Author Manuscript
Faculty of Biology and Medicine Publication

This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Published in final edited form as:

1   Review

2   *SIMKANIA NEGEVENSIS,* **AN INSIGHT INTO THE BIOLOGY AND CLINICAL**

3   **IMPORTANCE OF A NOVEL MEMBER OF THE *CHLAMYDIALES* ORDER**

4   Manon Vouga[1,2], David Baud[1*], Gilbert Greub[2,3*#]

5   * Equally contributed

6

7   #Address correspondence to Gilbert Greub, gilbert.greub@chuv.ch

8   [1]Materno-fetal and Obstetrics Research Unit, Department of Obstetrics and Gynecology,

9   Maternity, University Hospital, Lausanne, Switzerland

10   [2]Center for Research on Intracellular Bacteria, Institute of Microbiology, Faculty of

11   Biology and Medicine, University of Lausanne and University Hospital, Lausanne,

12   Switzerland

13   [3]Infectious Disease Service, University Hospital, Lausanne, Switzerland

14

15   Running-head: *Simkania*, new insights

16   **Key-words**: *Chlamydia,* intracellular bacteria, pneumonia, bronchiolitis

17

18   **ABSTRACT**

19   *Simkania negevensis* is a *Chlamydia*-related bacterium discovered in 1993 and represents

20   the founding member of the *Simkaniaceae* family within the *Chlamydiales* order. As

21   other *Chlamydiales*, it is an obligate intracellular bacterium characterized by a biphasic

22   developmental cycle. Its similarities with the pathogenic *Chlamydia trachomatis* and

23   *Chlamydia pneumoniae* make it an interesting bacterium. So far, little is known about its

24   biology, but *S. negevensis* harbors various microbiological characteristics of interest

25   including a strong recruitment of the ER to the *Simkania*-containing vacuole and the

26   presence of an intron in the 23S rRNA encoding gene.

27   Evidence of human exposition has been reported worldwide. However, there is a lack of

28   robust clinical studies evaluating its implication in human diseases; current data suggest

29   an association with pneumonia and bronchiolitis making *S. negevensis* a potential

30   emerging pathogen. Owing to its fastidious growth requirements, the clinical relevance of

31   *S. negevensis* is probably underestimated. In this review, we summarize the current

32   knowledge   on   *S.   negevensis*   and   explore   future   research   challenges.

2

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.

3

55	In this review, we summarize the current knowledge on *S. negevensis* and explore future

56	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

5

80 *Parachlamydiaceae* (Everett et al., 1999a). These "Everett" cut-offs have been validated

81 by the International Subcommittee of Taxonomy (Greub, 2010) and have been recently

82 extended by Pillonel *et al.* to use 6 additional taxonomically informative genes (Pillonel

83 et al., 2015). Currently, the order is divided in nine family-level lineages, namely the

84 *Chlamydiaceae, Clavichlamydiaceae, Criblamydiaceae, Parachlamydiaceae,*

85 *Parilichlamydiaceae, Piscichlamydiaceae, , Rhabdochlamydiaceae, Simkaniaceae* and

86 *Waddliaceae* (Bavoil et al., 2013; Stride et al., 2013). Such division might however only

87 represent a small portion of the ecological diversity of this order as suggested by

88 Lagkouvardos *et al.*, who estimate that more than 180 potential family-level lineages

89 likely exist (Lagkouvardos et al., 2014).

90

91 **MICROBIOLOGY**

92 ***Simkaniaceae* diversity**

93 The *Simkania* genus belongs to the *Simkaniaceae* family, within the *Chlamydiales* order

94 and consists of a single species, *S. negevensis*. *Simkaniaceae* comprise 3 genera:

95 *Simkania*, *Fritschea* and *Syngnamydia*; the latter two genera are reported as *Candidatus*

96 strains, since they have not been yet recovered by culture. *Fritschea* were first identified

97 in 2003 as endosymbionts of arthropods and are subdivided in 2 *Candidatus* species: *F.*

98 *bemisiae* and *F. eriococci* (Everett et al., 2005; Thao et al., 2003; Xue et al., 2012).

99 *Syngnamydia,* which also includes 2 *Candidatus* species (*S. venezia* and *S. salmonis*),

100 were recently described as pathogens in fishes, causing epitheliocystis, a disease

101 characterized by large cysts in the gills (Fehr et al., 2013; Nylund et al., 2014).

102 Additionally, seven DNA sequences recovered in environmental samples were recently

6

103   described as putative members of the *Simkaniaceae* family based on their 16S rRNA and

104   23S rRNA encoding genes sequences. The first sequence (EF177461) was recovered in

105   gastrodermal cells of *Xenoturbella,* a primitive marine worm (Israelsson, 2007). Three

106   other sequences (FJ976094, FJ976095 and AF448723.3) were identified in environmental

107   water samples and grouped as the cvE9 cluster (cvE38, cvE41 and cvE9) (Corsaro et al.,

108   2002; Corsaro and Venditti, 2009). The last three were recovered from unicellular

109   eukaryotes present in the ocean crust and from citrus plants suffering from the Yellow

110   dragon disease (*Huanglongbing*), a mortal disease in citrus, caused by vector transmitted

111   Gram-negative bacteria (Sagaram et al., 2009; Santelli et al., 2008)

112

113   ***Simkania negevensis*: genome and evolution**

114   All *Chlamydiales* derived from a *Chlamydiae*/*Planctomycetes*/*Verrucomicrobia* common

115   ancestor (Budd and Devos, 2012). Three evolutionary clusters appear to exist: (i) the

116   *Chlamydiaceae*/*Clavichlamydiaceae* cluster, which harbors the smallest genomes and

117   seems   to   have   branched   the   earliest,   (ii)   the

118   *Parachlamydiaceae*/*Waddliaceae*/*Criblamydiaceae*   cluster   and   (iii)   the

119   *Simkaniaceae*/*Rhabdochlamydiaceae* cluster (Lagkouvardos et al., 2014). Indeed,

120   *Simkaniaceae* share an 86-87% homology in the 16S rRNA encoding gene sequence with

121   *Rhabdochlamydiaceae* (Kostanjsek et al., 2004) and only 82% homology with

122   *Chlamydiaceae* (Kahane et al., 1995). Comparative analysis of the full genome sequences

123   showed more than 400 conserved core genes preserved in all *Chlamydiales* members

124   (Bertelli et al., 2010; Collingro et al., 2011; Pillonel et al., 2015). Among them, 20 are

125   highly informative taxonomically and should be preferentially used to assign new strains

126   at species level (Pillonel et al., 2015).

127   *S. negevensis* genome is characterized by a 2-3 fold larger size compared to the

128   *Chlamydiaceae* members, but comparable to the genome size of *Waddliaceae* and

129   *Parachlamydiaceae* members (Collingro et al., 2011), as shown in table 1. This suggests

130   a large biosynthetic ability, allowing adaptation to various environments. The analysis of

131   *S. negevensis* genome revealed the presence of a type I intron localized in the 23S rRNA

132   gene (Everett et al., 1999b). Type I introns are self-splicing motile introns, which have so

133   far only been described in eukaryotic cells. To date, *S. negevensis* and *Coxiella burnetii*

134   are the only bacterial species, which possess such genetic structure (Nesbø and Doolittle,

135   2003). This molecular pattern is also present in the amoeba *Acanthamoeba castellanii* as

136   well as in the algal chloroplasts (Everett et al., 1999b) and therefore may have been

137   acquired from horizontal gene transfer. This discovery, along with the high proportion of

138   eukaryotic-like genes in chlamydial genomes, questions the possible relationship between

139   *Chlamydiales* and chloroplasts and their possible implication in the eukaryiogenesis

140   (Brinkman et al., 2002). The function of this intron remains unclear, but evidence showed

141   that the intron is not spliced from the 23S rRNA and might delay growth by reducing

142   ribosomal function (Everett et al., 1999b). It is not known whether this intron was also

143   present, at first, in other *Chlamydiales* growing in amoebae and was subsequently

144   eliminated to improve replication or whether it was horizontally acquired after the

145   divergence of *S. negevensis* from other *Chlamydiales*.

146   *S. negevensis* possess a 132kb plasmid, which carries a type IV secretion system (T4SS)

147   encoded by a *tra* operon. A similar *tra* operon is also present in the genome of

8

148 *Protochlamydia amoebophila* (Greub et al., 2004a) and of *Parachlamydia*

149 *acanthamoebae* (Greub et al., 2009)*, two members of the *Parachlamydiaceae* family.

150 These operons exhibit a striking similarity with the *tra* genes carried by the conjugative

151 F-plasmid of *Escherichia coli*. Thus, it is suspected that these chlamydial operons encode

152 a conjugative DNA transfer system. In addition, the *S. negevensis tra* operon also exhibits

153 genetic similarities to the T4SS conjugative system encoded in *Rickettsia belli*, where

154 conjugation has been demonstrated (Ogata et al., 2006), reinforcing the putative role of

155 the *S. negevensis* T4SS in DNA transfer. Independent of the presence of the T4SS, the

156 identification of a plasmid in the *S. negevensis* genome is of utmost interest, as it might

157 play a significant role in the pathogenesis of *S. negevensis* infection, as suggested for the

158 7'500kb *Chlamydia* spp. plasmid. Indeed, it has been shown both *in vivo* (Kari et al.,

159 2011; O'Connell et al., 2007) and *in vitro* (Porcella et al., 2015) that infections with a

160 plasmid deficient strain resulted in attenuated infections exhibiting a lower inflammatory

161 response. An adaptive immune response was however elicited enabling protection against

162 re-infection (Kari et al., 2011; O'Connell et al., 2007; Porcella et al., 2015). The

163 mechanisms by which the chlamydial plasmid modulates the pathogenesis are still

164 unclear, but they are probably related to: (i) direct effect of proteins encoded by the

165 plasmid (Liu et al., 2014, p. 201; Li et al., 2008) and (ii) regulation of the expression of

166 other virulence factors encoded by the chromosome (Carlson et al., 2008; Song et al.,

167 2013). Interestingly, the *S. negevensis* plasmid encodes various proteins of interest,

168 among which one copy of the Macrophage Infectivity Potentiator (Mip) (Collingro et al.,

169 2011). This lipoprotein is highly similar to the Mip protein of *Legionella pneumophila*. In

170 *Chlamydia,* it has been shown to be essential for an optimal intracellular infection and to

9

171 induce the production of pro-inflammatory cytokines (Bas et al., 2008; Lundemose et al.,

172 1993a, 1993b).

173

174 **Biochemical characteristics**

175 **Cell wall and surface proteins.** The composition of the outer membrane is of significant

176 importance for the pathogenesis of *Chlamydiales*. In the EB state, membrane proteins are

177 implicated in the entry within the host and serve as major antigens for the immune

178 response. In the RB state, membrane proteins may be part of the secretion systems used

179 to corrupt the host cell whereas others may serve as porins to uptake nutrients from the

180 host cytoplasm. A comparison of the structure of the membrane of various members of

181 the *Chlamydiales* order is shown in table 1.

182 The Chlamydial Outer Membrane Complex (COMC) of the *Chlamydiaceae* is mainly

183 composed by the MOMP (*ompA*) protein and by 2 cystein-rich proteins, OmcA and

184 OmcB (Caldwell et al., 1981). MOMP (*ompA*) represents 60% of the surface proteins, at

185 least in purified EBs and serves as a porin (Bavoil et al., 1984). Aistleitner *et al*. have

186 recently studied the structure of *S. negevensis* outer membrane (Aistleitner et al., 2014).

187 Using a computerized proteomic approach, 65 OMPs were identified as major

188 components of the *S. negevensis* EB's membrane. Among them 37 were MOMP-like

189 proteins, which resemble the MOMP (*ompA*) of *Chlamydiaceae*. Out of the 37 MOMP-

190 like proteins, 30 contained beta-sheet conformations, suggesting a role as porins. Two of

191 them demonstrated high similarity to MOMP proteins of veterinary *Chlamydia* species

192 (Collingro et al., 2011). *S. negevensis* outer membrane lacks the cysteine-rich proteins

193 (OmcA and OmcB) present in other *Chlamydiales*. These cysteine-rich proteins are

10

194　sensitive to the redox state of the environment and they might provide some rigidity and

195　resistance to osmotic pressure through the formation of disulfide bridges. Some authors

196　suggested that they may replace the peptidoglycan layer present in the periplasm of other

197　Gram-negative bacteria (Everett and Hatch, 1995; Sun et al., 2007). No homologues of

198　OmcA and OmcB were identified in *S. negevensis* genome and none of the identified

199　MOMP-like proteins could serve this function as their content in cysteine was extremely

200　low (Aistleitner et al., 2014). Such differences may result in a more flexible envelope and

201　a lack of surface proteins regulated by the redox state. Such regulations are associated

202　with modifications of the function of several surface proteins in *Chlamydiaceae* through

203　reduction and reoxidation of the cysteine residues and could play a role in the switch

204　from the EB to the RB state. For example, the permeability of MOMP is increased once

205　reduced (Bavoil et al., 1984) and the type III secretion system (T3SS) in *Chlamydia* is

206　also described as influenced by the redox state (Betts-Hampikian and Fields, 2011).

207　Studying the morphology of EBs and RBs particles is difficult and has been the subject of

208　various works. As an example, crescent bodies and star bodies were reported as infectious

209　EB-like particles of *Parachlamydiaceae* and *Criblamydiaceae*, respectively (Lienard et

210　al., 2011b; Rusconi et al., 2013; Thomas et al., 2006). Current knowledge suggests that

211　these observations are largely influenced by the buffer and the fixatives used and

212　therefore do not allow taxonomic discrimination, even if they correspond to some

213　structural differences in the cell wall of the different bacterial families (Pilhofer et al.,

214　2014; Rusconi et al., 2013). Noteworthy, *S. negevensis* EBs were described as highly

215　pleomorphic particles, especially once located in the cytoplasm (Pilhofer et al., 2014).

11

216   Such observation reinforces the hypothesis of a relative high cell wall flexibility due to

217   the lack of cysteine-rich surface proteins in *S. negevensis*.

218

219   The inner membrane of most bacteria is protected by a cell wall composed of

220   peptidoglycan. This structure is important for membrane stability as well as cell division.

221   Until recently, it was thought that *Chlamydiales* were lacking peptidoglycan and their

222   partial sensitivity to cell wall inhibitors such as penicillin was therefore not understood

223   and considered as a paradox called the *Chlamydiales* anomaly. However, the presence of

224   peptidoglycan was recently demonstrated in some members of the *Chlamydiales*, namely

225   *C. trachomatis*, in which they were present as a ring structure at the division septum

226   (Liechti et al., 2014) and *P. amoebophila,* which synthesizes sacculi that contain a

227   modified form of peptidoglycan (Pilhofer et al., 2013). Additionally, a recent work

228   identified two peptidoglycan-remodeling enzymes (the AmiA amidase and the NlpD

229   endopeptidase) implicated in coordinated cell wall constriction during division. Such

230   enzymes were shown to recognize peptidoglycan-like peptides in *Chlamydiales* (Frandi et

231   al., 2014). Most of this work was done on *Waddlia chondrophila,* a *Chlamydiales*

232   member that is susceptible to high dose of penicillin and also to fosfomycin, a drug active

233   on MurA (Frandi et al., 2014; Jacquier et al., 2014). The MurA enzyme is implicated in

234   peptidoglycan biosynthesis, suggesting the presence of peptidoglycan-like structures in at

235   least 3 family-level lineages of the *Chlamydiales* order, namely the *Chlamydiaceae,*

236   *Waddliaceae* and *Parachlamydiaceae* (Frandi et al., 2014; Jacquier et al., 2015, 2014). In

237   their first report, Kahane *et al.* described *S. negevensis* as being resistant to 10μg/ml of

238   penicillin, as opposed to what was observed for *C. trachomatis*. It was, however,

12

239   sensitive to cycloserine, an inhibitor of the peptidoglycan synthesis, similarly to other

240   *Chlamydiaceae.* In addition, activity of the AmiA amidase and the NlpD endopeptidase

241   was, recently, documented in *S. negevensis*, suggesting the presence of peptidoglycan-

242   like structures in *S. negevensis* (Frandi et al., 2014)*, despite failure to isolate

243   peptidoglycan containing sacculi in *S. negevensis* (Liechti et al., 2014). Their detection

244   might be prevented by a restricted time-frame synthesis (Jacquier et al., 2015) or might

245   require an additional method, similar to the one recently described by Packlam *et al.*,

246   which allowed the isolation of peptidoglycan fragments in *C. trachomatis* (Packiam et al.,

247   2015).

248

249   **Secretion systems.** Intracellular bacteria parasitize their host's metabolic pathways to

250   their own advantage. In parallel, they must inhibit the trigger of the host's immune

251   system. One strategy consists in secreting various proteins into the host cytoplasm or

252   environment through secretion systems (Greub, 2013). *S. negevensis* encodes a Type III

253   secretion system (T3SS) (Collingro et al., 2011). Hsia *el al.* first suggested the existence

254   of a type III secretion system in *Chlamydia* (Hsia et al., 1997)*, which was likely present

255   in the *Chlamydiales* common ancestor given its presence in every genome sequenced so

256   far (Bertelli et al., 2015, 2010; Greub et al., 2009; Horn et al., 2004; Hovis et al., 2013;

257   Stephens et al., 1998), as summarized in table 1. The T3SS appears as a needle inserted in

258   the inner and outer membranes of the bacterium, which protrudes into the cytoplasm of

259   the cell through the inclusion membrane (Pilhofer et al., 2013). Although, the structure of

260   the T3SS is highly conserved among *Chlamydiales*, effectors secreted through this

261   system largely vary among families and genera (Collingro et al., 2011). Interestingly, *S.*

13

262   *negevensis* does not possess genes encoding for major chaperones of the T3SS, namely

263   the SctF, CdsF, CT666 and the Specific *Chlamydiales* Chaperon 3 (SCC3), which are

264   conserved in *Waddliaceae*, *Parachlamydiaceae* and *Chlamydiaceae* (Collingro et al.,

265   2011).

266   As mentioned above, *S. negevensis* plasmid encodes a T4SS. In addition to its likely role

267   in plasmid propagation and DNA exchange, it may also allow secretion of proteins and

268   may represent an important virulence factor.

269

270   **Metabolism.** Strict intracellular bacteria are often characterized by their important lack

271   of metabolic pathways and the necessity to parasitize their hosts for nutrients and energy.

272   However, genomic studies have revealed extended metabolic capabilities of *S. negevensis*

273   compared to *Chlamydiaceae* (Collingro et al., 2011; Omsland et al., 2014)*.* This extended

274   metabolic capacity has also been observed for other *Chlamydia*-related bacteria

275   exhibiting large genomes (Bertelli et al., 2010; Collingro et al., 2011; Greub et al., 2009;

276   Horn et al., 2004), as shown in Table 1. Among major differences, we note their ability to

277   directly use glucose for the glycolysis pathway due to the presence of a glucokinase,

278   making them independent from the host cell glucose-6-phosphate (Bertelli et al., 2010;

279   Collingro et al., 2011; Greub et al., 2009; Omsland et al., 2014). Additionally, they

280   possess a complete citric acid cycle as opposed to *Chlamydiaceae,* which lack important

281   enzymes and need a constant exchange with the host cell to produce energy by oxidation

282   of acetyl-CoA (Omsland et al., 2014). In *S. negevensis*, entry in the citrate cycle can

283   occur: (a) through acetyl-CoA produced from pyruvate following glycolysis and (b)

284   through the transformation of asparagine in fumarate (Collingro et al., 2011; Omsland et

14

285   al., 2014). Moreover, *Waddliaceae*, *Parachlamydiaceae* and *Simkaniaceae* are able to

286   synthetize NAD+ from nicotinamide (Bertelli et al., 2010; Omsland et al., 2014) and *S.*

287   *negevensis* can, additionally, use asparagine as a source of NAD+ (Collingro et al., 2011;

288   Knab et al., 2011; Omsland et al., 2014). Finally, an *in vitro* experiment by Kahane *et al.*

289   suggests that *S. negevensis* is able to grow in a glucose free medium; indeed the number

290   of infectious forming units was similar using a Roswell Park Memorial Institute (RPMI)

291   media depleted in glucose and supplemented with 1% glucose (Kahane et al., 1999).

292   Taken together, these findings suggest that *S. negevensis* is less dependent from the host

293   cell in term of energy production compared to other *Chlamydiales* (Omsland et al., 2014).

294   Similarly to *Chlamydiaceae*, *S. negevensis* is unable to synthesize nucleotides *de novo*

295   and relies on nucleotides transporters for their uptake. Four isoforms of nucleotides

296   transporters have been identified in *S. negevensis*: SnSTT1, an ADP/ATP antiporter;

297   SnSTT2, a guanine/ATP/H+ symporter; SnSTT3 a global nucleotide triphosphate

298   antiporter, also able to transport deoxy-CTP (dCTP). The function of the last transporter,

299   SnSTT4, is unknown (Knab et al., 2011). The ability to transport dCTP is unique among

300   prokaryotes and might represent a selective metabolic advantage for *S. negevensis*,

301   favoring its relative growth in environments rich in cytosine.

302   Regarding amino-acid biosynthesis, *S. negevensis* is able to synthesize tyrosine,

303   phenylalanine, proline, alanine, aspartate and glutamate (Collingro et al., 2011; Omsland

304   et al., 2014). In addition, *S. negevensis* possesses a complete tryptophan operon (*trp*

305   operon) (Collingro et al., 2011; Omsland et al., 2014). Some pathogenic *Chlamydiaceae*,

306   notably the genitourinary strains of *C. trachomatis*, are partially capable of synthesizing

307   this metabolite but enzymes required in this pathway are completely absent in other

15

308   *Chlamydia*-related bacteria (Carlson et al., 2006; Omsland et al., 2014). It appears that

309   tryptophan plays an important role in the pathogenesis of *Chlamydia* spp. infections.

310   Tryptophan is, indeed, an important regulator of the chlamydial development cycle

311   (Bonner et al., 2014). In case of tryptophan depletion, the expression pattern changes and

312   forces the bacteria into a persistent form (Baud et al., 2008; Brunham and Rey-Ladino,

313   2005; Ibana et al., 2011). Tryptophan depletion can be induced in humans by IFN-γ

314   production (Taylor and Feng, 1991). Thus, *S. negevensis* might be less sensitive do IFN-γ

315   than *Chlamydiaceae,* which could play a role in the pathogenesis.

316

317   **Culture characteristics**

318   **Cell permissiveness and growth cycle.** *S. negevensis* is able to grow and survive *in vitro*

319   in mammalian cells such as Vero cells (Kahane et al., 1993), epithelial cells from the

320   respiratory and genitourinary tract, endothelial cells (Kahane et al., 2007a) as well as

321   macrophages (Kahane et al., 2008). Growth is also described in arthropods (both *in vitro*

322   and *in vivo*) (Croxatto et al., 2014; Sixt et al., 2012), and amoebae (Kahane et al., 2001).

323   Similarly to other *Chlamydiales*, *S. negevensis* growth cycle is characterized by the

324   presence of two developmental stages: one large replicative form (Reticulate Body, RB)

325   and one small compact form, which resembles to electron dense (Elementary Body, EB)

326   form of *Chlamydiaceae*. These two forms can be selectively fractionated on density

327   gradients (Kahane et al., 1999, 1993). The high density of EBs is likely due to the

328   presence of condensed DNA as shown by the presence of filamentous material on

329   electron microscopy tomography (EMT). Even though gene expression might be

16

330 downregulated through DNA condensation, EB particles are likely to be metabolically

331 active as suggested by the presence of ribosomes (Pilhofer et al., 2014).

332

333 The growth cycle of *S. negevensis* is characterized by a production phase lasting

334 approximately 3 days, during which particles are produced in an exponential way. During

335 that time, the cytoplasm of the host cells shows increasing dark perinuclear patches. This

336 is followed by a plateau phase, during which no or few particles are produced, but instead

337 the particles previously produced are stored in multiple large cytoplasmic vacuoles of the

338 host cells and cytopathic changes are observed. Based on culture observations, *S.*

339 *negevensis* does not seem to induce cell lysis, or at least not to the same extent as what is

340 observed for other members of the *Chlamydiales* order, such as *W. chondrophila* (Kebbi-

341 Beghdadi et al., 2011) In comparison, *C. trachomatis* particles are released between 30 to

342 68 hours post infection depending on the strain (Miyairi et al., 2006), *W. chondrophila*

343 particles are released 48-72 hours upon cell lysis (Goy et al., 2008) and *P.*

344 *acanthamoebae* induces lysis of infected amoebae after 72-96 hours depending on the

345 initial Multiplicity Of Infection (MOI) and incubation temperature (Greub et al., 2003b;

346 Greub and Raoult, 2002b). However, despite the apparent absence of cell lysis, some

347 particles seem to be released in the extracellular environment, as suggested by the

348 presence of *S. negevensis* particles in the supernatant of infected insect cell cultures

349 capable of inducing a second round of infection (Sixt et al., 2012). Recently, Hybiske *et*

350 *al.* showed that *C. trachomatis* may exit from the host cell through the extrusion of a

351 membrane-bound compartment (Hybiske and Stephens, 2007). This extrusion depended

352 on actin polymerization, as well as on various proteins implicated in the remodeling of

17

353   the cytoskeleton. A similar mechanism might be implicated in the extrusion of *S.*

354   *negevensis***.**

355   Interestingly, it has been proposed that *S. negevensis* RBs might be infectious, an aspect

356   that has not been observed for other *Chlamydiales* so far. Indeed, Kahane *et al*, observed

357   similar growth kinetics of *S. negevensis* purified EBs and RBs (Kahane et al., 2002).

358   However, this has not been confirmed yet and further investigations are needed before

359   making any definite conclusions.

360

361   **The *Simkania* containing vacuole and its interaction with cellular organelles.** *S.*

362   *negevensis* replicates in a single intracellular vacuole hereafter called the *Simkania*

363   Containing Vacuole (SCV). Interestingly, the SCV is closely associated with the

364   endoplasmic reticulum (ER) of the host cell, being almost completely surrounded by the

365   typical ribosmes-covered ER membrane (Mehlitz et al., 2014; Pilhofer et al., 2014). Such

366   ER recruitment was observed in infected epithelial cells, macrophages-derived cells and

367   amoebae, emphasizing the strong conservation of the phenotype. Intracellular traffic of *S.*

368   *negevensis* clearly differs from *P. acanthamoebae*, which survives in the late endosome

369   (Greub et al., 2005) and from *C. trachomatis*, which intercepts Golgi-derived vesicles and

370   causes Golgi apparatus fragmentation (Heuer et al., 2009). Recruitment of the ER also

371   occurs upon infection with other members of the *Chlamydiales* order, such as *C.*

372   *trachomatis* and *W. chondrophila*, but to a lesser extent than what is observed following

373   *S. negevensis* infection (reviewed in Derré, 2015). The mechanisms by which *S.*

374   *negevensis* induces this tight association remain to be elucidated, but might rely on

375   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

19

399   specific mechanism to recruit the mitochondria, or if their association to the SCV is

400   related to their natural association with the ER

401

402 **EPIDEMIOLOGY**

403 **Prevalence**

404 Scarce data on the prevalence of *Simkania* are available in the literature, most of them

405 provided by old studies. Caution should be taken when interpreting these results. The

406 seroprevalence appears high in the Middle East (Israel and Jordanian), ranging from 55-

407 65% among healthy adults and reaching 80% in Bedouins (Al-Younes and Paldanius,

408 2014; Friedman et al., 1999). Serological evidence of past infection was documented in

409 46% of pregnant women in Cornwall, UK (Friedman et al., 2006) and in 41% of Danish

410 blood donors (Johnsen et al., 2005). In contrast, an overall prevalence of only 23.5% was

411 observed among children and adults in Brooklyn, USA (Kumar et al., 2005) and only

412 4.5% of the population tested in Japan were seropositive, a low prevalence observed

413 despite the low cut-off titer of 1:8 used in the study (Yamaguchi et al., 2005). The large

414 discrepancy between these results could be explained by geographical differences in term

415 of exposure and risk factors. However, the heterogeneity of diagnostic tools used between

416 the studies makes comparisons difficult. Indeed, the prevalence observed in the Danish

417 and Jordanian population was obtained using an immunofluorescence assay with a cut-off

418 titer of 1:16 and 1:8, respectively, which probably overestimated the prevalence (Al-

419 Younes and Paldanius, 2014; Johnsen et al., 2005). Nevertheless, the relatively high

420 prevalence in Israel, England and USA was observed using the same ELISA assay based

421 on purified EBs (Friedman et al., 2006, 1999; Kumar et al., 2005). Authors tested a 1/100

422 diluted serum and considered an OD (optical density) higher than 0.5 as positive. In

423 conclusion, serological evidence of human exposure to *Simkania* infection was

21

424 documented worldwide and prevalence seems to increase with increasing age (Friedman

425 et al., 2006; Johnsen et al., 2005; Kumar et al., 2005).

426

427 **Transmission and reservoir**

428 *S. negevensis* was discovered as a cell culture contaminant. However, the natural host of

429 *Simkaniaceae* remains unknown. As mentioned above, *S. negevensis* is able to grow *in*

430 *vitro* in a wide range of hosts (i.e. mammalian cells, arthropods, amoebae) (Kahane et al.,

431 2008, 2007a, 2001; Sixt et al., 2012). Evidence of *Simkaniaceae* was documented *in vivo*

432 in ticks (Croxatto et al., 2014; Pilloux et al., 2015) and in granulomatous lesions in

433 reptiles (Soldati et al., 2004). *Fritschea* were discovered in arthropods (Everett et al.,

434 2005) and *Syngdamydia* in fish (Fehr et al., 2013; Nylund et al., 2014). Additionally,

435 various *Simkania*-related DNA sequences were amplified from marine environments

436 (Israelsson, 2007; Santelli et al., 2008) and in one case in vegetal cells (Sagaram et al.,

437 2009). This wide host range is quite impressive and contrasts with the narrow host range

438 of *Chlamydia trachomatis* (human-specific) and *Chlamydia felis* (cat-specific).

439

440 Various hypotheses on the reservoir of *Simkaniaceae* and their mode of transmission to

441 humans have been proposed. Firstly, contaminated water might be a source of human

442 infection, either as a free-living organism or through infected amoebae. Indeed, a report

443 showed that *S. negevensis* infectivity *in vitro* was still preserved after a 7 days incubation

444 of free particles in distilled water and was even increased in cases of co-infection with

445 amoebae (Kahane et al., 2004). In comparison, the infectivity of *C. trachomatis,*

446 considered as a sexually transmitted disease, was completely abolished after 5 hours of

22

447 incubation in the same conditions (Kahane et al., 2004). Moreover, co-culture of

448 macrophages with *S. negevensis*-infected amoebae resulted in the rapid death of the

449 amoebae and infection of the macrophages (Kahane et al., 2008). Amoebae could

450 therefore serve as reservoir and contribute to the selection of virulence traits (Greub and

451 Raoult, 2004; Lamoth and Greub, 2010). This is in agreement with the identification of

452 *Simkania* and amoebae's antigens in drinking water and in reclaimed wastewater used to

453 irrigate crop in Israel (Donati et al., 2015; Kahane et al., 2004). Similarly, *Simkania* DNA

454 was amplified from environmental water in Western Europe (Corsaro et al., 2002;

455 Corsaro and Venditti, 2009) and, recently, in Italian swimming pools (Donati et al., 2015;

456 Pérez et al., 2012, 2011). Transmission to humans through water is also suggested by a

457 prospective study, in which it was possible to identify the presence of *Simkania* in the

458 corresponding drinking water of children suffering from a *Simkania*-associated

459 pneumonia in as much as 76.5% of cases. In this study, results were only considered as

460 positive when congruent results were obtained by at least two different approaches

461 (Nested PCR, culture in Vero cells and/or Membrane Enzyme Immuno-Assay (MEIA))

462 (Kahane et al., 2007b). Nevertheless, these results should be treated with caution as (i)

463 only 34 children were included in the study and (ii) nested PCR contamination cannot be

464 excluded due to the very high proportions of positive results observed in the study.

465 Finally, an oral transmission of *S. negevensis* is likely since (i) a significant IgA response

466 to *Simkania* was observed in patients with gastro-intestinal symptoms (Donati et al.,

467 2013) and (ii) *S. negevensis* may replicate *in vitro* within gastro-intestinal cell lines

468 (Kahane et al., 2007a).

23

469   Arthropods, such as ticks, might represent another source of infection. Vector

470   transmission is quite common for other intracellular bacteria such as *Rickettsia* (Walker

471   and Ismail, 2008). Moreover, *Simkania*-associated DNA was amplified from Swiss

472   *Ixodes ricinus* ticks, though with a lower prevalence and amount than

473   *Rhabdochlamydiaceae* (Croxatto et al., 2014; Pilloux et al., 2015).

474   Additional hypotheses of transmission include direct human to human transmission

475   through aerosols or droplets due to the association with respiratory diseases (see

476   pathogenic potential, hereafter), as well as sexual contacts or contacts with animals,

477   which were described for other *Chlamydiales* (Baud et al., 2007; Baud and Greub, 2011;

478   Gottlieb et al., 2013; Knittler and Sachse, 2015; Schachter, 1986). Sexual contact seems

479   unlikely due to the high prevalence in children and the absence of cross-prevalence with

480   *C. trachomatis* (Friedman et al., 1999). Finally, a zoonotic transmission has so far not

481   been documented, but cannot be excluded.

482

24

483 **PATHOGENIC POTENTIAL**

484 *In vitro*

485 Several *in vitro* aspects of *S. negevensis* infection suggest an important pathogenic

486 potential. First*, S. negevensis* is able to grow and survive within macrophage-type cell

487 line U937 (Kahane et al., 2008). Moreover, uninfected epithelial cells can be actively

488 infected after co-culture with infected macrophages (Kahane et al., 2008). Interestingly,

489 *S. negevensis* is resistant *in vitro* to LL-37 cathelicidin human peptide present in

490 phagocyte cells, but is highly sensitive to 5 other animal cathelicidins (Donati et al.,

491 2011). Moreover, Karunakaran *et al*. demonstrated that *S. negevensis* prevents TNF-α-

492 induced apoptosis in host cells through the inhibition of the release of mitochondrial

493 caspase-activating proteins (Karunakaran et al., 2011). This is similar to what is known

494 for the pathogenic *C. trachomatis* and *C. pneumoniae* (Fan et al., 1998). However, the

495 phenotype seems to be stronger in *S. negevensis* as it could be observed even at a very

496 low MOI (MOI=0.5) (Fan et al.*,* 1998; Karunakaran et al., 2011). This anti-apoptotic

497 capability represents an important virulence factor and is not observed in all

498 *Chlamydiales*. For example, *Parachlamydiaceae* appear to instead induce apoptosis in

499 macrophages (Greub et al., 2003c) and in insect cell lines (Sixt et al., 2012). The ability

500 to inhibit apoptosis and the absence of cell lysis might allow *S. negevensis* to persist

501 within its host and establish a persistent latent infection. Such infection might then be

502 reactivated and might represent an important pathogenic mechanism.

503 Two types of *S. negevensis* infection have been described: (i) an active infection with

504 production of new bacterial particles and observation of cytopathic effects in the host,

505 and (ii) a persistent infection without any cytopathic effects (Kahane et al., 2007a). In

25

506 persistent infection, mostly RBs of atypical morphology were visualized within the

507 infected cells. Persistence of infectious particles within the host was demonstrated by

508 inoculation of uninfected Vero cells with persistently infected cells, which produced an

509 active infection. Infection was however less efficient compared to inoculation with

510 actively infected cells.

511 In *Chlamydia* spp., persistence may be induced by iron depletion (Raulston, 1997), IFN-γ

512 (Beatty et al., 1994; Rottenberg et al., 2002) and penicillin treatment (Matsumoto and

513 Manire, 1970). With *S. negevensis,* a latent infection can be observed either

514 spontaneously in specific cell lines, namely intestinal or genitourinary lines, or could be

515 induced by iron depletion. Reactivation was then possible after serial trypsin treatment

516 (Kahane et al., 2007a). Interestingly, reactivation was also observed when persistently

517 infected cells were cultured with macrophages-derived cells. The mechanism, by which

518 reactivation is induced, is still unclear, but might rely on recognition of the infection by

519 the macrophages and production of cytokines, which could promote the release of

520 infectious *S. negevensis* particles (Kahane et al., 2008). In addition, an inflammatory

521 response was observed in both types of infections, mostly through the production of IL-8

522 and IL-6 (Kahane et al., 2007a).

523

524 **Lessons from the clinic**

525 Since the discovery of *S. negevensis*, only few studies have been conducted to evaluate

526 the implication of *Simkania* spp. in human diseases (see list of all studies in

527 supplementary materials). An implication in respiratory diseases, particularly in

528 bronchiolitis and pneumonia in children and young adults, is likely, as shown in tables 2

26

529 and 3, respectively, which summarize the findings of major clinical studies. Thus, a

530 significant association between *Simkania* and acute bronchiolitis was shown in children

531 from Israel (Kahane et al., 1998). The authors used both a PCR and a cell culture

532 approach to detect the organism in nasopharyngeal samples. A total of 239 patients and

533 78 controls were included over a period of two years. Of the 116 patients, from whom a

534 possible etiologic agent was isolated, 60 were positive for *Simkania* by at least one of the

535 techniques used. This was the second most common agent identified after respiratory

536 syncitial virus *(*RSV) and statistically different from controls. Clinical findings were

537 similar to patients infected by RSV. Similar results were also obtained in a study

538 performed on English children with bronchiolitis. In 26% of the patients, evidence of a

539 *Simkania* infection was provided by both PCR and culture. This rate reached 45% when

540 considering only samples positive by PCR. However no controls were included in this

541 study (Friedman et al., 2006).

542 Various works showed evidence of an acute infection with *Simkania* in cases of

543 pneumonia in children and young adults (Donati et al., 2011; Fasoli et al., 2008;

544 Heiskanen-Kosma et al., 2008; Kahane et al., 2007b; Lieberman et al., 1997; Lienard et

545 al., 2011a; Nascimento-Carvalho et al., 2009). Acute infection was indicated either by a

546 rise in paired sera of IgG, IgA or IgM defined as a 4 fold increase of antibody titers

547 observed by micro-immunofluorescence (Fasoli et al., 2008; Heiskanen-Kosma et al.,

548 2008; Nascimento-Carvalho et al., 2009) or a rise of 0.5 OD in the ELISA signal

549 (Lieberman et al., 1997), or by elevated titers of IgM or IgA or direct identification of

550 *Simkania* through PCR, culture or antigen detection (Kahane et al., 2007b). Globally, a

551 low prevalence of acute infection was observed (2-10%), except in the study performed

27

552   by Kahane *et al.,* in which *Simkania* was identified in almost all samples, including

553   controls. Evidence of co-infections with other putative pathogens was found in 40 to 60%

554   of the cases; the most commonly associated pathogens were *Mycoplasma pneumoniae,*

555   RSV, *Streptococcus pneumoniae* and *Chlamydia pneumoniae* (Fasoli et al., 2008;

556   Lieberman et al., 1997; Nascimento-Carvalho et al., 2009). Noteworthy, the association

557   of *Simkania* infection with the occurrence of bronchiolitis or pneumonia was not

558   confirmed in a study performed on children, in Brooklyn, USA (Kumar et al., 2005).

559   An association between *Simkania* infection and other respiratory diseases was also

560   studied. However, no association was found with asthma (Korppi et al., 2006; Kumar et

561   al., 2005), nor with exacerbation of Chronic Obstructive Pulmonary Disease (COPD)

562   (Lieberman et al., 2002), chronic cough (Johnsen et al., 2005) or acute lung rejection

563   (Husain et al., 2007). Results are difficult to interpret owing to low statistical power of

564   these studies. However, a significant correlation with serological evidence of an acute

565   infection was shown in unspecified respiratory tract infection among adults, in England

566   (Friedman et al., 2006) and a higher prevalence of *Simkania* was documented in patients

567   with a lung transplant as compared to other immunocompromised patients or

568   immunocompetent patients (Husain et al., 2007).

569

570   Caution should be taken when interpreting the results since only few studies included a

571   control group. In addition, the same group from the Ben-Gurion University of the Negev,

572   in Israel, discovered *S. negevensis* and performed most of the studies suggesting a

573   pathogenic role. The specificity of the diagnostic tools might have not been optimal and

574   contamination could have occurred within the laboratory. Indeed, the prevalence

28

575   observed in the studies performed by this group (26% in Israel (Kahane et al., 1998),

576   63.6% in Inuit patients (Greenberg et al., 2003) and 80 to 97.5% in lung transplants

577   (Husain et al., 2007)) are strikingly high, even in the control groups (Kahane et al.,

578   2007b; Kumar et al., 2005).

579   Moreover, recent studies performed by other groups have failed to confirm such high

580   prevalence. Indeed, using a specific quantitative PCR, Niemi *et al.* could not identify any

581   positive case of *Simkania* infection in 97 respiratory samples taken from adult patients

582   with a suspected respiratory tract infection (Niemi et al., 2011). Similarly, only two

583   samples from Swiss children with bronchiolitis were identified as positive for a

584   *Simkaniaceae* infection by a validated pan-*Chlamydiales* quantitative PCR (Lienard et al.,

585   2011a) and only one case of a co-infection with *Simkania* and *C. psittaci* was identified in

586   a large German study investigating community acquired pneumonia (Dumke et al., 2015).

587   This discrepancy between the results could either be due to differences in the reliability

588   of the diagnostic techniques used or to geographical variations of the prevalence of the

589   infection.

590 **DIAGNOSIS**

591 Various techniques have been described to diagnose a *S. negevensis* infection, reviewed

592 by Corsaro *et al.* (Corsaro and Greub, 2006). Most of them were developed in the early

593 2000's. They may now lack specificity due to the recent discovery of novel *Chlamydiales*

594 species.

595

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

597 **PCR.** PCR is currently the technique of choice to diagnose *C. trachomatis* or *C.*

598 *pneumoniae* infections. Diagnostic real-time PCRs have also been developed and are

599 routinely used in our laboratory to detect *W. chondrophila, P. acanthamoebae* and *P.*

600 *neagleriophila* in clinical samples (Casson et al., 2008a, 2008b; Goy et al., 2009). By

601 analogy, this technique should be preferentially used to detect *Simkania* spp.. Most of the

602 primers and probes developed earlier, which are listed in Table 4, are based on the

603 detection of amplicons on agarose gel, a technique with a lower sensitivity and specificity

604 than real-time PCR, at risk of contamination by amplicons. Thus, we currently

605 recommend the use of a pan-*Chlamydiales* PCR (Lienard et al., 2011a) followed by

606 sequencing to detect putative cases of infection.

607

608 **Culture.** Isolation of organisms through culture is crucial to prove the presence of viable

609 bacteria in clinical samples and to provide a reference strain for a precise downstream

610 characterization, as well as to provide antigens for serological assays. *Simkania* spp. may

611 be difficult to recover from clinical samples through culture, as it requires cell culture,

612 which is at risk of contamination by other bacteria present in the flora of non-sterile

30

613   samples such as sputa, nasopharyngeal swabs or vaginal swabs. Owing to these culture

614   difficulties, the sensitivity of culture seems to be lower than PCR (Corsaro and Greub,

615   2006; Kahane et al., 1998).

616   Culture in Vero cells is currently the method of choice to isolate *Simkania* spp. in clinical

617   samples (Corsaro and Greub, 2006), although amoebal co-culture might represent an

618   ideal alternative for respiratory samples, given the non-susceptibility of amoebae to

619   *Mycoplasma* and to most bacteria present in the physiological oropharynx flora (Greub et

620   al., 2004b; Jacquier et al., 2013). Testing the cells used for cell culture for the presence of

621   a possible contamination should be routinely done; this screening should be able to detect

622   *Mycoplasma* spp. as well as *Chlamydiales,* including *S. negevensis,* since the later was

623   first isolated as a cell culture contaminant. We recommend microscopy examination of

624   cell lines and a Pan-*Chlamydiales* PCR (Lienard et al., 2011a), when contamination is

625   suspected.

626

627   **Others.** Immunohistochemistry based on rabbit or mouse anti-*S. negevensis* antibodies

628   may be used to visualize this bacterium in lesions when biopsies are available. By

629   analogy with other *Chlamydiales*, Gram staining should be avoided since the results are

630   inconsistent, as EBs result generally Gram-positive, whereas RBs result Gram-negative

631   (Lamoth and Greub, 2010).

632

633   **Serological studies**

634   Micro-immunofluorescence (MIF) is currently the technique of choice to detect anti-*C.*

635   *pneumoniae* specific antibodies (Dowell et al., 2001). By analogy, this technique should

31

636   also be applied to detect anti-*Simkania* spp. specific antibodies. Current recommendations

637   for *Chlamydia* and *Chlamydia*-related bacteria propose a cut-off titer of ≥1:64 for IgG to

638   confirm a past infection and a cut-off of ≥1:32 for IgM for an acute infection (Corsaro

639   and Greub, 2006). Acute infections may also be documented based on seroconversion

640   defined as an increase in IgG titer from 0 to ≥1:64 in paired sera or a ≥4-fold rise in the

641   IgG titer between acute- and convalescent-phase sera (Corsaro and Greub, 2006). Such

642   recommendations should also be applied to detect a *Simkania* infection in order to enable

643   inter-laboratories comparison of obtained results. Alternatively, an ELISA assay

644   developed in our laboratory to detect *W. chondrophila* (Lienard et al., 2014) could be

645   adapted for *Simkania* infections. Such ELISA might be a good alternative, as the results

646   are not biased by subjective analysis. Nevertheless, we do not recommend using the assay

647   developed by Lieberman *et al.* (Lieberman et al., 1997) since it has not been properly

648   validated and may overestimate true seroprevalence.

649 **TREATMENT AND PREVENTION**

650 **Antibiotic susceptibility**

651 So far, *Simkania* infections have been treated empirically with macrolides, similarly to

652 other *Chlamydia* infections. Pneumonia was indeed successfully treated with a regimen

653 of erythromycin (Lieberman et al., 1997). However, *in vitro* experiments evaluating

654 antibiotic susceptibility are lacking. Long-term *in vitro* treatment (4 months) with either

655 rifampicin or azithromycin was shown to eliminate infectivity; however, DNA was still

656 detectable and reactivation of the infection was possible with trypsin treatment after

657 exposure to rifampicin (Kahane et al., 2007a). A treatment by doxycycline might

658 alternatively be proposed for *Simkania* spp. infections based on the fact that it is one of

659 the recommended treatment of *Chlamydia* and *Chlamydia*-related infections (de Barsy et

660 al., 2014; Goy and Greub, 2009; Hammerschlag and Kohlhoff, 2012; Vouga et al., 2015).

661 Similarly to *P. acanthamoebae* and *N. hartmannellae* (de Barsy et al., 2014; Vouga et al.,

662 2015)*, S. negevensis* is resistant to quinolones (Casson and Greub, 2006). This resistance

663 is probably due to a mutation in two quinolones resistance-determining regions (QRDR)

664 as shown by genomic analysis, namely in the QRDR of GyrA and ParC, respectively

665 (Casson and Greub, 2006; de Barsy et al., 2014). Studies reported a resistant phenotype to

666 β-lactams derivatives (Kahane et al., 1993). However, high concentrations of β-lactams

667 induce the development of aberrant bodies in *W. chondrophila* (Jacquier et al., 2014),

668 despite the fact that it is traditionally considered resistant to β-lactams (de Barsy et al.,

669 2014). The recent discovery of peptidoglycan among *Chlamydiales* may explain this

670 finding (Jacquier et al., 2015). A similar phenotype might be observed upon exposure of

671 *S. negevensis* to high dose of β-lactams and might induce a partial bacteriostatic effect

672 despite apparent resistance at lower concentrations.

673

674 **FUTURE CHALLENGES AND CONCLUSIONS**

675 ***Simkania negevensis,* an emerging pathogen?**

676 So far the pathogenic role of *S. negevensis* is difficult to hammer out, due to the lack of

677 standardized studies. Although a high serological prevalence was often observed, direct

678 detection of the pathogen or evidence of acute infections was rare. These findings are

679 similar to what is observed for *C. pneumoniae*, whose pathogenic role is nevertheless

680 commonly accepted. Recent studies reported a prevalence of *C. pneumoniae* infection of

681 <2% in patients suffering from community acquired pneumonia (Dumke et al., 2015;

682 Pletz et al., 2011; Senn et al., 2011; Wellinghausen et al., 2006). In addition, the

683 correlation between serology and direct identification of *C. pneumoniae* through PCR is

684 not good (Wellinghausen et al., 2006) and might be explained by a delay (2-3 weeks) in

685 the apparition of IgM (Kuo et al., 1995). Thus, recent studies evaluating the role of *S.*

686 *negevensis* infection in pneumonia using MIF might have missed some of the cases.

687 Therefore, an implication of *Simkania* in respiratory diseases, especially in children and

688 young adults, cannot be ruled out and requires further investigations. Despite the

689 questionable reliability of some of the older studies that did not include controls, cases of

690 *Simkania* infection were identified even in recent studies, either as the only putative

691 pathogen or with other organisms (Dumke et al., 2015; Fasoli et al., 2008; Heiskanen-

692 Kosma et al., 2008; Nascimento-Carvalho et al., 2009). The identification of a few

693 patients with either a specific *Simkania*-associated pneumonia or bronchiolitis suggests

34

694 that it might be effectively a true pathogen and might be problematic in endemic regions.

695 Table 5 summarizes the clinical findings of these patients. Physicians practicing in such

696 regions, especially in Middle-east, should be aware of the existence of *Simkania*. In

697 addition, infection might be promoted in cases of reduction of host defenses, either by a

698 co-infection or by reduction of the mucociliary function. No information on the

699 prevalence of *Simkania* infection in hospitalized patient is available, but, similarly to *C.*

700 *pneumoniae* (Steinhoff et al., 1996), the prevalence might be higher due to increased

701 proportion of immunocompromised patients.

702

703 Since other members of the *Chlamydiales* order including *C. trachomatis* and *W.*

704 *chondrophila* have been associated with miscarriages (Baud et al., 2011b, 2007), a

705 possible association of *S. negevensis* with adverse pregnancy outcomes should be

706 investigated. In addition, pregnancy represents an immunotolerant state where *S.*

707 *negevensis* could find an opportunity to reactivate (Alijotas-Reig et al., 2014; Kropf et al.,

708 2007).

709

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

711 *S. negevensis* harbors specificities not present in other *Chlamydiales*, notably, (i) the

712 existence of a conjugative plasmid, which could promote genetic variations in this strict

713 intracellular bacterium and (ii) a type-I intron suggesting a common evolutionary history

714 with amoebae and/or plant plastids. In addition, the distinct features of *S. negevensis*

715 membrane and the specificities of its metabolic pathways could help understanding the

716 biology and evolution of this fascinating order.

35

717

718 In conclusion, *S. negevensis* represents a *Chlamydia*-related bacterium of high interest as

719 it provides new insights into the biology and evolution of the *Chlamydiales* order. An

720 implication in human diseases, particularly in respiratory tract infections in children and

721 young adults, is suspected. Clinicians should thus consider *S. negevensis* in the

722 differential diagnosis of respiratory tract infections and should search this pathogen using

723 a pan-*Chlamydiales* PCR (Lienard et al., 2011a) or using another specific molecular test,

724 that will be available in the near future. We recommend an antibiotic treatment with

725 macrolides based on previous empirical observations, but doxycycline might represent a

726 good alternative.

727 **SEARCH STRATEGY AND SELECTION CRITERIA**

728 We searched PubMed for articles published from January 1st, 1990 to September 30th,

729 2015 with the term "*Simkania**" to identify both "*Simkania*" and "*Simkaniaceae*" and

730 identified 81 published articles. We did not search for articles older than this date range

731 as the first report describing *Simkaniaceae* was published in May 1993. We also reviewed

732 relevant references cited in these articles, as well as articles referring to the

733 "microorganism Z", which was the first name of *S. negevensis*. In addition, we searched

734 the NCBI nucleotide database with the terms "*Simkania*" and "*Simkaniaceae*" to recover

735 potential related sequences.

736

737

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.

38

761

DECLARATION OF INTEREST

Funding

Statement

The authors report no declarations of interest.

773

39

REFERENCES

Aistleitner, K., Anrather, D., Schott, T., Klose, J., Bright, M., Ammerer, G., Horn, M., 2014. Conserved features and major differences in the outer membrane protein composition of *Chlamydiae*. Environ. Microbiol.

Alijotas-Reig, J., Llurba, E., Gris, J.M., 2014. Potentiating maternal immune tolerance in pregnancy: a new challenging role for regulatory T cells. Placenta 35, 241–248.

Al-Younes, H.M., Paldanius, M., 2014. High seroprevalence of *Simkania negevensis* in Jordan. Braz. J. Microbiol. 45, 1433–1437.

Asner, S.A., Jaton, K., Kyprianidou, S., Nowak, A.-M.L., Greub, G., 2014. *Chlamydia pneumoniae*: possible association with asthma in children. Clin. Infect. Dis. 58, 1198–1199.

Bartlett, E.C., Levison, W.B., Munday, P.E., 2013. Pelvic inflammatory disease. BMJ 346, f3189.

Bas, S., Neff, L., Vuillet, M., Spenato, U., Seya, T., Matsumoto, M., Gabay, C., 2008. The proinflammatory cytokine response to *Chlamydia trachomatis* elementary bodies in human macrophages is partly mediated by a lipoprotein, the macrophage infectivity potentiator, through TLR2/TLR1/TLR6 and CD14. J. Immunol. Baltim. Md 1950 180, 1158–1168.

Baud, D., Goy, G., Jaton, K., Osterheld, M.-C., Blumer, S., Borel, N., Vial, Y., Hohlfeld, P., Pospischil, A., Greub, G., 2011a. Role of *Chlamydia trachomatis* in miscarriage. Emerg. Infect. Dis. 17, 1630–1635.

Baud, D., Goy, G., Osterheld, M.-C., Borel, N., Vial, Y., Pospischil, A., Greub, G., 2011b. *Waddlia chondrophila*: from bovine abortion to human miscarriage. Clin. Infect. Dis. 52, 1469–1471.

Baud, D., Goy, G., Osterheld, M.-C., Croxatto, A., Borel, N., Vial, Y., Pospischil, A., Greub, G., 2014. Role of *Waddlia chondrophila* placental infection in miscarriage. Emerg. Infect. Dis. 20, 460–464.

Baud, D., Greub, G., 2011. Intracellular bacteria and adverse pregnancy outcomes. Clin. Microbiol. I 17, 1312–1322.

Baud, D., Regan, L., Greub, G., 2008. Emerging role of *Chlamydia* and *Chlamydia*-like organisms in adverse pregnancy outcomes. Curr. Opin. Infect. Dis. 21, 70–76.

Baud, D., Thomas, V., Arafa, A., Regan, L., Greub, G., 2007. *Waddlia chondrophila*, a potential agent of human fetal death. Emerg. Infect. Dis. 13, 1239–1243.

Bavoil, P., Kaltenboeck, B., Greub, G., 2013. In *Chlamydia* veritas. Pathog. Dis. 67, 89–90.

Bavoil, P., Ohlin, A., Schachter, J., 1984. Role of disulfide bonding in outer membrane structure and permeability in *Chlamydia trachomatis*. Infect. Immun. 44, 479–485.

Beatty, W.L., Morrison, R.P., Byrne, G.I., 1994. Persistent *Chlamydiae*: from cell culture to a paradigm for chlamydial pathogenesis. Microbiol. Rev. 58, 686–699.

Bertelli, C., Aeby, S., Chassot, B., Clulow, J., Hilfiker, O., Rappo, S., Ritzmann, S., Schumacher, P., Terrettaz, C., Benaglio, P., Falquet, L., Farinelli, L., Gharib, W.H., Goesmann, A., Harshman, K., Linke, B., Miyazaki, R., Rivolta, C., Robinson-Rechavi, M., van der Meer, J.R., Greub, G., 2015. Sequencing and

40

818   characterizing the genome of *Estrella lausannensis* as an undergraduate project:
819   training students and biological insights. Front. Microbiol. 6, 101.

820 Bertelli, C., Collyn, F., Croxatto, A., Rückert, C., Polkinghorne, A., Kebbi-Beghdadi, C.,
821   Goesmann, A., Vaughan, L., Greub, G., 2010. The *Waddlia* genome: a window
822   into chlamydial biology. PloS One 5, e10890.

823 Betts-Hampikian, H.J., Fields, K.A., 2011. Disulfide bonding within components of the
824   *Chlamydia* type III secretion apparatus correlates with development. J. Bacteriol.
825   193, 6950–6959.

826 Birkelund, S., Morgan-Fisher, M., Timmerman, E., Gevaert, K., Shaw, A.C.,
827   Christiansen, G., 2009. Analysis of proteins in *Chlamydia trachomatis* L2 outer
828   membrane complex, COMC. FEMS Immunol. Med. Microbiol. 55, 187–195.

829 Blasi, F., Tarsia, P., Aliberti, S., 2009. *Chlamydophila pneumoniae.* Clin. Microbiol.
830   Infect. 15, 29–35.

831 Bonner, C.A., Byrne, G.I., Jensen, R.A., 2014. *Chlamydia* exploit the mammalian
832   tryptophan-depletion defense strategy as a counter-defensive cue to trigger a
833   survival state of persistence. Front. Cell. Infect. Microbiol. 4, 17.

834 Brinkman, F.S.L., Blanchard, J.L., Cherkasov, A., Av-Gay, Y., Brunham, R.C.,
835   Fernandez, R.C., Finlay, B.B., Otto, S.P., Ouellette, B.F.F., Keeling, P.J., Rose,
836   A.M., Hancock, R.E.W., Jones, S.J.M., Greberg, H., 2002. Evidence that plant-
837   like genes in *Chlamydia* species reflect an ancestral relationship between
838   *Chlamydiaceae*, cyanobacteria, and the chloroplast. Genome Res. 12, 1159–1167.

839 Brunham, R.C., Rey-Ladino, J., 2005. Immunology of *Chlamydia* infection: implications
840   for a *Chlamydia trachomatis* vaccine. Nat. Rev. Immunol. 5, 149–161.

841 Budd, A., Devos, D.P., 2012. Evaluating the Evolutionary Origins of Unexpected
842   Character Distributions within the Bacterial Planctomycetes-Verrucomicrobia-
843   Chlamydiae Superphylum. Front. Microbiol. 3, 401.

844 Caldwell, H.D., Kromhout, J., Schachter, J., 1981. Purification and partial
845   characterization of the major outer membrane protein of *Chlamydia trachomatis*.
846   Infect. Immun. 31, 1161–1176.

847 Carlson, J.H., Whitmire, W.M., Crane, D.D., Wicke, L., Virtaneva, K., Sturdevant, D.E.,
848   Kupko, J.J., Porcella, S.F., Martinez-Orengo, N., Heinzen, R.A., Kari, L.,
849   Caldwell, H.D., 2008. The *Chlamydia trachomatis* plasmid is a transcriptional
850   regulator of chromosomal genes and a virulence factor. Infect. Immun. 76, 2273–
851   2283.

852 Carlson, J.H., Wood, H., Roshick, C., Caldwell, H.D., McClarty, G., 2006. *In vivo* and *in*
853   *vitro* studies of *Chlamydia trachomatis* TrpR:DNA interactions. Mol. Microbiol.
854   59, 1678–1691.

855 Casson, N., Greub, G., 2006. Resistance of different *Chlamydia*-like organisms to
856   quinolones and mutations in the quinoline resistance-determining region of the
857   DNA gyrase A- and topoisomerase-encoding genes. Int. J. Antimicrob. Agents 27,
858   541–544.

859 Casson, N., Michel, R., Müller, K.-D., Aubert, J.D., Greub, G., 2008a. *Protochlamydia*
860   *naegleriophila* as etiologic agent of pneumonia. Emerg. Infect. Dis. 14, 168–172.
861   doi:10.3201/eid1401.070980

862 Casson, N., Posfay-Barbe, K.M., Gervaix, A., Greub, G., 2008b. New diagnostic real-
863   time PCR for specific detection of *Parachlamydia acanthamoebae* DNA in
864   clinical samples. J. Clin. Microbiol. 46, 1491–1493.
865 Collingro, A., Tischler, P., Weinmaier, T., Penz, T., Heinz, E., Brunham, R.C., Read,
866   T.D., Bavoil, P.M., Sachse, K., Kahane, S., Friedman, M.G., Rattei, T., Myers,
867   G.S.A., Horn, M., 2011. Unity in variety--the pan-genome of the *Chlamydiae*.
868   Mol. Biol. Evol. 28, 3253–3270.
869 Corsaro, D., Greub, G., 2006. Pathogenic potential of novel *Chlamydiae* and diagnostic
870   approaches to infections due to these obligate intracellular bacteria. Clin.
871   Microbiol. Rev. 19, 283–297.
872 Corsaro, D., Venditti, D., 2009. Detection of *Chlamydiae* from freshwater environments
873   by PCR, amoeba coculture and mixed coculture. Res. Microbiol. 160, 547–552.
874 Corsaro, D., Venditti, D., Valassina, M., 2002. New chlamydial lineages from freshwater
875   samples. Microbiol. Read. Engl. 148, 343–344.
876 Croxatto, A., Greub, G., 2010. Early intracellular trafficking of *Waddlia chondrophila* in
877   human macrophages. Microbiol. Read. Engl. 156, 340–355.
878 Croxatto, A., Rieille, N., Kernif, T., Bitam, I., Aeby, S., Péter, O., Greub, G., 2014.
879   Presence of *Chlamydiales* DNA in ticks and fleas suggests that ticks are carriers
880   of Chlamydiae. Ticks Tick-Borne Dis. 5, 359–365.
881 de Barsy, M., Bottinelli, L., Greub, G., 2014. Antibiotic susceptibility of *Estrella*
882   *lausannensis*, a potential emerging pathogen. Microbes Infect. 16, 746–754.
883 Derré, I., 2015. *Chlamydiae* interaction with the endoplasmic reticulum: contact, function
884   and consequences. Cell. Microbiol. 17, 959–966.
885 Derré, I., Swiss, R., Agaisse, H., 2011. The lipid transfer protein CERT interacts with the
886   Chlamydia inclusion protein IncD and participates to ER-*Chlamydia* inclusion
887   membrane contact sites. PLoS Pathog. 7, e1002092.
888 Donati, M., Cremonini, E., Di Francesco, A., Dallolio, L., Biondi, R., Muthusamy, R.,
889   Leoni, E., 2015. Prevalence of *Simkania negevensi*s in chlorinated water from spa
890   swimming pools and domestic supplies. J. Appl. Microbiol. n/a–n/a.
891 Donati, M., Di Francesco, A., Di Paolo, M., Fiani, N., Benincasa, M., Gennaro, R.,
892   Nardini, P., Foschi, C., Cevenini, R., 2011. Activity of Cathelicidin Peptides
893   against *Simkania negevensis*. Int. J. Pept. 2011, 708710.
894 Donati, M., Fiani, N., Di Francesco, A., Di Paolo, M., Vici, M., Cevenini, R., 2013. IgG
895   and IgA response to *Simkania negevensis* in sera of patients with respiratory and
896   gastrointestinal symptoms. New Microbiol. 36, 303–306.
897 Dowell, S.F., Peeling, R.W., Boman, J., Carlone, G.M., Fields, B.S., Guarner, J.,
898   Hammerschlag, M.R., Jackson, L.A., Kuo, C.C., Maass, M., Messmer, T.O.,
899   Talkington, D.F., Tondella, M.L., Zaki, S.R., C. pneumoniae Workshop
900   Participants, 2001. Standardizing *Chlamydia pneumoniae* assays:
901   recommendations from the Centers for Disease Control and Prevention (USA)
902   and the Laboratory Centre for Disease Control (Canada). Clin. Infect. Dis.. 33,
903   492–503.
904 Dumke, R., Schnee, C., Pletz, M.W., Rupp, J., Jacobs, E., Sachse, K., Rohde, G., Group,
905   C.S., 2015. *Mycoplasma pneumoniae* and *Chlamydia* spp. Infection in
906   Community-Acquired Pneumonia, Germany, 2011–2012. Emerg. Infect. Dis. 21,
907   426–434.

42

908 Efron, B., Halloran, E., Holmes, S., 1996. Bootstrap confidence levels for phylogenetic
909   trees. Proc. Natl. Acad. Sci. U. S. A. 93, 13429–13434.

910 Everett, K.D., Andersen, A.A., 1997. The ribosomal intergenic spacer and domain I of
911   the 23S rRNA gene are phylogenetic markers for *Chlamydia* spp. Int. J. Syst.
912   Bacteriol. 47, 461–473.

913 Everett, K.D., Bush, R.M., Andersen, A.A., 1999a. Emended description of the order
914   *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam.
915   nov., each containing one monotypic genus, revised taxonomy of the family
916   *Chlamydiaceae*, including a new genus and five new species, and standards for
917   the identification of organisms. Int. J. Syst. Bacteriol. 49 Pt 2, 415–440.

918 Everett, K.D.E., Thao, M., Horn, M., Dyszynski, G.E., Baumann, P., 2005. Novel
919   *Chlamydiae* in whiteflies and scale insects: endosymbionts "Candidatus *Fritschea*
920   *bemisiae*" strain Falk and "Candidatus *Fritschea eriococci*" strain Elm. Int. J.
921   Syst. Evol. Microbiol. 55, 1581–1587.

922 Everett, K.D., Hatch, T.P., 1995. Architecture of the cell envelope of *Chlamydia psittaci*
923   6BC. J. Bacteriol. 177, 877–882.

924 Everett, K.D., Kahane, S., Bush, R.M., Friedman, M.G., 1999b. An unspliced group I
925   intron in 23S rRNA links *Chlamydiales*, chloroplasts, and mitochondria. J.
926   Bacteriol. 181, 4734–4740.

927 Fan, T., Lu, H., Hu, H., Shi, L., McClarty, G.A., Nance, D.M., Greenberg, A.H., Zhong,
928   G., 1998. Inhibition of apoptosis in *Chlamydia*-infected cells: blockade of
929   mitochondrial cytochrome c release and caspase activation. J. Exp. Med. 187,
930   487–496.

931 Fasoli, L., Paldanius, M., Don, M., Valent, F., Vetrugno, L., Korppi, M., Canciani, M.,
932   2008. *Simkania negevensis* in community-acquired pneumonia in Italian children.
933   Scand. J. Infect. Dis. 40, 269–272.

934 Fehr, A., Walther, E., Schmidt-Posthaus, H., Nufer, L., Wilson, A., Svercel, M., Richter,
935   D., Segner, H., Pospischil, A., Vaughan, L., 2013. Candidatus *Syngnamydia*
936   *venezia*, a novel member of the phylum *Chlamydiae* from the broad nosed
937   pipefish, *Syngnathus typhle*. PloS One 8, e70853.

938 Forhan, S.E., Gottlieb, S.L., Sternberg, M.R., Xu, F., Datta, S.D., McQuillan, G.M.,
939   Berman, S.M., Markowitz, L.E., 2009. Prevalence of sexually transmitted
940   infections among female adolescents aged 14 to 19 in the United States. Pediatrics
941   124, 1505–1512.

942 Frandi, A., Jacquier, N., Théraulaz, L., Greub, G., Viollier, P.H., 2014. FtsZ-independent
943   septal recruitment and function of cell wall remodelling enzymes in chlamydial
944   pathogens. Nat. Commun. 5.

945 Friedman, M.G., Galil, A., Greenberg, S., Kahane, S., 1999. Seroprevalence of IgG
946   antibodies to the chlamydia-like microorganism "*Simkania Z*" by ELISA.
947   Epidemiol. Infect. 122, 117–123.

948 Friedman, M.G., Kahane, S., Dvoskin, B., Hartley, J.W., 2006. Detection of *Simkania*
949   *negevensis* by culture, PCR, and serology in respiratory tract infection in
950   Cornwall, UK. J. Clin. Pathol. 59, 331–333.

951 Gimenes, F., Souza, R.P., Bento, J.C., Teixeira, J.J.V., Maria-Engler, S.S., Bonini, M.G.,
952   Consolaro, M.E.L., 2014. Male infertility: a public health issue caused by sexually
953   transmitted pathogens. Nat. Rev. Urol. 11, 672–687.

43

Gottlieb, S.L., Xu, F., Brunham, R.C., 2013. Screening and treating *Chlamydia trachomatis* genital infection to prevent pelvic inflammatory disease: interpretation of findings from randomized controlled trials. Sex. Transm. Dis. 40, 97–102.

Goy, G., Croxatto, A., Greub, G., 2008. *Waddlia chondrophila* enters and multiplies within human macrophages. Microbes Infect. Inst. Pasteur 10, 556–562.

Goy, G., Croxatto, A., Posfay-Barbe, K.M., Gervaix, A., Greub, G., 2009. Development of a real-time PCR for the specific detection of *Waddlia chondrophila* in clinical samples. Eur. J. Clin. Microbiol. Infect. Dis. 28, 1483–1486.

Goy, G., Greub, G., 2009. Antibiotic susceptibility of *Waddlia chondrophila* in *Acanthamoeba castellanii* amoebae. Antimicrob. Agents Chemother. 53, 2663–2666.

Greenberg, D., Banerji, A., Friedman, M.G., Chiu, C.-H., Kahane, S., 2003. High rate of *Simkania negevensis* among Canadian inuit infants hospitalized with lower respiratory tract infections. Scand. J. Infect. Dis. 35, 506–508.

Greub, G., 2013. Pathogenesis and cell corruption by intracellular bacteria. Microbes Infect. Inst. Pasteur 15, 969–970.

Greub, G., 2010. International Committee on Systematics of Prokaryotes. Subcommittee on the taxonomy of the *Chlamydiae*: minutes of the inaugural closed meeting, 21 March 2009, Little Rock, AR, USA. Int. J. Syst. Evol. Microbiol. 60, 2691–2693.

Greub, G., 2009. *Parachlamydia acanthamoebae*, an emerging agent of pneumonia. Clin. Microbiol. Infect. 15, 18–28.

Greub, G., Boyadjiev, I., La Scola, B., Raoult, D., Martin, C., 2003a. Serological hint suggesting that *Parachlamydiaceae* are agents of pneumonia in polytraumatized intensive care patients. Ann. N. Y. Acad. Sci. 990, 311–319.

Greub, G., Collyn, F., Guy, L., Roten, C.-A., 2004a. A genomic island present along the bacterial chromosome of the *Parachlamydiaceae* UWE25, an obligate amoebal endosymbiont, encodes a potentially functional F-like conjugative DNA transfer system. BMC Microbiol. 4, 48.

Greub, G., Kebbi-Beghdadi, C., Bertelli, C., Collyn, F., Riederer, B.M., Yersin, C., Croxatto, A., Raoult, D., 2009. High throughput sequencing and proteomics to identify immunogenic proteins of a new pathogen: the dirty genome approach. PloS One 4, e8423.

Greub, G., La Scola, B., Raoult, D., 2004b. Amoebae-resisting bacteria isolated from human nasal swabs by amoebal coculture. Emerg. Infect. Dis. 10, 470–477.

Greub, G., La Scola, B., Raoult, D., 2003b. *Parachlamydia acanthamoeba* is endosymbiotic or lytic for *Acanthamoeba polyphaga* depending on the incubation temperature. Ann. N. Y. Acad. Sci. 990, 628–634.

Greub, G., Mege, J.-L., Gorvel, J.-P., Raoult, D., Méresse, S., 2005. Intracellular trafficking of *Parachlamydia acanthamoebae*. Cell. Microbiol. 7, 581–589.

Greub, G., Mege, J.-L., Raoult, D., 2003c. *Parachlamydia acanthamoebae* enters and multiplies within human macrophages and induces their apoptosis [corrected]. Infect. Immun. 71, 5979–5985.

Greub, G., Raoult, D., 2004. Microorganisms resistant to free-living amoebae. Clin. Microbiol. Rev. 17, 413–433.

44

999   Greub, G., Raoult, D., 2002a. Parachlamydiaceae: potential emerging pathogens. Emerg.
1000   Infect. Dis. 8, 625–630.
1001   Greub, G., Raoult, D., 2002b. Crescent bodies of *Parachlamydia acanthamoeba* and its
1002   life cycle within *Acanthamoeba polyphaga*: an electron micrograph study. Appl.
1003   Environ. Microbiol. 68, 3076–3084.
1004   Hammerschlag, M.R., Kohlhoff, S.A., 2012. Treatment of chlamydial infections. Expert
1005   Opin. Pharmacother. 13, 545–552.
1006   Heiskanen-Kosma, T., Paldanius, M., Korppi, M., 2008. *Simkania negevensis* may be a
1007   true cause of community acquired pneumonia in children. Scand. J. Infect. Dis.
1008   40, 127–130.
1009   Herweg, J.-A., Pons, V., Becher, D., Hecker, M., Krohne, G., Barbier, J., Berger, H.,
1010   Rudel, T., Mehlitz, A., 2015. Proteomic analysis of the *Simkania*-containing
1011   vacuole: the central role of retrograde transport. Mol. Microbiol.
1012   Heuer, D., Rejman Lipinski, A., Machuy, N., Karlas, A., Wehrens, A., Siedler, F.,
1013   Brinkmann, V., Meyer, T.F., 2009. *Chlamydia* causes fragmentation of the Golgi
1014   compartment to ensure reproduction. Nature 457, 731–735.
1015   Horn, M., 2008. *Chlamydiae* as symbionts in eukaryotes. Annu. Rev. Microbiol. 62, 113–
1016   131.
1017   Horn, M., Collingro, A., Schmitz-Esser, S., Beier, C.L., Purkhold, U., Fartmann, B.,
1018   Brandt, P., Nyakatura, G.J., Droege, M., Frishman, D., Rattei, T., Mewes, H.-W.,
1019   Wagner, M., 2004. Illuminating the evolutionary history of *Chlamydiae*. Science
1020   304, 728–730.
1021   Hovis, K.M., Mojica, S., McDermott, J.E., Pedersen, L., Simhi, C., Rank, R.G., Myers,
1022   G.S.A., Ravel, J., Hsia, R., Bavoil, P.M., 2013. Genus-optimized strategy for the
1023   identification of chlamydial type III secretion substrates. Pathog. Dis. 69, 213–
1024   222.
1025   Hsia, R.C., Pannekoek, Y., Ingerowski, E., Bavoil, P.M., 1997. Type III secretion genes
1026   identify a putative virulence locus of *Chlamydia*. Mol. Microbiol. 25, 351–359.
1027   Husain, S., Kahane, S., Friedman, M.G., Paterson, D.L., Studer, S., McCurry, K.R., Wolf,
1028   D.G., Zeevi, A., Pilewski, J., Greenberg, D., 2007. *Simkania negevensis* in
1029   bronchoalveolar lavage of lung transplant recipients: a possible association with
1030   acute rejection. Transplantation 83, 138–143.
1031   Hybiske, K., Stephens, R.S., 2007. Mechanisms of host cell exit by the intracellular
1032   bacterium *Chlamydia*. Proc. Natl. Acad. Sci. U. S. A. 104, 11430–11435.
1033   Ibana, J.A., Belland, R.J., Zea, A.H., Schust, D.J., Nagamatsu, T., AbdelRahman, Y.M.,
1034   Tate, D.J., Beatty, W.L., Aiyar, A.A., Quayle, A.J., 2011. Inhibition of
1035   indoleamine 2,3-dioxygenase activity by levo-1-methyl tryptophan blocks gamma
1036   interferon-induced *Chlamydia trachomatis* persistence in human epithelial cells.
1037   Infect. Immun. 79, 4425–4437.
1038   Israelsson, O., 2007. *Chlamydial* symbionts in the enigmatic *Xenoturbella*
1039   (Deuterostomia). J. Invertebr. Pathol. 96, 213–220.
1040   Jacquier, N., Aeby, S., Lienard, J., Greub, G., 2013. Discovery of new intracellular
1041   pathogens by amoebal coculture and amoebal enrichment approaches. J. Vis. Exp.
1042   JoVE e51055.
1043   Jacquier, N., Frandi, A., Pillonel, T., Viollier, P., Greub, G., 2014. Cell wall precursors
1044   are required to organize the chlamydial division septum. Nat. Commun. 5, 3578.
45

1045 Jacquier, N., Viollier, P., Greub, G., 2015. The role of peptidoglycans in chlamydial cell
1046   division : towards resolving the chlamydial anomaly. FEMS Microbiol. Rev.

1047 Johnsen, S., Birkebaek, N., Andersen, P.L., Emil, C., Jensen, J.S., Østergaard, L., 2005.
1048   Indirect immunofluorescence and real time PCR for detection of *Simkania*
1049   *negevensis* infection in Danish adults with persistent cough and in healthy
1050   controls. Scand. J. Infect. Dis. 37, 251–255.

1051 Kahane, S., Dvoskin, B., Friedman, M.G., 2008. The role of monocyte/macrophages as
1052   vehicles of dissemination of *Simkania negevensis*: an in vitro simulation model.
1053   FEMS Immunol. Med. Microbiol. 52, 219–227.

1054 Kahane, S., Dvoskin, B., Mathias, M., Friedman, M.G., 2001. Infection of *Acanthamoeba*
1055   *polyphaga* with *Simkania negevensis* and *S. negevensis* survival within amoebal
1056   cysts. Appl. Environ. Microbiol. 67, 4789–4795.

1057 Kahane, S., Everett, K.D., Kimmel, N., Friedman, M.G., 1999. *Simkania negevensis*
1058   strain ZT: growth, antigenic and genome characteristics. Int. J. Syst. Bacteriol. 49
1059   Pt 2, 815–820.

1060 Kahane, S., Fruchter, D., Dvoskin, B., Friedman, M.G., 2007a. Versatility of *Simkania*
1061   *negevensis* infection in vitro and induction of host cell inflammatory cytokine
1062   response. J. Infect. 55, e13–21.

1063 Kahane, S., Gonen, R., Sayada, C., Elion, J., Friedman, M.G., 1993. Description and
1064   partial characterization of a new *Chlamydia*-like microorganism. FEMS
1065   Microbiol. Lett. 109, 329–333.

1066 Kahane, S., Greenberg, D., Friedman, M.G., Haikin, H., Dagan, R., 1998. High
1067   prevalence of "*Simkania Z*," a novel *Chlamydia*-like bacterium, in infants with
1068   acute bronchiolitis. J. Infect. Dis. 177, 1425–1429.

1069 Kahane, S., Greenberg, D., Newman, N., Dvoskin, B., Friedman, M.G., 2007b. Domestic
1070   water supplies as a possible source of infection with *Simkania*. J. Infect. 54, 75–
1071   81.

1072 Kahane, S., Kimmel, N., Friedman, M.G., 2002. The growth cycle of *Simkania*
1073   *negevensis*. Microbiol. Read. Engl. 148, 735–742.

1074 Kahane, S., Metzer, E., Friedman, M.G., 1995. Evidence that the novel microorganism
1075   "Z" may belong to a new genus in the family *Chlamydiaceae*. FEMS Microbiol.
1076   Lett. 126, 203–207.

1077 Kahane, S., Platzner, N., Dvoskin, B., Itzhaki, A., Friedman, M.G., 2004. Evidence for
1078   the presence of *Simkania negevensis* in drinking water and in reclaimed
1079   wastewater in Israel. Appl. Environ. Microbiol. 70, 3346–3351.

1080 Kari, L., Whitmire, W.M., Olivares-Zavaleta, N., Goheen, M.M., Taylor, L.D., Carlson,
1081   J.H., Sturdevant, G.L., Lu, C., Bakios, L.E., Randall, L.B., Parnell, M.J., Zhong,
1082   G., Caldwell, H.D., 2011. A live-attenuated chlamydial vaccine protects against
1083   trachoma in nonhuman primates. J. Exp. Med. 208, 2217–2223.

1084 Karunakaran, K., Mehlitz, A., Rudel, T., 2011. Evolutionary conservation of infection-
1085   induced cell death inhibition among *Chlamydiales*. PloS One 6, e22528.

1086 Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S.,
1087   Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P.,
1088   Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop
1089   software platform for the organization and analysis of sequence data. Bioinforma.
1090   Oxf. Engl. 28, 1647–1649.

Kebbi-Beghdadi, C., Cisse, O., Greub, G., 2011. Permissivity of Vero cells, human pneumocytes and human endometrial cells to *Waddlia chondrophila*. Microbes Infect. Inst. Pasteur 13, 566–574.

Kebbi-Beghdadi, C., Domröse, A., Becker, E., Cisse, O.H., Hegemann, J.H., Greub, G., 2015. OmpA family proteins and Pmp-like autotransporter: new adhesins of *Waddlia chondrophila*. Pathog. Dis. 73, ftv035.

Knab, S., Mushak, T.M., Schmitz-Esser, S., Horn, M., Haferkamp, I., 2011. Nucleotide parasitism by *Simkania negevensis* (*Chlamydiae*). J. Bacteriol. 193, 225–235.

Knittler, M.R., Sachse, K., 2015. *Chlamydia psittaci*: update on an underestimated zoonotic agent. Pathog. Dis. 73, 1–15.

Korppi, M., Paldanius, M., Hyvärinen, A., Nevalainen, A., 2006. *Simkania negevensis* and newly diagnosed asthma: a case-control study in 1- to 6-year-old children. Respirol. Carlton Vic 11, 80–83.

Kostanjsek, R., Strus, J., Drobne, D., Avgustin, G., 2004. "Candidatus *Rhabdochlamydia porcellionis*", an intracellular bacterium from the hepatopancreas of the terrestrial isopod Porcellio scaber (Crustacea: Isopoda). Int. J. Syst. Evol. Microbiol. 54, 543–549.

Kropf, P., Baud, D., Marshall, S.E., Munder, M., Mosley, A., Fuentes, J.M., Bangham, C.R.M., Taylor, G.P., Herath, S., Choi, B.-S., Soler, G., Teoh, T., Modolell, M., Müller, I., 2007. Arginase activity mediates reversible T cell hyporesponsiveness in human pregnancy. Eur. J. Immunol. 37, 935–945.

Kumar, S., Kohlhoff, S.A., Gelling, M., Roblin, P.M., Kutlin, A., Kahane, S., Friedman, M.G., Hammerschlag, M.R., 2005. Infection with *Simkania negevensis* in Brooklyn, New York. Pediatr. Infect. Dis. J. 24, 989–992.

Kuo, C.C., Jackson, L.A., Campbell, L.A., Grayston, J.T., 1995. *Chlamydia pneumoniae* (TWAR). Clin. Microbiol. Rev. 8, 451–461.

Lagkouvardos, I., Weinmaier, T., Lauro, F.M., Cavicchioli, R., Rattei, T., Horn, M., 2014. Integrating metagenomic and amplicon databases to resolve the phylogenetic and ecological diversity of the *Chlamydiae*. ISME J. 8, 115–125.

Lamoth, F., Greub, G., 2010. Amoebal pathogens as emerging causal agents of pneumonia. FEMS Microbiol. Rev. 34, 260–280. d

Lieberman, D., Dvoskin, B., Lieberman, D.V., Kahane, S., Friedman, M.G., 2002. Serological evidence of acute infection with the *Chlamydia*-like microorganism *Simkania negevensis* (Z) in acute exacerbation of chronic obstructive pulmonary disease. Eur. J. Clin. Microbiol. Infect. Dis. 21, 307–309.

Lieberman, D., Kahane, S., Lieberman, D., Friedman, M.G., 1997. Pneumonia with serological evidence of acute infection with the *Chlamydia*-like microorganism "Z." Am. J. Respir. Crit. Care Med. 156, 578–582.

Liechti, G.W., Kuru, E., Hall, E., Kalinda, A., Brun, Y.V., VanNieuwenhze, M., Maurelli, A.T., 2014. A new metabolic cell-wall labelling method reveals peptidoglycan in *Chlamydia trachomatis*. Nature 506, 507–510.

Lienard, J., Croxatto, A., Aeby, S., Jaton, K., Posfay-Barbe, K., Gervaix, A., Greub, G., 2011a. Development of a new chlamydiales-specific real-time PCR and its application to respiratory clinical samples. J. Clin. Microbiol. 49, 2637–2642.

Lienard, J., Croxatto, A., Gervaix, A., Posfay-Barbe, K., Baud, D., Kebbi-Beghdadi, C., Greub, G., 2014. Undressing of *Waddlia chondrophila* to enrich its outer

47

membrane proteins to develop a new species-specific ELISA. New Microbes New Infect. 2, 13–24.

Lienard, J., Croxatto, A., Prod'hom, G., Greub, G., 2011b. *Estrella lausannensis*, a new star in the *Chlamydiales* order. Microbes Infect. Inst. Pasteur 13, 1232–1241.

Liu, Y., Huang, Y., Yang, Z., Sun, Y., Gong, S., Hou, S., Chen, C., Li, Z., Liu, Q., Wu, Y., Baseman, J., Zhong, G., 2014. Plasmid-encoded Pgp3 is a major virulence factor for Chlamydia muridarum to induce hydrosalpinx in mice. Infect. Immun. 82, 5327–5335.

Li, Z., Chen, D., Zhong, Y., Wang, S., Zhong, G., 2008. The chlamydial plasmid-encoded protein pgp3 is secreted into the cytosol of *Chlamydia*-infected cells. Infect. Immun. 76, 3415–3428.

Lundemose, A.G., Kay, J.E., Pearce, J.H., 1993a. *Chlamydia trachomatis* Mip-like protein has peptidyl-prolyl cis/trans isomerase activity that is inhibited by FK506 and rapamycin and is implicated in initiation of chlamydial infection. Mol. Microbiol. 7, 777–783.

Lundemose, A.G., Rouch, D.A., Penn, C.W., Pearce, J.H., 1993b. The *Chlamydia trachomatis* Mip-like protein is a lipoprotein. J. Bacteriol. 175, 3669–3671.

Matsumoto, A., Manire, G.P., 1970. Electron microscopic observations on the effects of penicillin on the morphology of *Chlamydia psittaci*. J. Bacteriol. 101, 278–285.

Mehlitz, A., Karunakaran, K., Herweg, J.-A., Krohne, G., van de Linde, S., Rieck, E., Sauer, M., Rudel, T., 2014. The chlamydial organism *Simkania negevensis* forms ER vacuole contact sites and inhibits ER-stress. Cell. Microbiol. 16, 1224–1243.

Miyairi, I., Mahdi, O.S., Ouellette, S.P., Belland, R.J., Byrne, G.I., 2006. Different growth rates of *Chlamydia trachomatis* biovars reflect pathotype. J. Infect. Dis. 194, 350–357.

Nascimento-Carvalho, C.M., Cardoso, M.-R.A., Paldanius, M., Barral, A., Araújo-Neto, C.A., Saukkoriipi, A., Vainionpää, R., Leinonen, M., Ruuskanen, O., 2009. *Simkania negevensis* infection among Brazilian children hospitalized with community-acquired pneumonia. J. Infect. 58, 250–253.

Nesbø, C.L., Doolittle, W.F., 2003. Active self-splicing group I introns in 23S rRNA genes of hyperthermophilic bacteria, derived from introns in eukaryotic organelles. Proc. Natl. Acad. Sci. U. S. A. 100, 10806–10811.

Niemi, S., Greub, G., Puolakkainen, M., 2011. *Chlamydia*-related bacteria in respiratory samples in Finland. Microbes Infect. Inst. Pasteur 13, 824–827.

Nylund, S., Steigen, A., Karlsbakk, E., Plarre, H., Andersen, L., Karlsen, M., Watanabe, K., Nylund, A., 2014. Characterization of "Candidatus *Syngnamydia salmonis*" (*Chlamydiales, Simkaniaceae*), a bacterium associated with epitheliocystis in Atlantic salmon (*Salmo salar* L.). Arch. Microbiol.

O'Connell, C.M., Ingalls, R.R., Andrews, C.W., Scurlock, A.M., Darville, T., 2007. Plasmid-deficient *Chlamydia muridarum* fail to induce immune pathology and protect against oviduct disease. J. Immunol. Baltim. Md 1950 179, 4027–4034.

Ogata, H., La Scola, B., Audic, S., Renesto, P., Blanc, G., Robert, C., Fournier, P.-E., Claverie, J.-M., Raoult, D., 2006. Genome sequence of *Rickettsia bellii* illuminates the role of amoebae in gene exchanges between intracellular pathogens. PLoS Genet. 2, e76.

48

1183 Omsland, A., Sixt, B.S., Horn, M., Hackstadt, T., 2014. *Chlamydial metabolism* revisited:
1184   interspecies metabolic variability and developmental stage-specific physiologic
1185   activities. FEMS Microbiol. Rev. 38, 779–801.
1186 Packiam, M., Weinrick, B., Jacobs, W.R., Maurelli, A.T., 2015. Structural
1187   characterization of muropeptides from *Chlamydia trachomatis* peptidoglycan by
1188   mass spectrometry resolves "chlamydial anomaly." Proc. Natl. Acad. Sci. 112,
1189   11660–11665.
1190 Pellati, D., Mylonakis, I., Bertoloni, G., Fiore, C., Andrisani, A., Ambrosini, G.,
1191   Armanini, D., 2008. Genital tract infections and infertility. Eur. J. Obstet.
1192   Gynecol. Reprod. Biol. 140, 3–11.
1193 Pérez, L.M., Codony, F., Ríos, K., Adrados, B., Fittipaldi, M., De Dios, G., Peñuela, G.,
1194   Morató, J., 2011. Prevalence study of *Simkania negevensis* in cooling towers in
1195   Spain. J. Water Health 9, 312–316.
1196 Pérez, L.M., Codony, F., Ríos, K., Peñuela, G., Adrados, B., Fittipaldi, M., de Dios, G.,
1197   Morató, J., 2012. Searching *Simkania negevensis* in environmental waters. Folia
1198   Microbiol. (Praha) 57, 11–14.
1199 Pilhofer, M., Aistleitner, K., Biboy, J., Gray, J., Kuru, E., Hall, E., Brun, Y.V.,
1200   VanNieuwenhze, M.S., Vollmer, W., Horn, M., Jensen, G.J., 2013. Discovery of
1201   chlamydial peptidoglycan reveals bacteria with murein sacculi but without FtsZ.
1202   Nat. Commun. 4.
1203 Pilhofer, M., Aistleitner, K., Ladinsky, M.S., König, L., Horn, M., Jensen, G.J., 2014.
1204   Architecture and host interface of environmental *Chlamydiae* revealed by electron
1205   cryotomography. Environ. Microbiol. 16, 417–429.
1206 Pillonel, T., Bertelli, C., Salamin, N., Greub, G., 2015. Taxogenomics of the
1207   Chlamydiales. Int. J. Syst. Evol. Microbiol.
1208 Pilloux, L., Aeby, S., Gaümann, R., Burri, C., Beuret, C., Greub, G., 2015. The High
1209   Prevalence and Diversity of *Chlamydiales* DNA within *Ixodes ricinus* Ticks
1210   Suggest a Role for Ticks as Reservoirs and Vectors of *Chlamydia*-Related
1211   Bacteria. Appl. Environ. Microbiol. 81, 8177–8182.
1212 Pletz, M.W., Rohde, G., Schütte, H., Bals, R., von Baum, H., Welte, T., CAPNETZ-
1213   Studiengruppe, 2011. [Epidemiology and Aetiology of Community-acquired
1214   Pneumonia (CAP)]. Dtsch. Med. Wochenschr. 1946 136, 775–780.
1215 Porcella, S.F., Carlson, J.H., Sturdevant, D.E., Sturdevant, G.L., Kanakabandi, K.,
1216   Virtaneva, K., Wilder, H., Whitmire, W.M., Song, L., Caldwell, H.D., 2015.
1217   Transcriptional profiling of human epithelial cells infected with plasmid-bearing
1218   and plasmid-deficient *Chlamydia trachomatis*. Infect. Immun. 83, 534–543.
1219 Principi, N., Esposito, S., 2001. Emerging role of *Mycoplasma pneumoniae* and
1220   *Chlamydia pneumoniae* in paediatric respiratorytract infections. Lancet Infect.
1221   Dis. 1, 334–344.
1222 Raulston, J.E., 1997. Response of *Chlamydia trachomatis* serovar E to iron restriction in
1223   vitro and evidence for iron-regulated chlamydial proteins. Infect. Immun. 65,
1224   4539–4547.
1225 Rottenberg, M.E., Gigliotti-Rothfuchs, A., Wigzell, H., 2002. The role of IFN-γ in the
1226   outcome of chlamydial infection. Curr. Opin. Immunol. 14, 444–451.
1227 Rusconi, B., Greub, G., 2013. Discovery of Catalases in Members of the *Chlamydiales*
1228   Order. J. Bacteriol. 195, 3543–3551.

49

1229 Rusconi, B., Lienard, J., Aeby, S., Croxatto, A., Bertelli, C., Greub, G., 2013. Crescent
1230   and star shapes of members of the *Chlamydiales* order: impact of fixative
1231   methods. Antonie Van Leeuwenhoek 104, 521–532.
1232 Sagaram, U.S., DeAngelis, K.M., Trivedi, P., Andersen, G.L., Lu, S.-E., Wang, N., 2009.
1233   Bacterial diversity analysis of Huanglongbing pathogen-infected citrus, using
1234   PhyloChip arrays and 16S rRNA gene clone library sequencing. Appl. Environ.
1235   Microbiol. 75, 1566–1574.
1236 Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing
1237   phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
1238 Santelli, C.M., Orcutt, B.N., Banning, E., Bach, W., Moyer, C.L., Sogin, M.L., Staudigel,
1239   H., Edwards, K.J., 2008. Abundance and diversity of microbial life in ocean crust.
1240   Nature 453, 653–656.
1241 Sauvadet, A.L., Le Panse, S., Roussel, E.G., Bigeard, E.M.C., Schrevel, J., Guillou, L.,
1242   2011. Tripartite interactions between Cirratulidae (Polychaeta),
1243   Durchoniella (Ciliophora, Astomatia) and Bacteria: A Russian Doll complex in
1244   anoxic coastal environments.
1245 Schachter, J., 1986. *Chlamydia psittaci* — Reemergence of a Forgotten Pathogen. N.
1246   Engl. J. Med. 315, 189–191.
1247 Senn, L., Jaton, K., Fitting, J.-W., Greub, G., 2011. Does Respiratory Infection Due to
1248   *Chlamydia pneumoniae* Still Exist? Clin. Infect. Dis. 53, 847–848.
1249 Sixt, B.S., Hiess, B., König, L., Horn, M., 2012. Lack of effective anti-apoptotic activities
1250   restricts growth of *Parachlamydiaceae* in insect cells. PloS One 7, e29565.
1251 Soldati, G., Lu, Z.H., Vaughan, L., Polkinghorne, A., Zimmermann, D.R., Huder, J.B.,
1252   Pospischil, A., 2004. Detection of mycobacteria and *Chlamydiae* in
1253   granulomatous inflammation of reptiles: a retrospective study. Vet. Pathol. 41,
1254   388–397.
1255 Song, L., Carlson, J.H., Whitmire, W.M., Kari, L., Virtaneva, K., Sturdevant, D.E.,
1256   Watkins, H., Zhou, B., Sturdevant, G.L., Porcella, S.F., McClarty, G., Caldwell,
1257   H.D., 2013. Chlamydia trachomatis plasmid-encoded Pgp4 is a transcriptional
1258   regulator of virulence-associated genes. Infect. Immun. 81, 636–644.
1259 Steinhoff, D., Lode, H., Ruckdeschel, G., Heidrich, B., Rolfs, A., Fehrenbach, F.J.,
1260   Mauch, H., Höffken, G., Wagner, J., 1996. *Chlamydia pneumoniae* as a cause of
1261   community-acquired pneumonia in hospitalized patients in Berlin. Clin. Infect.
1262   22, 958–964.
1263 Stephens, R.S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., Mitchell, W.,
1264   Olinger, L., Tatusov, R.L., Zhao, Q., Koonin, E.V., Davis, R.W., 1998. Genome
1265   sequence of an obligate intracellular pathogen of humans: *Chlamydia
1266   trachomatis*. Science 282, 754–759.
1267 Stride, M.C., Polkinghorne, A., Miller, T.L., Groff, J.M., Lapatra, S.E., Nowak, B.F.,
1268   2013. Molecular characterization of "Candidatus *Parilichlamydia carangidicola*,"
1269   a novel *Chlamydia*-like epitheliocystis agent in yellowtail kingfish, Seriola lalandi
1270   (Valenciennes), and the proposal of a new family, "Candidatus
1271   *Parilichlamydiaceae*" fam. nov. (order *Chlamydiales*). Appl. Environ. Microbiol.
1272   79, 1590–1597.

50

1273   Sun, G., Pal, S., Sarcon, A.K., Kim, S., Sugawara, E., Nikaido, H., Cocco, M.J., Peterson,
1274   E.M., de la Maza, L.M., 2007. Structural and functional analyses of the major
1275   outer membrane protein of *Chlamydia trachomatis*. J. Bacteriol. 189, 6222–6235.

1276   Tamura, K., 1992. Estimation of the number of nucleotide substitutions when there are
1277   strong transition-transversion and G+C-content biases. Mol. Biol. Evol. 9, 678–
1278   687.

1279   Taylor, M.W., Feng, G.S., 1991. Relationship between interferon-gamma, indoleamine
1280   2,3-dioxygenase, and tryptophan catabolism. FASEB J. 5, 2516–2522.

1281   Thao, M.L., Baumann, L., Hess, J.M., Falk, B.W., Ng, J.C.K., Gullan, P.J., Baumann, P.,
1282   2003. Phylogenetic evidence for two new insect-associated *Chlamydia* of the
1283   family *Simkaniaceae*. Curr. Microbiol. 47, 46–50.

1284   Thomas, V., Casson, N., Greub, G., 2006. *Criblamydia sequanensis*, a new intracellular
1285   Chlamydiales isolated from Seine river water using amoebal co-culture. Environ.
1286   Microbiol. 8, 2125–2135.

1287   Vouga, M., Diabi, H., Boulos, A., Baud, D., Raoult, D., Greub, G., 2015. Antibiotic
1288   susceptibility of *Neochlamydia hartmanellae* and *Parachlamydia acanthamoebae*
1289   in amoebae. Microbes Infect. Inst. Pasteur 17, 761–765.

1290   Walker, D.H., Ismail, N., 2008. Emerging and re-emerging rickettsioses: endothelial cell
1291   infection and early disease events. Nat. Rev. Microbiol. 6, 375–386.

1292   Wellinghausen, N., Straube, E., Freidank, H., Baum, H. von, Marre, R., Essig, A., 2006.
1293   Low prevalence of *Chlamydia pneumoniae* in adults with community-acquired
1294   pneumonia. Int. J. Med. Microbiol. 296, 485–491.

1295   Xue, X., Li, S.-J., Ahmed, M.Z., De Barro, P.J., Ren, S.-X., Qiu, B.-L., 2012. Inactivation
1296   of *Wolbachia* reveals its biological roles in whitefly host. PloS One 7, e48148.

1297   Yamaguchi, T., Yamazaki, T., Inoue, M., Mashida, C., Kawagoe, K., Ogawa, M., Shiga,
1298   S., Nakagawa, Y., Kishimoto, T., Kurane, I., Ouchi, K., Ohzeki, T., 2005.
1299   Prevalence of antibodies against *Simkania negevensis* in a healthy Japanese
1300   population determined by the microimmunofluorescence test. FEMS Immunol.
1301   Med. Microbiol. 43, 21–27.

1302

1303

1304 **TABLES AND FIGURE**

1305 **Table 1:** General features of *Simkania negevensis* compared to other *Chlamydiales*

1306 members

1307 Table 1 highlights various biological similarities and differences of *S. negevensis* and

1308 other *Chlamydiales*.

1309 Abbreviations: CPAF, Chlamydia Protease-like Activity Factor; MOMP, Major Outer

1310 Membrane Protein; n.a., not applicable; nt, Nucleotides; PG, Peptidoglycan; Pmp,

1311 Polymorphic membrane protein; T3SS, Type 3 Secretion System ; T4SS, Type 4

1312 Secretion System

1313 [1] (Collingro et al., 2011)

1314 [2] (Carlson et al., 2008; Stephens et al., 1998)

1315 [3] (Bertelli et al., 2010)

1316 [4] (Horn et al., 2004)

1317 [5] (Bertelli et al., 2010; Carlson et al., 2008; Collingro et al., 2011; Horn et al., 2004;

1318 Stephens et al., 1998)

1319 [6] (Birkelund et al., 2009; Frandi et al., 2014; Jacquier et al., 2014; Kebbi-Beghdadi et al.,

1320 2015; Liechti et al., 2014; Pilhofer et al., 2014, 2013)

1321 [7] (Collingro et al., 2011; Rusconi and Greub, 2013)

1322 [8] (Bertelli et al., 2010; Carlson et al., 2006; Collingro et al., 2011; Horn et al., 2004; Knab

1323 et al., 2011; Omsland et al., 2014)

1324 [9] Two of them are considered as homologous of OmpA

1325 [10] Strain 2032/99 does not have a plasmid

1326 [11] Putative Pmp encoded in the genome, but not isolated at the membrane

52

1327   [12] Putative Pom encoded in the genome, but not isolated at the membrane

1328   [13] One homologue encoded in the genome, but not isolated at the membrane

1329

53

Table 1:
General features of *Simkania negevensis* compared to other *Chlamydiales* members

	S. negevensis Z[1]	*C. trachomatis* D/UW-3/CX[2]	*W. chondrophila* WSU 86-1044[3]	*P. acanthamoebae* UV-7[4]
Genetic features[5]				
Chromosome size (nt)	2'496'337	1'042'519	2'141'577	3'072'383
Plasmid size (nt)	132'038	7'493	15'593[10]	no
rRNA encoding genes				
16S	1	2	2	3
23S	1	2	2	3
Outer membrane structure[6]				
Major membrane protein	MOMP-like	MOMP	MOMP-like	Hypothetical protein (puv)
Cysteine-rich proteins (membrane stabilization)	absent	OmcA/OmcB	OmcA/OmcB/wcw_0272	OmcB / puv_11160
Porins	MOMP-like	MOMP/ PorB/OprB	MOMP-like	Puv_27500/Puv_07550[12]
MOMP (*ompA*)	2	1	no	no
MOMP like	35 [9]	n.a.	11	1[13]
Pmp (auto transporter/cell adhesion)	yes (PmpB)	yes	yes	?[11]
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[7]				
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[8]				
Glucokinase	yes	no	yes	yes
Citric acid cycle	complete	incomplete	complete	complete
Nucleotides transporters	4	2	5	5
Tryptophan operon	complete	incomplete	absent	absent

1331   **Table 2**: Prevalence of an acute *Simkania* infection in patients suffering from

1332   bronchiolitis

1333   Abbreviations: PCR, Polymerase Chain Reaction; NS, not significant

Country, patients	Diagnostic method	Patients (%)		Controls (%)		p values	Co-infections (%)		Reference
Israel, 239	PCR	60/239	(25)	0/78	(0)	<0.001	22/60	(36)	Kahane, 1998
	Immunoperoxidase	14/92	(15)	1/78	(1)	<0.001			
Inuit Canada, 22	PCR	14/22	(63)				12/14	(85)	Greenberg, 2003
USA, 66	PCR	9/55	(16)	2/28	(7)	NS			Kumar, 2005
UK, 222	Nested PCR	100/222	(45)						Friedman, 2006

1334

1335

1337 **Table 3**: Prevalence of an acute *Simkania* infection in patients suffering from pneumonia

1338 Abbreviations: ELISA, enzyme-linked immunosorbent assay; MIA, membrane immune-

1339 assay; MIF, Micro Immunofluorescence; NS, not significant; PCR, Polymerase chain

1340 reaction

1341 [1] High signal for IgA (> 1.2 OD) or an increase of 0.5 OD units between paired sera

1342 levels for IgA (1:30) or IgG (1:100)

1343 [2] IgM $\geq$ 1:10 or a 4-fold increase between paired sera

1344 [3] When adjusted for recommended IgM cut-off titers $\geq$ 1:32, results are 12/174

1345 [4] After adjustment 4

1346 [5] IgA $\geq$ 1:8

1347 [6] pan-*Chlamydiales* PCR results are only specific at the family-level lineage. These cases

1348 should therefore be considered as *Simkaniaceae* infections."

1349

1350

Table 3:
Prevalence of an acute *Simkania* infection in patients suffering from pneumonia

Population, country, patients	Diagnostic method	Patients (%)	Controls (%)	p values	Simkania as only pathogen	Reference
Adults, Israel, 308	ELISA[1]	8/308 (3)			4	Lieberman, 1997
Children, USA, 25	PCR ELISA[1]	5/22 (22)		NS		Kumar, 2005
Children, Israel, 34	Nested PCR MIA	31/34 (91) 17/34 (50)	34/35 (97)	NS	20	Kahane, 2007
Children, Finland, 174	MIF[2]	18/174[3] (10)			6[4]	Heiskanen-Kosma, 2008
Children, Italy, 101	MIF[2]	5/101 (5)			2	Fasoli, 2008
Children, Brazil, 183	MIF[2]	3/184 (2)			1	Nascimento-Carvalho, 2009
Children, Switzerland, 265	PCR pan-*Chlamydia*[6]	2/265 (1)				Liénard, 2011
Adults, Italy, 102	MIF[5]	13/102 (13)	2/104 (2)	<0.05		Donati, 2013

1351   **Table 4**: PCRs used to detect *Simkania* in clinical and environmental samples

1352   [1] Amplicon length varies according to *Chlamydiales* species

1353

Table 4:

PCR used to detect *Simkania* in clinical and environmental samples

Gene	Primers and probes set	Amplicon length	References
***Simkania*-specific**			
16S	Zpf 5' AAAGGTAACGAATAATTCCCT-3' ZpR 5' GCACAGTCGGGGTTGAGACCGACT-3'	398	Kahane, 1998
	16F2 5'CAA GAA AAG GTA ACG AAT AAT TGCC3' 16R2 5' GAG CTC CGG AAT TTC ACA TCT G' 16S2 5'FAM-AAG GGC GCG TAG GCG GGT AAG C-BHQ1'3'	171	Niemi, 2011
23S			
	AF 5'-CACAGGTAGGCATGATGA-3' BR 5'-CTAGCTGCGGGTAAACG-3'	1099	Everett, 1999
	INTF 5'-TTAGATGCACAATGGATAGTTGGA-3' INTR 5'-CCATCAGCGCTCATGTGCTCA-3'	338	Everett, 1999
Pan-Chlamydiales			
16S			
	ccF 5'-CTT CGG GTT GTA AAG CAC TTT CGC-3' ccR 5'-CCC CGT CAA TTC TTT TGA GTT T-3'	512	Kahane, 2004
	panCh16F2 5'-CCGCCAACACTGGGACT-3' panCh16R2 5'-GGAGTTAGCCGGTGCTTCTTTAC-3' panCh16S 5'-FAM-CTACGGGAGGCTGCAGTCGAGAATC-BHQ1-3'	207-215[1]	Liénard, 2011

57

1354 **Table 5**: Patients with *Simkania*-caused pneumonia

1355 Abbreviations: CRP, C-reactive protein; id., indeterminate; FMF, familial Mediterranean

1356 fever; d., days; m.o., months old; PCT, procalcitonin; WBC, white blood cells; y.o., years

1357 old

1358 [1] WBC > 11'000 is considered significant for a serious bacterial infection

1359 [2] CRP >0.5mg/l is considered significant for a serious bacterial infection

1360 [3] PCT >0.5 microg/l is considered significant for a serious bacterial infection

1361

1362

1363

1364

Table 5 :
Patients with *Simkania*-caused pneumonia

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Age	31 y.o	32 y.o	32 y.o	28 y.o	57 m.o	52 m.o	58 m.o
Sex	F	M	M	M	M	M	F
Medical History	None	None	FMF Colchicine treatment	None	id.	id.	none
Country				Italy	Italy	Italy	Brazil
Smoking	5 pack-year	20 pack-year	15 pack-year	id.	id.	id.	none
Clinical							
Fever	38.5 °C	36.8 °C	39.2 °C	39.4 °C	id.	id.	37.5 °C
Symptoms	unproductive cough, abdominal pain, diarrhea	unproductive cough, chest pain, diarrhea, vomiting	unproductive cough chest pain	unproductive cough, chest pain	id.	id.	cough, dyspnoea
WBC^1(cells/ml)	7'400	10'800	15'000	6'300	14'850	11'870	29'200
CRP^2 (mg/l)	< 5	< 5	< 5	< 5	id.	id.	id.
PCT^3 (microg/l)	id.	id.	id.	id.	0.34	0.21	id.
Treatment							
Hospitalization	in patient 4d.	in patient 5d.	in patient 3d.	in patient 4d.	outpatient	outpatient	in patient
Antibiotic regimen	Erythromycin	Penicillin after a trial erythromycin as an outpatient	Erythromycin	Erythromycin	id.	id.	Penicillin
Reference	Lieberman, 1997	Lieberman, 1997	Lieberman, 1997	Lieberman, 1997	Fasoli, 2008	Fasoli, 2008	Nascimento-Carvalho, 2009

59

1365 **Figure 1**: Evolutionary relationships of *Simkaniaceae*

1366 The evolutionary history was inferred using the Neighbor-Joining method (Saitou and

1367 Nei, 1987) based on 1548 nucleotides from 15 16S rRNA sequences. Sequences were

1368 retrieved from the NCBI database using "Simkania*" as a research word. The percentage

1369 of replicate trees, in which the associated taxa clustered together in the bootstrap test (100

1370 replicates) are shown next to the branches (Efron et al., 1996). The evolutionary distances

1371 were computed using the Tamura 3-parameter method (Tamura, 1992) and are shown as

1372 number of base substitutions per site. The rate variation among sites was modelled with a

1373 gamma distribution. Evolutionary analyses were conducted in MEGA5 after sequence

1374 analysis on Geneious 7.1.7 (Kearse et al., 2012)

1375

1376

SUPPLEMENTARY MATERIALS

1378 Supplementary table: Clinical studies with *Simkania*

1379 Abbreviations: CAP, Community acquired pneumonia; COPD, Chronic obstructive

1380 pulmonary disease; ELISA, enzyme-linked immunosorbent assay; MIA, membrane

1381 immune-assay; MIF, Micro Immunofluorescence; NS, Not significant; PCR, Polymerase

1382 chain reaction; qPCR, quantitative PCR; RTI, Respiratory tract infection

1383

1384 [1] Past infections determined by IgG levels

1385 [2] Recent infections determined by high IgM levels, augmentation of IgG or IgM levels

1386 between two paired sera or PCR

1387 [3] IgG ≥ 1:16

1388 [4] IgG ≥ 1:8, IgM ≥1:10

1389 [5] 51 broncho-alveolar lavages and concomitant biopsies obtained from 19 patients

1390 included

1391 [6] When adjusted for recommended IgM cut-off titers ≥ 1:32, results are 12/174

1392 [7] After adjustment 8/12

1393 [8] Only a 4-fold increase or decrease between paired sera was considerer as positive

1394 [9] IgG ≥ 1:16, IgA ≥1:8

1395

Supplementary table:
Clinical studies with *Simkania*

Population, country, patients	Disease	Diagnostic method	past infections[1] (%)	Controls (%)	p values	Recent infections[2] (%)	Controls (%)	p values	Co-infections (%)	Reference
Adults, Israel, 308	CAP	ELISA	114/308 (37)			8/308 (3)				Lieberman, 1997
Children, Israel, 239	Bronchiolitis	PCR				60/239 (25)	0/78 (0)	<0.001	4/8 (50)	Kahane, 1998
		Immunoperoxidase				14/92 (15)	1/78 (1)	<0.001	22/60 (37)	
Adults, smokers, Israel, 190	Exacerbation COPD	ELISA	120/190 (63)	69/100 (69)	NS	5/217 (2)			4/5 (80)	Lieberman, 2002
Children, Inuit, Canada, 22	Bronchiolitis	PCR 16S (Kahane)				14/22 (63)			12/14 (86)	Greenberg, 2003
Adults, Denmark, 197	Persistent cough	MIF[3]	80/185 (43)	41/100 (41)	NS	0/176 (0)				Johnsen, 2005
		PCR 16S (Kahane)				6/37 (16)				
Adults, USA, 37	Asthma	PCR 16S (Kahane)	8/31 (26)	16/42 (38)	NS	6/37 (16)	6/37 (16)	NS		Kumar, 2005
		ELISA (Liebermann)								
Children, USA, 66	Bronchiolitis	ELISA (Liebermann)	0/6 (0)	3/17 (18)	NS	6/37 (16)	9/42 (21)	NS		Kumar, 2005
		PCR 16S (Kahane)								
Children, USA, 25	CAP	ELISA (Liebermann)	1/9 (11)		NS	5/22 (22)		NS		Kumar, 2005
		PCR 16S (Kahane)								
Children, USA, 60	Asthma	ELISA (Liebermann)	5/33 (15)	5/24 (21)	NS	9/55 (16)	12/31 (39)	NS		
Adults, UK, 29[5]	RTI	ELISA (Liebermann)	18/29 (62)	92/200 (46)	NS	5/29 (17)	6/200 (3)	0.004		Friedman, 2006
Children, UK, 222	Bronchiolitis	Nested PCR (Kahane)				100/222 (45)				Friedman, 2006
Children, Finland, 104	Asthma	MIF[4]	12/104 (12)	12/122 (10)	NS	2/104 (2)	9/120 (8)	NS		Korppi, 2006
Children, Israel, 34	CAP	Nested PCR (Kahane)				31/34 (91)	34/35 (97)	NS	14/34 (41)	Kahane, 2007
		MIA				17/34 (50)	(50)			
Adults, USA, 19[5]	Acute rejection lung transplant	Nested PCR (Kahane)				24/27 (88)	17/24 (71)	NS	8/51 (16)	Husain, 2007
Children, Finland, 174	CAP	MIF (Korppi)[4]				18/174[6] (10)			12/18[7] (67)	Heiskanen-Kosma, 2008
Children, Italy, 101	CAP	MIF (Korppi)[4]				5/101[8] (5)			2/5 (40)	Fasoli, 2008
Children, Brazil, 183	CAP	MIF (Korppi)[4]				3/184 (2)			2/3 (67)	Nascimento-Carvalho, 2009
Children, Switzerland, 265	CAP	PCR pan-*Chlamydia*				2/265 (1)				Liénard, 2011
Adults, Finland, 541	RTI	qPCR 16S				0/531 (0)				Niemi, 2011
Adults, Italy, 102	Lower RTI	MIF[9]	51/102 (50)	36/104 (35)	0.03	13/102 (13)	2/104 (2)	<0.05		Donati, 2013
Adults, Italy, 224	Gastro-intestinal symptoms	MIF[9]	152/224 (68)	36/104 (35)	<0.001	40/224 (18)	2/104 (2)	<0.05		Donati, 2013