222 (45)</td>
<td></td>
<td></td>
<td></td>
<td>Friedman, 2006</td>
</tr>
</tbody>
</table>
Table 3: Prevalence of an acute *Simkania* infection in patients suffering from pneumonia

Abbreviations: ELISA, enzyme-linked immunosorbent assay; MIA, membrane immunoassay; MIF, Micro Immunofluorescence; NS, not significant; PCR, Polymerase chain reaction

1. High signal for IgA (> 1.2 OD) or an increase of 0.5 OD units between paired sera levels for IgA (1:30) or IgG (1:100)

2. IgM ≥ 1:10 or a 4-fold increase between paired sera

3. When adjusted for recommended IgM cut-off titers ≥ 1:32, results are 12/174

4. After adjustment

5. IgA ≥ 1:8

6. pan-*Chlamydiales* PCR results are only specific at the family-level lineage. These cases should therefore be considered as *Simkaniaceae* infections.”

<table>
<thead>
<tr>
<th>Population, country, patients</th>
<th>Diagnostic method</th>
<th>Patients (%)</th>
<th>Controls (%)</th>
<th>p values</th>
<th><em>Simkania</em> as only pathogen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults, Israel, 308</td>
<td>ELISA&lt;sup&gt;1&lt;/sup&gt;</td>
<td>8/308 (3)</td>
<td></td>
<td></td>
<td>4</td>
<td>Lieberman, 1997</td>
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<tr>
<td>Children, USA, 25</td>
<td>PCR ELISA&lt;sup&gt;1&lt;/sup&gt;</td>
<td>5/22 (22)</td>
<td></td>
<td>NS</td>
<td></td>
<td>Kumar, 2005</td>
</tr>
<tr>
<td>Children, Israel, 34</td>
<td>Nested PCR MIA</td>
<td>31/34 (91)</td>
<td>34/35 (97)</td>
<td>NS</td>
<td>20</td>
<td>Kahane, 2007</td>
</tr>
<tr>
<td>Children, Finland, 174</td>
<td>MIF&lt;sup&gt;2&lt;/sup&gt;</td>
<td>18/174&lt;sup&gt;4&lt;/sup&gt; (10)</td>
<td></td>
<td></td>
<td>6&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Heiskanen-Kosma, 2008</td>
</tr>
<tr>
<td>Children, Italy, 101</td>
<td>MIF&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5/101 (5)</td>
<td></td>
<td></td>
<td>2</td>
<td>Fasoli, 2008</td>
</tr>
<tr>
<td>Children, Brazil, 183</td>
<td>MIF&lt;sup&gt;2&lt;/sup&gt;</td>
<td>3/184 (2)</td>
<td></td>
<td></td>
<td>1</td>
<td>Nascimento-Carvalho, 2009</td>
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<tr>
<td>Children, Switzerland, 265</td>
<td>PCR pan-*Chlamydia&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2/265 (1)</td>
<td></td>
<td></td>
<td></td>
<td>Liénard, 2011</td>
</tr>
<tr>
<td>Adults, Italy, 102</td>
<td>MIF&lt;sup&gt;5&lt;/sup&gt;</td>
<td>13/102 (13)</td>
<td>2/104 (2)</td>
<td>&lt;0.05</td>
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<td>Donati, 2013</td>
</tr>
</tbody>
</table>
Table 4: PCRs used to detect *Simkania* in clinical and environmental samples

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers and probes set</th>
<th>Amplicon length</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Simkania-specific</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>16S</td>
<td>Zpf 5’ AAAGGTAACGAATAATTCCCT-3’</td>
<td>398</td>
<td>Kahane, 1998</td>
</tr>
<tr>
<td></td>
<td>ZpR 5’ GCACAGTCGGGTTAGACCCGAC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16F2 5’ CAA GAA AAG GTA ACG AAT AAT TGCC3’</td>
<td>171</td>
<td>Niemi, 2011</td>
</tr>
<tr>
<td></td>
<td>16R2 5’ GAG CTC CGG AAT TTC ACA TCT G’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16S2 5’FAM-AAG GGC GCG TAG GCG GGT AAG C-BHQ1’-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23S</td>
<td>AF 5’-CACAGGTAGCCATGATGA-3’</td>
<td>1099</td>
<td>Everett, 1999</td>
</tr>
<tr>
<td></td>
<td>BR 5’-CTAGCTGCAGTGATGAAACG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>INTF 5’-TTAGATGCACAATGAGATGTGGA-3’</td>
<td>338</td>
<td>Everett, 1999</td>
</tr>
<tr>
<td></td>
<td>INTR 5’-CCATACGCGTCTATGTGCTCA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pan-Chlamydiales</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S</td>
<td>ccF 5’-CTT CGG GTT GTA AAG CAC TTT CGC-3’</td>
<td>512</td>
<td>Kahane, 2004</td>
</tr>
<tr>
<td></td>
<td>ccR 5’-CCC CGTCAA TTT TGA GTT T-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>panCh16F2 5’-CCGCAACTGGAAGACT-3’</td>
<td>207-215\textsuperscript{1}</td>
<td>Liénard, 2011</td>
</tr>
<tr>
<td></td>
<td>panCh16R2 5’-GGAGTTAGCCGGTCTTTTAC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>panCh16S 5’-FAM-CTACGGAGGCTCAGTGGAATC-BHQ1-3’</td>
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<td></td>
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</table>
Table 5: Patients with *Simkania*-caused pneumonia

Abbreviations: CRP, C-reactive protein; id., indeterminate; FMF, familial Mediterranean fever; d., days; m.o., months old; PCT, procalcitonin; WBC, white blood cells; y.o., years old

1 WBC > 11'000 is considered significant for a serious bacterial infection
2 CRP > 0.5 mg/l is considered significant for a serious bacterial infection
3 PCT > 0.5 microg/l is considered significant for a serious bacterial infection
Table 5:
Patients with Simkania-caused pneumonia

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
<th>Patient 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>31 y.o</td>
<td>32 y.o</td>
<td>32 y.o</td>
<td>28 y.o</td>
<td>57 m.o</td>
<td>52 m.o</td>
<td>58 m.o</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td><strong>Medical History</strong></td>
<td>None</td>
<td>None</td>
<td>FMF Colchicine treatment</td>
<td>None</td>
<td>id.</td>
<td>id.</td>
<td>Brazil</td>
</tr>
<tr>
<td><strong>Country</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td>5 pack-year</td>
<td>20 pack-year</td>
<td>15 pack-year</td>
<td>Id.</td>
<td>Id.</td>
<td>Id.</td>
<td>Id.</td>
</tr>
<tr>
<td><strong>Clinical</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fever</strong></td>
<td>38.5 °C</td>
<td>36.8 °C</td>
<td>39.2 °C</td>
<td>39.4 °C</td>
<td>Id.</td>
<td>Id.</td>
<td>Id.</td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td>unproductive cough, abdominal pain, diarrhea</td>
<td>unproductive cough, chest pain, diarrheea, vomiting</td>
<td>unproductive cough, chest pain</td>
<td>unproductive cough, chest pain</td>
<td>Id.</td>
<td>Id.</td>
<td>37.5 °C cough, dyspnœa</td>
</tr>
<tr>
<td><strong>WBC (cells/ml)</strong></td>
<td>7400</td>
<td>10'800</td>
<td>15'000</td>
<td>6'300</td>
<td>14'850</td>
<td>11'870</td>
<td>29'200</td>
</tr>
<tr>
<td><strong>CRP (mg/l)</strong></td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td>id.</td>
<td>id.</td>
<td>id.</td>
</tr>
<tr>
<td><strong>PCT (microg/l)</strong></td>
<td>id.</td>
<td>id.</td>
<td>id.</td>
<td>id.</td>
<td>id.</td>
<td>id.</td>
<td>id.</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td>Hospitalization</td>
<td>Antibiotic regimen</td>
<td>in patient 4d.</td>
<td>Erythromycin</td>
<td>in patient 3d.</td>
<td>Erythromycin</td>
<td>outpatient</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Erythromycin</td>
<td>Erythromycin</td>
<td>Erythromycin</td>
<td>Erythromycin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Penicillin after a trial</td>
<td>erythromycin</td>
<td>id.</td>
<td>id.</td>
<td>Penicillin</td>
</tr>
</tbody>
</table>
Figure 1: Evolutionary relationships of Simkaniaceae

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) based on 1548 nucleotides from 15 16S rRNA sequences. Sequences were retrieved from the NCBI database using “Simkania*” as a research word. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Efron et al., 1996). The evolutionary distances were computed using the Tamura 3-parameter method (Tamura, 1992) and are shown as number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution. Evolutionary analyses were conducted in MEGA5 after sequence analysis on Geneious 7.1.7 (Kearse et al., 2012)
SUPPLEMENTARY MATERIALS

Supplementary table: Clinical studies with Simkania

Abbreviations: CAP, Community acquired pneumonia; COPD, Chronic obstructive pulmonary disease; ELISA, enzyme-linked immunosorbent assay; MIA, membrane immune-assay; MIF, Micro Immunofluorescence; NS, Not significant; PCR, Polymerase chain reaction; qPCR, quantitative PCR; RTI, Respiratory tract infection

1 Past infections determined by IgG levels
2 Recent infections determined by high IgM levels, augmentation of IgG or IgM levels between two paired sera or PCR
3 IgG ≥ 1:16
4 IgG ≥ 1:8, IgM ≥1:10
5 51 broncho-alveolar lavages and concomitant biopsies obtained from 19 patients included
6 When adjusted for recommended IgM cut-off titers ≥ 1:32, results are 12/174
7 After adjustment 8/12
8 Only a 4-fold increase or decrease between paired sera was considerer as positive
9 IgG ≥ 1:16, IgA ≥1:8
<table>
<thead>
<tr>
<th>Population, country, patients</th>
<th>Disease</th>
<th>Diagnostic method</th>
<th>past infections (%)</th>
<th>Controls (%)</th>
<th>p values</th>
<th>Recent infections (%)</th>
<th>Controls (%)</th>
<th>p values</th>
<th>Co-infections (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults, Israel, 308</td>
<td>CAP</td>
<td>Bacterial culture</td>
<td>114/308 (37)</td>
<td>83/308</td>
<td>37/0.001</td>
<td>8/8</td>
<td>(0)</td>
<td>&lt;0.001</td>
<td>22/60</td>
<td>(37)</td>
</tr>
<tr>
<td>Children, Israel, 239</td>
<td>Bronchitis</td>
<td>EUSA</td>
<td>66/239 (25)</td>
<td>0/78 (0)</td>
<td>&lt;0.001</td>
<td>4/8</td>
<td>(0)</td>
<td>&lt;0.001</td>
<td>22/60</td>
<td>(37)</td>
</tr>
<tr>
<td>Adults, smokers, Israel, 190</td>
<td>Exacerbation COPD</td>
<td>Bacterial culture</td>
<td>120/190 (63)</td>
<td>69/100 (69)</td>
<td>NS</td>
<td>5/217 (2)</td>
<td>0.05</td>
<td>80</td>
<td>12/14</td>
<td>86</td>
</tr>
<tr>
<td>Children, Inuit, Canada, 22</td>
<td>Bronchitis</td>
<td>EUSA</td>
<td>149/22 (6)</td>
<td>1/78 (1)</td>
<td>&lt;0.001</td>
<td>4/5</td>
<td>80</td>
<td>12/14</td>
<td>86</td>
<td>Greenberg, 2003</td>
</tr>
<tr>
<td>Adults, Denmark, 197</td>
<td>Persistent cough</td>
<td>EUSA</td>
<td>80/185 (43)</td>
<td>41/100 (41)</td>
<td>NS</td>
<td>0/76 (0)</td>
<td>0.05</td>
<td>80</td>
<td>12/14</td>
<td>86</td>
</tr>
<tr>
<td>Adults, USA, 37</td>
<td>Asthma</td>
<td>PCR 105 (Kahane)</td>
<td>8/17 (5)</td>
<td>6/37 (16)</td>
<td>NS</td>
<td>4/27 (15)</td>
<td>0.05</td>
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<td>12/14</td>
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<tr>
<td>Children, USA, 66</td>
<td>Bronchitis</td>
<td>EUSA</td>
<td>4/6 (0)</td>
<td>3/17 (18)</td>
<td>NS</td>
<td>2/22 (22)</td>
<td>0.05</td>
<td>80</td>
<td>12/14</td>
<td>86</td>
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<tr>
<td>Children, USA, 25</td>
<td>CAP</td>
<td>EUSA (Kahane)</td>
<td>2/9 (11)</td>
<td>8/31 (26)</td>
<td>NS</td>
<td>2/22 (22)</td>
<td>0.05</td>
<td>80</td>
<td>12/14</td>
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<td>Children, USA, 60</td>
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<td>EUSA (Kahane)</td>
<td>5/33 (15)</td>
<td>5/24 (21)</td>
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<td>5/55 (16)</td>
<td>0.004</td>
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<td>12/14</td>
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<tr>
<td>Adults, UK, 29</td>
<td>RTI</td>
<td>EUSA (Kahane)</td>
<td>18/29 (62)</td>
<td>12/22 (54)</td>
<td>NS</td>
<td>5/33 (15)</td>
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<tr>
<td>Children, UK, 222</td>
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<td>Nested PCR (Kahane)</td>
<td>12/104 (11)</td>
<td>12/122 (10)</td>
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<td>Children, Finland, 104</td>
<td>Asthma</td>
<td>Nested PCR (Kahane)</td>
<td>31/54 (57)</td>
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<td>17/54 (31)</td>
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<td>12/14</td>
<td>86</td>
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<tr>
<td>Children, Israel, 34</td>
<td>CAP</td>
<td>Nested PCR (Kahane)</td>
<td>17/34 (50)</td>
<td>17/24 (71)</td>
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<td>8/51 (16)</td>
<td>0.004</td>
<td>80</td>
<td>12/14</td>
<td>86</td>
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<tr>
<td>Adults, USA, 17</td>
<td>Acute rejection lung transplant</td>
<td>Nested PCR (Kahane)</td>
<td>34/37 (92)</td>
<td>12/28 (43)</td>
<td>NS</td>
<td>1/128 (0)</td>
<td>0.004</td>
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<td>12/14</td>
<td>86</td>
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<tr>
<td>Children, Finland, 174</td>
<td>CAP</td>
<td>MIF (Korpipäät)</td>
<td>14/174 (8)</td>
<td>12/178 (7)</td>
<td>NS</td>
<td>1/174 (8)</td>
<td>0.004</td>
<td>80</td>
<td>12/14</td>
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<td>Children, Italy, 101</td>
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<td>MIF (Korpipäät)</td>
<td>5/101 (5)</td>
<td>2/2 (10)</td>
<td>NS</td>
<td>1/101 (5)</td>
<td>0.004</td>
<td>80</td>
<td>12/14</td>
<td>86</td>
</tr>
<tr>
<td>Children, Brazil, 183</td>
<td>CAP</td>
<td>MIF (Korpipäät)</td>
<td>3/184 (2)</td>
<td>2/3 (67)</td>
<td>NS</td>
<td>3/184 (2)</td>
<td>0.004</td>
<td>80</td>
<td>12/14</td>
<td>86</td>
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<tr>
<td>Children, Switzerland, 265</td>
<td>CAP</td>
<td>PCR pan-Catamody</td>
<td>2/265 (1)</td>
<td>2/3 (67)</td>
<td>NS</td>
<td>2/265 (1)</td>
<td>0.004</td>
<td>80</td>
<td>12/14</td>
<td>86</td>
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<tr>
<td>Adults, Finland, 541</td>
<td>RTI</td>
<td>PCR pan-Catamody</td>
<td>5/541 (1)</td>
<td>2/3 (67)</td>
<td>NS</td>
<td>5/541 (1)</td>
<td>0.004</td>
<td>80</td>
<td>12/14</td>
<td>86</td>
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<tr>
<td>Adults, Italy, 102</td>
<td>Lower RTI</td>
<td>MIF</td>
<td>51/102 (50)</td>
<td>38/104 (35)</td>
<td>0.05</td>
<td>11/102 (13)</td>
<td>2/104 (2)</td>
<td>&lt;0.05</td>
<td>2/104 (2)</td>
<td>&lt;0.05</td>
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<tr>
<td>Adults, Italy, 124</td>
<td>Gastro-intestinal symptoms</td>
<td>MIF</td>
<td>15/124 (12)</td>
<td>36/104 (35)</td>
<td>&lt;0.001</td>
<td>40/124 (32)</td>
<td>2/104 (2)</td>
<td>&lt;0.05</td>
<td>2/104 (2)</td>
<td>&lt;0.05</td>
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