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Presence of Chlamydiales DNA in ticks and fleas suggests that ticks are carriers of Chlamydiae.

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Abstract

The Chlamydiales order includes the Chlamydiaceae, Parachlamydiaceae, Waddliaceae, Simkaniaceae, Criblamydiaceae, Rhabdochlamydiaceae, Clavichlamydiaceae, and Piscichlamydiaceae families. Members of the Chlamydiales order are obligate intracellular bacteria that replicate within eukaryotic cells of different origins including humans, animals, and amoebae. Many of these bacteria are pathogens or emerging pathogens of both humans and animals, but their true diversity is largely underestimated, and their ecology remains to be investigated. Considering their potential threat on human health, it is important to expand our knowledge on the diversity of Chlamydiae, but also to define the host range colonized by these bacteria. Thus, using a new pan-Chlamydiales PCR, we analyzed the prevalence of Chlamydiales DNA in ticks and fleas, which are important vectors of several viral and bacterial infectious diseases. To conduct this study, 1340 *Ixodes ricinus* ticks prepared in 192 pools were collected in Switzerland and 55 other ticks belonging to different tick species and 97 fleas belonging to different flea species were harvested in Algeria. In Switzerland, the prevalence of Chlamydiales DNA in the 192 pools was equal to 28.1% (54/192) which represents an estimated prevalence in the 1340 individual ticks of between 4.0 to 28.4%. The pan-Chlamydiales qPCR was positive for 45.5% (25/55) of tick samples collected in Algeria. The sequencing of the positive qPCR amplicons revealed a high diversity of Chlamydiales species. Most of them belonged to the Rhabdochlamydiaceae and Parachlamydiaceae families. Thus, ticks may carry Chlamydiales and should thus be considered as possible vectors for Chlamydiales propagation to both humans and animals.

Keywords: Arthropods; Ticks; Carriers; Chlamydiae; Intracellular bacteria

Introduction

The Chlamydiales order (Everett et al., 1999) currently includes the Chlamydiaceae, Parachlamydiaceae, Waddliaceae, Simkaniaceae, Criblamydiaceae, Rhabdochlamydiaceae, Clavichlamydiaceae, and Piscichlamydiaceae families (Corsaro and Greub, 2006; Greub, 2013). Members of the Chlamydiales order are strict intracellular bacteria that replicate within eukaryotic cells of different origins including humans, animals, and amoebae (Corsaro and Greub, 2006; Horn, 2008). Chlamydiales are characterized by a biphasic development cycle comprising infectious metabolically inactive elementary bodies and non-infectious metabolically active and replicating reticulate bodies (Moulder, 1991).

Chlamydiales bacteria have been identified in hosts covering the whole animal kingdom. Several Chlamydiaceae such as *Chlamydia trachomatis* and *C. pneumoniae* colonize humans. *Waddlia chondrophila* was isolated from an aborted bovine fetus (Rurangirwa et al., 1999), whereas *Waddlia malaysiensis* was isolated from fruit bats (Chua et al., 2005). Bacteria belonging to the Piscichlamydiaceae and the Clavichlamydiaceae families were detected in gills from fish exhibiting signs of epitheliocystis (Draghi et al., 2004; Karlsen et al., 2008). The 2 members of the Rhabdochlamydiaceae family, *Candidatus* Rhabdochlamydia porcellionis and *Candidatus* Rhabdochlamydia crassificans, were identified in arthropods by 16 sRNA gene sequence analysis and electron microscopy (Corsaro et al., 2007; Kostanjsek et al., 2004), but were never recovered and isolated. Similarly, 2 candidatus Chlamydiales species belonging to the Simkaniaceae family were detected in insects by DNA and electron microscopy analysis, but were not isolated from their hosts (Everett et al., 2005). One member of the Simkaniaceae family, *Simkania negevensis* was isolated as a culture contaminant of human and simian cells (Kahane et al., 1999). Finally, several Chlamydiales bacteria belonging mainly to the Parachlamydiaceae and Criblamydiaceae families are considered symbionts of amoebae (Amann et al., 1997; Greub and Raoult, 2002; Horn et al., 2000) or associated with amoebae (Lienard et al., 2011b; Thomas et al., 2006), indicating the important role that these latter organisms play in the ecology of these obligate intracellular bacteria (Fritsche et al., 1993; Horn, 2008). However, the diversity of Chlamydiales bacteria is still likely underestimated, and their ecological distribution remains to be further investigated. Such investigations are especially warranted since many Chlamydiales have been recognized as human and animal pathogens or are seriously considered pathogenic microorganisms (Corsaro and Venditti, 2004; Corsaro and Greub, 2006; Longbottom and Coulter, 2003; Senn et al., 2005). *Chlamydia trachomatis* is the causative agent of trachoma, the most frequent infectious cause of blindness (Burton, 2007) and is the most common cause of bacterial sexually transmitted diseases (Beagley and Timms, 2000). *Chlamydia pneumoniae* is a causative agent of pneumonia, and *Chlamydia psittaci* is the causative agent of the zoonotic infection called psittacosis which is often characterized by an interstitial pneumonia (Lamoth and Greub, 2010). There is also clear evidence supporting the role of *Parachlamydia acanthamoebae* as a human respiratory pathogen (Greub, 2009). Thus, several serological and molecular studies have demonstrated a pathogenic role of *P. acanthamoebae* mainly in immunocompromised and intensive-care patients suffering from pneumonia (reviewed in Lamoth and Greub, 2010). Finally, *Waddlia chondrophila* is an emerging pathogen which is considered a possible causative agent of abortion in both ruminants (Dilbeck-Robertson et al.,

2003) and humans (Baud et al., 2007; Baud et al., 2011). Due to the intracellular lifestyle of chlamydiae, classic culture methods are ineffective to identify any members of the Chlamydiales order. Thus, the pathogenic potential of several of these bacteria still remains largely unexplored.

Chlamydiales bacteria belonging to the Rhabdochlamydiaceae family have been identified in arthropods including the cockroach *Blatta orientalis* and the terrestrial isopod *Porcellio scaber* (Corsaro et al., 2007; Kostanjsek et al., 2004). Arthropods represent thus a possible important reservoir for Chlamydiales bacteria that need to be investigated. Among arthropods, fleas and ticks are important vectors of both viral and bacterial infectious diseases. Lyme borreliosis caused by *Borrelia burgdorferi* sensu lato and tick-borne encephalitis (TBE) are the major tick-borne diseases affecting humans. In addition, several less frequent additional tick-borne infectious agents can cause severe diseases in humans including *Francisella tularensis* (tularemia), *Rickettsia* spp. (spotted fever), and *Anaplasma phagocytophilum* (anaplasmosis) (Brouqui et al., 2004). Similarly, fleas have been identified as vectors of transmission of numerous important human diseases including bubonic plague caused by *Yersinia pestis* (Wimsatt and Biggins, 2009). Finally, 2 studies suggested that ticks could play a role in the transmission of chlamydiae to cattle (Caldwell and Belden, 1973; McKercher et al., 1980). Thus, using a pan-Chlamydiales PCR, the prevalence and sequence diversity of Chlamydiales 16S rDNA were analyzed in *Ixodes ricinus* ticks collected in Switzerland and in several tick and flea species collected in Algeria.

Material and methods

Tick collecting in Switzerland

The field work was conducted from May to July 2010. *Ixodes ricinus* ticks (adults and nymphs, n=1340) were collected using a 1-m^2 white cotton towel which was dragged over the vegetation. Every 10 m, the operator stopped to count and put attached ticks into tubes, which were stored at –80°C until further analysis. Ticks were collected on the site of Mutt-Rarogne (Fig. 1), which was chosen because of its small size and its clear demarcation due to the topology of the area.

Tick pooling and DNA extraction in Switzerland

The 3-step high-throughput method was applied with ticks from Rarogne as described by Gäumann et al., 2010). Frozen ticks were prepared in pools of 5 adults, 10 nymphs, and different amounts of mixed adults and nymphs with 600 µl of buffer solution at 4°C. The

buffer solution was composed of PBS solution supplemented with InhibitEX tablets (1 tablet/20 ml of buffer; Qiagen). One 3-mm tungsten carbide bead (Qiagen) was added to each tube (collection microtubes; Qiagen), and tick pools were immediately homogenized using the TissueLyser system (Qiagen) for 4 min at 30 Hz. After a short step of centrifugation of 5 s at 3200 × g, 200 µl of supernatant was inactivated in 800 µl of AVL viral lysis buffer (Qiagen) supplemented with 3 µg of carrier RNA (Qiagen). Simultaneous DNA and RNA extraction was performed using the QIAsymphony SP system (Qiagen) and the QIAsymphony Virus/Bacteria Midi kit (Qiagen) with a specially adapted protocol (CP Complex 920 FIX v1; Qiagen). DNA and RNA were eluted in a final volume of 60 µl and stored at –80°C for further use.

Estimation of Chlamydiales DNA prevalence in individual *Ixodes ricinus* ticks collected in Switzerland

The estimation of the Chlamydiales DNA prevalence in the 1340 individual *I. ricinus* ticks was calculated from the measured prevalence obtained in the 192 pools as followed: The minimum prevalence was calculated by considering that only one tick per positive pool is infected, whereas the maximum prevalence was calculated by considering that all the ticks present in a positive pool are infected (Table 1).

Tick and fleas collecting and DNA extraction in Algeria

In February 2006 and September 2010, ticks were captured by the flagging technique on vegetation close to the road and olive trees in the mountains of the Blida region (Chréa Mountain) and Skikda in northern Algeria (Bitam et al., 2006). Fleas were collected by the technique of candle trapping which consists of a candle placed in the middle of a plate filled with water. This device attracts fleas which are trapped in the water, but remain alive for a few hours (Bitam et al., 2010; Roucher et al., 2012).

Fleas and ticks were identified at the species level and were then crushed individually in sterile Eppendorf tubes with the tip of a sterile pipette. DNA was extracted by using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Real-time PCR assay

PCR assays were performed in 20 µl, with iTaq supermix with ROX (BioRad, Reinach, Switzerland), 0.1 µM of each primer (Eurogentec, Seraing, Belgium), 0.1 µM of probe (Eurogentec), molecular biology grade water (Sigma-Aldrich, Buchs, Switzerland), and 5 µl

of DNA sample. Primers forward panCh16F2 (5'-CCGCCAACACTGGGACT-3'), primer reverse panCh16R2 (5'-GGAGTTAGCCGGTGCTTCTTTAC-3') and a probe panCh16S (5'-FAM [6-carboxyfluorescein]-CTACGGGAGGCTGCAGTCGAGAATC-BHQ1 [Black Hole Quencher]-3'), targeting a fragment of about 207–215 bp in the 16S ribosomal RNA gene (length variable according to the species), were used for the pan-Chlamydiales PCR as described earlier (Lienard et al., 2011a). Underlined bases represent locked nucleic acids (LNA). Cycling conditions were 3 min at 95°C, followed by 50 times 3-step cycles of 15 s at 95°C, 15 s at 67°C, and 15 s at 72°C. PCR products, tested in duplicate, were detected with a StepOne Plus instrument (Applied Biosystems, Zug, Switzerland). Water was used as a negative PCR control.

A plasmid carrying a 16S rRNA gene fragment amplified with primers Pacstd16SF2 (5'-CTGACGGCGTGGATGAGGC-3') and Pacstd16SR2 (5'-CCTACGCGCCCTTTACGCCC-3') as previously reported (Lienard et al., 2011a) was used for quantification and as a positive control. Quantification of the recombinant plasmid was done on a Nanodrop ND-1000 (Witech, Littau, Switzerland), and serial dilutions (10^5 to 100 copies/µl) were used to establish a standard curve for quantification.

Sequencing of positive samples

Amplicons of positive samples were purified using the MSB Spin PCRapace kit (Invitek, Berlin, Germany). A sequencing PCR was performed with specifically designed inner primers panFseq (5'-CCAACACTGGGACTGAGA-3') and panRseq (5'-GCCGGTGCTTCTTTAC-3') (Lienard et al., 2011a). The sequencing PCR assay was done using the BigDye® Terminator v 1.1 Cycle seq kit (Applied Biosystems). The sequencing reaction was purified using SigmaSpin sequencing reaction clean-up (Sigma-Aldrich, USA). Sequences of Chlamydiales bacteria obtained from flea and tick samples have been deposited on the NCBI website (http://www.ncbi.nlm.nih.gov/genbank/). Accession numbers are JQ860006 to JQ860084.

Results

Prevalence of Chlamydiales bacteria in *Ixodes ricinus* ticks collected in Switzerland

A total of 1340 *I. ricinus* ticks was collected in the area of Rarogne in the canton of Valais, Switzerland (Fig. 1). The 1340 ticks were collected and pooled in 192 samples prior to DNA extraction. Thus, 129 pools of 5 adult ticks/pool representing 645 single adult ticks, 45 pools of 10 nymph ticks/pool representing 450 single nymphal ticks, and 18 pools of various

amounts of mixed adults and nymphal ticks/pool representing 245 single adults and nymphs were prepared. The 192 pools representing 1340 ticks were screened by quantitative pan-Chlamydiales qPCR in duplicates. The qPCR was positive for 54 samples giving a Chlamydiales prevalence of 28.1% (54/192) when considering all pools and a prevalence of 25.6% (33/129) in adult ticks pools, of 31.1% (14/45) in nymph pools and 38.9% (7/18) in mixed adult/nymph pools (Table 1). The proportion of individual Chlamydiales DNA-positive ticks from the 192 pools was estimated to be between 4.0% (considering that only one tick per pool was positive) and 28.4% (considering that all the ticks per pool were positive). Similarly, the rates of pan-Chlamydiales qPCR-positive ticks were calculated in adult ticks (5.1–25.6%), nymphs (3.1–31.1%), and mixed adults+nymphs (2.8–31%) (Table 1).

The 54 pan-Chlamydiales qPCR-positive samples were sequenced. The sequencing failed for 17 samples. Among the remaining 37 samples, 4 samples provided 2 different sequences from single wells of each duplicate, resulting in a total of 41 sequences (Supplementary Table 1). A percentage of identity by best BLAST superior to 90% was obtained for the 41 sequences allowing identification at the family level (Table 2). Of the 41 sequences, 16/41 (39%) belonged to the Rhabdochlamydiaceae family, 12/41 (29%) belonged to the Parachlamydiaceae family, 1/41 (2%) belonged to the Criblamydiaceae (genus *Criblamydia*), the Clavichlamydiaceae (genus *Clavichlamydia*), the Simkaniaceae, and the Waddliaceae, respectively, and 9/41 (22%) provided a best BLAST hit with unclassified Chlamydiales bacteria. Among the 12 sequences corresponding to the Parachlamydiaceae, 6 gave a best Blast hit with the genus *Neochlamydia*, 3 with the genus *Parachlamydia*, and 3 with the genus *Protochlamydia*. The distribution and the prevalence of Chlamydiales families in tick samples collected in different geographical areas in Rarogne, Switzerland, was determined (Fig. 1). Except for one distinct collection area where the highest prevalence was observed for the Parachlamydiaceae, DNA from the Rhabdochlamydiaceae was predominantly detected among the positive-sequenced samples.

Most of the pan-Chlamydiales qPCR were found to be positive with a relative low DNA copy number per microliter (<5 copies/µl) except for 3 samples (Supplementary Table 1). Two samples corresponding to the Rhabdochlamydiaceae contained 1000 and 48 copies/µl, respectively. One sample showing the highest similarity with the Waddliaceae exhibited 10.5 copies/µl.

The sequencing results showed that 5/41 samples have a best identity below 97% which corresponds to putatively new species according to the Everett cutoff (Everett et al., 1999). Of the remaining 36 samples, 22 samples showed 100% identity with Chlamydiales DNA

sequences previously submitted to the NCBI databases, and the remaining 14 sequences likely correspond to new Chlamydiales strains.

Prevalence of Chlamydiales bacteria in various tick and flea species collected in Algeria

A total of 55 ticks belonging to different species was collected in several areas in Algeria including 7 *Dermacentor marginatus*, 10 *Hyalomma detritum detritum*, 20 *Hyalomma dromedarii*, 9 *Ixodes ricinus*, and 9 *Rhipicephalus sanguineus*. The pan-Chlamydiales qPCR was applied on these samples in quadruplicate and was positive for 25/55 (45%) tick samples (Table 3). The positive qPCR was sequenced (Supplementary Table 2). A total of 32 sequences was obtained since 3 samples provided 2 different sequences and 2 samples provided 3 different sequences from positive single wells of the qPCR performed in quadruplicate. Only one sequencing failed. Among the 31 sequences obtained, 23/31 (74%) showed a best BLAST hit with the Parachlamydiaceae family, 4/31 (13%) with the Rhabdochlamydiaceae, 2/31 (6%) with the Criblamydiaceae, and 1/31 (3%) corresponded to the Chlamydiaceae and to unclassified Chlamydiales, respectively (Table 3). Among the Parachlamydiaceae, 11/23 (48%) belonged to the genus *Neochlamydia*, 5/23 (22%) to the genus *Parachlamydia*, 3/23 (13%) to the genus *Protochlamydia*, 1/23 (4%) to the genus *Metachlamydia*, and for 3/23 (13%) no classification at the genus level could be determined. The sequence belonging to the Criblamydiaceae corresponded to the genus *Estrella*, and the sequence belonging to the Chlamydiaceae corresponded to the genus *Chlamydia*.

The highest prevalence of Chlamydiales DNA was observed in *H. dromedarii* ticks (14/20), whereas the prevalence was of 4/10, 4/9, and 3/9 in the tick species *H. d. detritum*, *I. ricinus*, and *R. sanguineus*, respectively. The pan-Chlamydiales qPCR performed on *D. marginatus* samples gave always a negative result (0/7).

In addition, DNA of 97 fleas composed of 19 *Stenoponia tripectinata*, 49 *Nosophyllus* spp., 3 *Xenopsylla cheopis*, 21 *Ctenocephalides felis*, and 5 *Archaeopsylla* spp. were analyzed. The pan-Chlamydiales qPCR was positive for 7/97 (7%) samples. The sequences showed that 4 of the positive samples belonged to genus *Protochlamydia* (family Parachlamydiaceae), 2 to the genus *Metachlamydia* (Parachlamydiaceae), and one sequence corresponded to unclassified Chlamydiales. The proportion of fleas that were positive for Chlamydiales DNA (7%) was significantly lower than the proportion observed in ticks (45%, $p<0.05$).

Discussion

There is increasing evidence demonstrating that the diversity of Chlamydiales bacteria and their host range is largely underestimated (Corsaro et al., 2009; Horn, 2008). Several new Chlamydiales families composed of multiple genera and species have been isolated and described in the past few years from various hosts distributed in very diverse geographical areas (Corsaro and Greub, 2006; Horn, 2008). However, the true dissemination of these novel Chlamydiales in environmental reservoirs, vectors, and hosts is still poorly investigated. Some of these bacteria, including *W. chondrophila* and *P. acanthamoebae*, represent new emerging pathogens likely causing miscarriage and respiratory tract infections (Baud et al., 2007; Baud et al., 2008; Baud et al., 2011; Lamoth and Greub, 2009, 2010), but the mode of human exposure to these *Chlamydia*-related bacteria is still unknown and remains only speculative. However, the understanding of bacterial pathogenicity and transmission capacity largely depends on the identification of possible bacterial vectors and of susceptible hosts. Thus, ticks and fleas, which represent well-recognized vectors of multiple bacterial and viral diseases, have been investigated for the presence of Chlamydiales DNA with a newly developed pan-Chlamydiales qPCR (Lienard et al., 2011a). In Switzerland, the prevalence of Chlamydiales DNA in 1340 adult and nymphal *I. ricinus* ticks prepared in 192 pools was 28.1% (54/192), which represents an estimated prevalence of about 16% (between 4.0 and 28.4%). No significant difference of Chlamydiales prevalence between adults and nymphal ticks was observed. However, differences in the prevalence and in the distribution of Chlamydiales families in tick populations collected in different areas in the region of Rarogne were observed (Fig. 1) suggesting that the prevalence of Chlamydiales may differ significantly between ticks collected in closely located niches.

On average, a 45% (25/55) prevalence of Chlamydiales DNA in various tick species collected in Algeria were observed. Even though differences in prevalence among different tick species were obtained ranging from 0% (0/7) prevalence in *H. d. detritum* to 70% (14/20) in *H. dromedarii*, the number of tick samples per species was too low to generate statistically significant data. The difference of Chlamydiales DNA prevalence between ticks collected in Switzerland and Algeria could originate from several ecological parameters such as tick species, geographical climatic differences, and the presence of susceptible hosts, but also from experimental parameters such as tick collecting and DNA extraction. The high sensitivity of the pan-Chlamydiales qPCR requires the use of a strictly standardized experimental procedure ensuring minimal contamination of the samples by external DNA, such as automated DNA extraction and processing of the experimental reactions in separate rooms (Lienard et al., 2011a). When DNA extraction is performed manually (as done in

Algeria), cross-contamination between extracted samples may occur and may result in an overestimation of the real Chlamydiales DNA prevalence in biological samples. However, despite this limitation and the need of further studies in Algeria using standardized sample collection and DNA preparation, the observed higher prevalence in ticks as compared to fleas supports the validity of the Algerian data and the role of ticks as carriers of Chlamydiales.
A total of 58 pan-Chlamydiales qPCR-positive samples of Rarogne and 32 samples of Algeria, respectively, were sequenced. The sequencing failed for 17 samples from Rarogne and for one sample from Algeria. Sequencing failure was mainly due to (i) no sequence generation because of insufficient amount of template DNA or due to (ii) to the generation of multiple sequencing peaks likely caused by the presence of several Chlamydiales DNA templates in a single sample, which could explain the relatively high rate of sequencing failure with the pooled samples of ticks collected in Switzerland (29.3%) compared to samples collected in Algeria (5%). Thus, positive sequencing results obtained from 41 samples from Switzerland and 38 samples from Algeria showed that a high diversity of Chlamydiales DNA was found in association with all tick and flea species tested except *D. marginatus* and *Archaeopsylla* spp., respectively. Among a total of 79 sequences obtained from tick and flea samples, 5 sequences from Rarogne (Switzerland) and 18 sequences from Algeria showed an identity of <97% with previously sequenced Chlamydiales 16S rDNA. According to Everett cutoff (Everett et al., 1999), a strain exhibiting a sequence similarity of the complete 16S rRNA gene of less than 97%, 95%, and 90% should be classified as a new species, new genus, or new family-level lineage, respectively. The pan-Chlamydiales qPCR targets a highly variable region of the 16S rRNA gene of Chlamydiales bacteria as described in Lienard et al. (2011a) and thus provides a high discriminative power for classification at the family-level lineage, but also with a high confidence at the genus level. Soergel et al. (2012) recently demonstrated that the choice of an optimal primer for short 16S rRNA sequences even below 200 bp can provide up to 100% of the confident genus classification available from longer reads. However, the 200-bp 16S rRNA gene sequences obtained with the pan-Chlamydiales qPCR do not ensure 100% accuracy for genus classification and thus do not allow definite classification at the genus level. Although only partial sequences of the 16S rRNA gene of about 200 bp were obtained in this study, Everett's criteria were thus used to provide some insights into the putative classification of the strains at the genus- and family-level lineages (Supplementary Fig. 1). Hence, 14 sequences from Rarogne and 13 sequences from Algeria exhibited an identity between 97.1% and 99.4% indicating that 27 sequences obtained in this study correspond to putative new Chlamydiales strains. Seven sequences from

Algeria and 22 sequences from Rarogne had a 100% identity with previously identified Chlamydiales DNA. Among those, 13 sequences obtained in Rarogne exhibit 100% identity with the uncultured Chlamydiales bacterium clone GE11093 which is a putative new *Candidatus* Rhabdochlamydia porcellionis strain (97.8% identity) previously identified in Switzerland (Lienard et al., 2011a). One of the remaining 9 sequences of Rarogne had 100% identity with *Rhabdochlamydia crassificans* strain CRIB01, one with an uncultured Chlamydiales bacterium clone belonging to the Parachlamydiaceae family, and 7 with unclassified Chlamydiales. Finally, 6 of the 7 sequences from Algeria showed 100% identity with members of the Parachlamydiaceae family and one with an unclassified Chlamydiales. Rhabdochlamydiaceae and Parachlamydiaceae were predominantly identified in this study. The Rhabdochlamydiaceae family comprises 2 known candidate species, *Candidatus* R. porcellionis and *Candidatus* R. crassificans, which were identified in the isopod *Porcellio scaber* and the cockroach *Blatta orientalis*, respectively (Corsaro et al., 2007; Kostanjsek et al., 2004). This study confirms the high prevalence and biodiversity of Rhabdochlamydiaceae among arthropods. Interestingly, high Rhabdochlamydiaceae DNA copy numbers were detected in 2 pooled tick samples collected in the same area in Rarogne suggesting that *I. ricinus* ticks could be heavily colonized by Chlamydiales bacteria belonging to this family. Chlamydiales species belonging to the *Parachlamydia*, *Protochlamydia*, and *Neochlamydia* genera of the Parachlamydiaceae family have been mainly identified as free amoebal symbionts isolated from water and soil samples (reviewed in Greub and Raoult, 2002; Horn, 2008). Free-living amoebae are ubiquitous microorganisms that likely play a major role in the ecology of *Chlamydia*-related bacteria and probably represent an important reservoir and a major vector of Chlamydiales bacteria with transmission to various hosts including arthropods such as ticks and fleas. Interestingly, other obligate intracellular bacteria such as *Rickettsia* spp. have been identified as symbionts of both amoebae and insects (arthropods) (Gottlieb et al., 2008; Thomas and Greub, 2010). Moreover, *Rickettsia bellii* is found in soft ticks and in hard ticks and is also able to survive in amoebae for several weeks (Ogata et al., 2006). The diversity of the microbiota associated with ticks is largely unexplored (Taylor et al., 2012), and this study suggests that the Parachlamydiaceae family could contain several species exhibiting a dual symbiosis or association with both amoebae and arthropods/insects. This might be important in terms of bacterial and eukaryotic evolution, especially considering possible horizontal gene transfer from symbionts to their hosts, from hosts to their symbionts, and between symbionts.

In conclusion, this study indicates that ticks and fleas are potentially associated with a high diversity of Chlamydiales bacteria belonging mainly to the Rhabdochlamydiaceae and Parachlamydiaceae families. However, a significant effort to isolate new Chlamydiales species and/or strains by coculture within various host cell types such as amoebae and tick cell lines should be initiated to better characterize the interactions between Chlamydiales bacteria and those putative arthropod hosts. Interestingly, more than 40 tick cell lines originating from 13 ixodid (hard) and one argasid (soft) tick species are available (Bell-Sakyi et al., 2007), and several of these cell lines have been used successfully to isolate or propagate pathogens and/or tick symbionts such as *Anaplasma* and *Rickettsia* species (Bell-Sakyi et al., 2007; Goodman et al., 1996; Munderloh et al., 2003; Pornwiroon et al., 2006). Similarly, the previously isolated strains of Parachlamydiaceae are natural amoebal endosymbionts which were mainly recovered by amoebal coculture from various environmental and clinical samples (Amann et al., 1997; Birtles et al., 1997; Greub and Raoult, 2002). Sixt et al. (2012) demonstrated that while *Protochlamydia amoebophila* UWE25 and *Parachlamydia acanthamoebae* UV7 exhibited limited intracellular growth in insect cell lines, *Simkania negevensis* could efficiently replicate within insect cells.Together, these data clearly indicate that amoebae and tick cell lines represent interesting biological tools to isolate bacteria from arthropod samples which could represent the first step for a thorough identification and characterization of new Chlamydiales bacterial strains.

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Legend of figure

Fig. 1. Prevalence and distribution of Chlamydiales families DNA in *Ixodes ricinus* ticks collected in different areas of Rarogne, Switzerland. The 1340 ticks were collected in different geographical areas (pine forest, meadow, and broad-leaved forest) in the region of Rarogne. The prevalence and the distribution of sequences belonging to different Chlamydiales families differed between the different areas of sample collection.

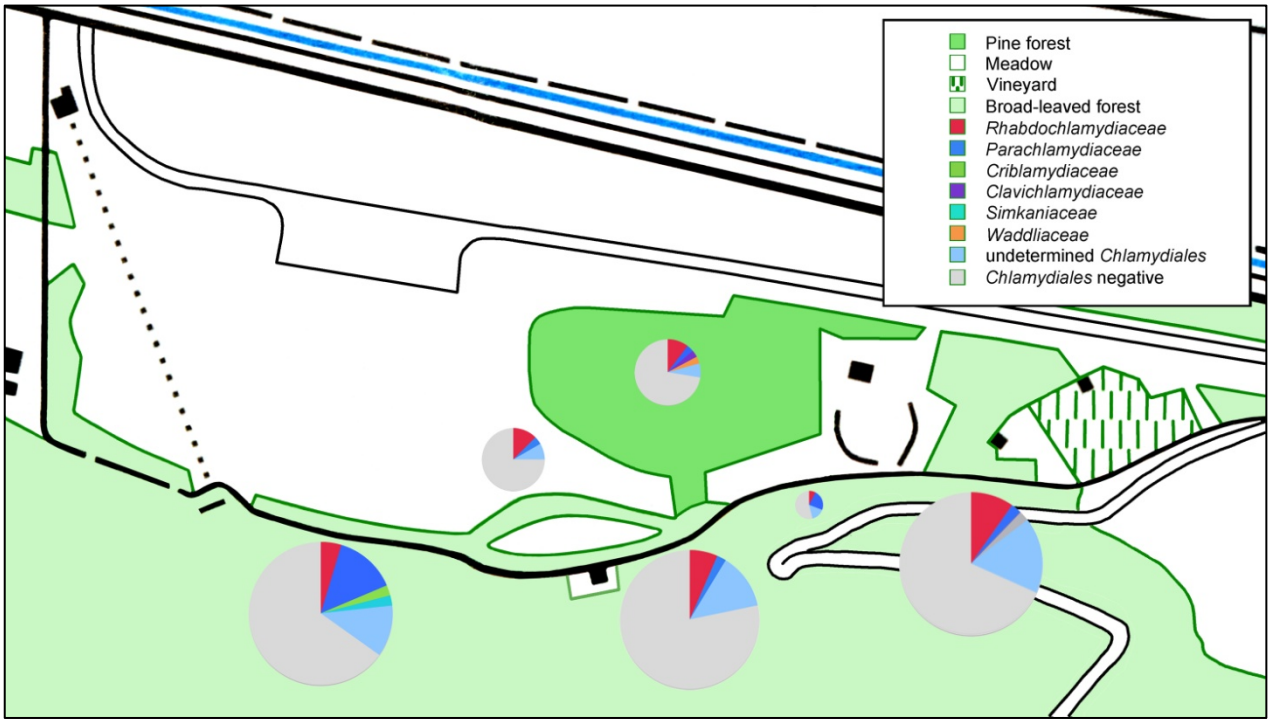

Pine forest
Meadow
Vineyard
Broad-leaved forest
Rhabdochlamydiaceae
Parachlamydiaceae
Criblamydiaceae
Clavichlamydiaceae
Simkaniaceae
Waddliaceae
undetermined Chlamydiales
Chlamydiales negative
100m

Figure 1

Supplementary Table 1. Sequencing results of positive pan-*Chlamydiales* qPCR on *Ixodes ricinus* ticks collected in Switzerland.

ID ADN	Copies / µl	Family-level lineage	16SrRNA gene Best Blast hit	Identity (%)	Accesion number	16SrRNA gene Best Blast hit with described species or candidate species	Identity (%)	Acces num
P1A7	0.27	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	97.8	AY223
P1A8	0.03	*Rhabdochlamydiaceae*	*Candidatus* Rhabdochlamydia crassificans strain CRIB01	100	AY928092	-		
P1A11	0.73	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone FW1013-189	96.1	EF693090	*Parachlamydia* sp. OEW1 partial 16S rRNA gene	94.8	AM412
P1A12	0.48	ND (seq. failure)						
P1B1	0.5	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone HE210050	98.2	HQ721227	Uncultured *Candidatus* Rhabdochlamydia sp. clone CN808	92.5	EU090
P1B6-1*	0.2	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone Upland_120_7966	96.7	JF988666	*Parachlamydiaceae* bacterium KV	94.1	JN112
P1B6-2*	0.2	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	97.8	AY223
P1B9	0.44	ND (seq. failure)						
P1B10	0.37	ND (seq. failure)						
P1B11	0.16	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	98.0	AY223
P1C4	0.4	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE10147	99.3	HQ721198	*Parachlamydia* sp. Hall's coccus	98.6	AF366
P1C5	0.2	*Criblamydiaceae*	Uncultured *Chlamydiales* bacterium clone HE20074	97.1	HQ721240	*Criblamydia sequanensis*	92.5	DQ12
P1C7	0.37	Unclassified *Chlamydiales*	Uncultured *Chlamydiales* bacterium clone GE10169	100	HQ721201	-		
P1C8	0.36	Unclassified *Chlamydiales*	Uncultured *Chlamydiales* bacterium clone GE10169	100	HQ721201	-		
P1C10	0.41	ND (seq. failure)						
P1C11	0.55	Unclassified *Chlamydiales*	Uncultured *Chlamydiales* bacterium clone HE210050	98.5	HQ721227	*Chlamydia psittaci* strain CPX0308	87.1	AB285
P1D1	0.5	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone F5K2Q4C04IBDKU	99.1	GU915305	*Protochlamydia naegleriophila* strain CRIB42	98.1	FJ5322
P1D2	0.4	ND (seq. failure)						

P1D4	0.32	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone 55A12h	100	HM599245	*Neochlamydia hartmannellae* strain A1Hsp	96.8	NR_02
P1D5	0.43	ND (seq. failure)						
P1D11	0.47	ND (seq. failure)						
P1D12	0.13	ND (seq. failure)						
P1E1	0.31	*Simkaniaceae*	Uncultured *Chlamydiales* clone PRPR83	99.3	DQ903996	*Simkania negevensis* strain Z	98.6	NR_02
P1E12	0.18	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	98.1	AY223
P1F5	0.5	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone HE210023_C12	98.4	HQ721223	*Protochlamydia naegleriophila* strain CRIB42	95.1	FJ5322
P1F7	0.23	Unclassified *Chlamydiales*	Uncultured *Chlamydiales* bacterium clone GE10169	100	HQ721201	-		
P1F11	1.8	Unclassified *Chlamydiales*	Uncultured *Chlamydiales* bacterium clone HE210050	100	HQ721227	-		
P1F12	0.49	*Clavichlamydiaceae*	Uncultured *Chlamydiales* bacterium clone HE20028	96.6	EU363464	*Candidatus* Clavichlamydia salmonicola	91	EF577
P1G2	0.3	ND (seq. failure)						
P1G8	0.11	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	98.2	AY223
P1G11	1.58	ND (seq. failure)						
P1G12	0.22	ND (seq. failure)						
P1H2	0.48	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GDIC2IK01A8V8N	98.9	JF660305	*Neochlamydia hartmannellae* strain A1Hsp	96.6	NR_02
P1H3	0.34	ND (seq. failure)						
P1H5	0.43	Unclassified *Chlamydiales*	Uncultured *Chlamydiales* bacterium clone GE10193	100	HQ721203	*Chlamydia pecorum* strain E58	89.2	CP002
P1H10	0.5	Unclassified *Chlamydiales*	Uncultured *Chlamydiales* bacterium clone GE10193	100	HQ721203	*Chlamydia pecorum* strain E58	89.2	CP002
P1H11	2.03	Unclassified *Chlamydiales*	Uncultured *Chlamydiales* bacterium clone HE210050	100	HQ721227	*Neochlamydia hartmannellae* strain A1Hsp	85.8	NR_02
P1H12	10.5	*Waddliaceae*	Uncultured *Chlamydiales* bacterium clone HE210050	98.7	HQ721227	*Waddliaceae* bacterium cvE65	91.2	JF7067
P2A9	0.32	*Parachlamydiaceae*	*Parachlamydiaceae* bacterium KV	95.8	JN112799	*Neochlamydia hartmannellae* strain A1Hsp	95	NR_02
P2B8-1*	0.43	*Parachlamydiaceae*	*Neochlamydia* sp. CRIB37	97.8	EU683885	-		
P2B8-2*	0.08	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	97.9	AY223

P2B12	0.13	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	98.3	AY223
P2C1	0.32	ND (seq. failure)						
P2C3	1000	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	97.8	AY223
P2C8	0.5	Unclassified *Chlamydiales*	Uncultured *Chlamydiales* bacterium clone KK135A0008	99.3	HM063023	*Chlamydiales bacterium* cvE21	98.6	FJ9760
P2D3	48	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	98.3	AY223
P2D4	0.25	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	98	AY223
P2D5	0.23	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	97.9	AY223
P2D11	0.07	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	98.3	AY223
P2D12	0.13	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11064	98	HQ721209	*Protochlamydia naegleriophila* strain CRIB42	93	FJ5322
P2E1	0.41	ND (seq. failure)						
P2E9	0.38	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone P-4	98.7	AF364569	*Neochlamydia hartmannellae* strain A1Hsp	97.3	NR_02
P2F2	0.41	ND (seq. failure)						
P2F10-1*	1.27	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	100	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	97.9	AY223
P2F10-2*	0.14	ND (seq. failure)						
P2H10-1*	0.34	ND (seq. failure)						
P2H10-2*	0.41	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone DS1-22	95.9	EU883174	*Neochlamydia hartmannellae* strain A1Hsp	92.3	NR_02
P2H11	0.13	*Rhabdochlamydiaceae*	Uncultured Candidatus *Rhabdochlamydia* sp. clone KF-9	98.7	EF445478	*Candidatus* Rhabdochlamydia porcellionis	96.8	AY223

* Different sequencing results were obtained from single wells of one pan-*Chlamydiales* qPCR performed in duplicate. ND (seq. Failed): Not determined due to sequencing failure.

5 Supplementary Table 2. Sequencing results of positive pan-*Chlamydiales* qPCR on tick and flea species collected in Algeria

Ticks and fleas Species	**ID ADN**	**Copies/ µl**	**Family-level lineage**	**16SrRNA gene Best Blast hit**	**Identity (%)**	**Accesion number**	**16SrRNA gene Best Blast hit with described species or candidate species**	**Identity (%)**	**Acce num**
Ticks (*R. sanguineus*)	ARS1	0.05	*Parachlamydiaceae*	Uncultured *Neochlamydia* sp. LTUNC09656	100	AY144295	-		
Ticks (*R. sanguineus*)	ARS2	0.82	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE10014	95.7	HQ721239	*Candidatus* Metachlamydia lacustris strain CHSL	95.1	GQ22
Ticks (*R. sanguineus*)	ARS3	0.63	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone FW1013-189	95.7	EF693090	*Parachlamydia acanthamoebae* UV-7	95.1	FR87
Ticks (*I. ricinus*)	AIR1	0.06	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	98.8	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	98.4	AY22
Ticks (*I. ricinus*)	AIR2	0.11	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	98.6	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	97.9	AY22
Ticks (*I. ricinus*)	AIR3	0.07	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone HE20074	96.8	HQ721240	*Neochlamydia* sp. CRIB37	96.7	EU68
Ticks (*I. ricinus*)*	AIR4-1	0.12	Unclassified *Chlamydiales*	Uncultured *Chlamydiales* clone PRPR85	100	DQ903997	*Estrella lausannensis* strain CRIB 30	89.9	EU07
	AIR4-2		*Criblamydiaceae*	Uncultured *Chlamydiales* bacterium clone EP912A0005	97.7	HM444977	*Estrella lausannensis* strain CRIB 30	95.3	EU07
Ticks (*H. d. detritum*)	AHDe1	0.02	ND (seq. failure)	-			-		
Ticks (*H. d. detritum*)*	AHDe2-1	0.44	*Parachlamydiaceae*	*Parachlamydia acanthamoebae* strain Bn9	97.3	NR_026357	-		
	AHDe2-2		*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE10014	96.1	HQ721239	Uncultured *Neochlamydia* sp. LTUNC08556	93.4	AY14
Ticks (*H. d. detritum*)	AHDe3	0.18	*Chlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11061	98.1	HQ721207	*Chlamydia pneumoniae* CWL029	91.2	AE00
Ticks (*H. d. detritum*)	AHDe4	3.13	*Parachlamydiaceae*	*Neochlamydia hartmannellae* strain A1Hsp	98.2	NR_025037	-		
Ticks (*H.dromedarii*)*	AHDr1-1	0.64	*Rhabdochlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11093	98.8	HQ721212	*Candidatus* Rhabdochlamydia porcellionis	98.1	AY22
	AHDr1-2		*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11061	95.6	HQ721207	*Parachlamydiaceae* bacterium KV	93.4	JN11
Ticks (*H.dromedarii*)	AHDr2	0.93	*Parachlamydiaceae*	*Protochlamydia naegleriophila* strain cvE27	93.9	FJ976101	-		
Ticks	AHDr3	0.5	*Parachlamydiaceae*	*Parachlamydiaceae* bacterium KV	96.7	JN112799	*Protochlamydia naegleriophila*	94.6	EU38

(*H.dromedarii*)							strain CRIB 36		
Ticks (*H.dromedarii*)	AHDr4	1.01	*Parachlamydiaceae*	*Chlamydiales* bacterium cvE18	99.2	FJ976098	*Neochlamydia hartmannellae* strain A1Hsp	96.7	NR_0 7
Ticks (*H.dromedarii*)*	AHDr5-1	0.76	*Parachlamydiaceae*	Uncultured *Chlamydiae* bacterium clone MD2896-0.1m.78	94.6	DQ996922	*Neochlamydia hartmannellae* strain A1Hsp	93	NR_0 7
	AHDr5-2		*Rhabdochlamydiaceae*	*Candidatus* Rhabdochlamydia crassificans clone P1s-222	94.3	GQ287585	-		
	AHDr5-3		*Parachlamydiaceae*	*Neochlamydia hartmannellae* strain A1Hsp	96.8	NR_025037	-		
Ticks (*H.dromedarii*)	AHDr6	0.41	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11061	97.5	HQ721207	*Neochlamydia hartmannellae* strain A1Hsp	96.3	NR_0 7
Ticks (*H.dromedarii*)	AHDr7	1.94	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE11061	96.8	HQ721207	*Neochlamydia hartmannellae* strain A1Hsp	95.8	NR_0 7
Ticks (*H.dromedarii*)	AHDr8	3.24	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone GE10014	96.7	HQ721239	*Parachlamydiaceae* bacterium CRIB38	95.2	EU68
Ticks (*H.dromedarii*)	AHDr9	0.71	*Parachlamydiaceae*	*Protochlamydia naegleriophila* strain CRIB 36	99.2	EU384664	-		
Ticks (*H.dromedarii*)	AHDr10	1.23	*Criblamydiaceae*	Uncultured *Chlamydiales* bacterium clone VS30055	97.8	HQ721236	*Estrella lausannensis* strain CRIB 30	96.7	EU07
Ticks (*H.dromedarii*)	AHDr11	0.09	*Parachlamydiaceae*	*Parachlamydia acanthamoebae* strain Bn9	100	NR_026357	-		
Ticks (*H.dromedarii*)	AHDr12	0.35	*Parachlamydiaceae*	*Parachlamydia acanthamoebae* strain Seine	100	DQ309029	-		
Ticks (*H.dromedarii*)	AHDr13	0.23	*Parachlamydiaceae*	*Parachlamydia acanthamoebae* strain Seine	100	DQ309029	-		
Ticks (*H.dromedarii*)*	AHDr14-1	2.12	*Parachlamydiaceae*	*Chlamydiales* bacterium cvE21	95.2	FJ976097	*Neochlamydia hartmannellae* strain A1Hsp	94.4	NR_0 7
	AHDr14-2		*Parachlamydiaceae*	Uncultured *Chlamydiales* organism clone SBZO_1546	96.3	JN530146	*Parachlamydiaceae* bacterium CRIB38	95.2	EU68
	AHDr14-3		*Parachlamydiaceae*	*Neochlamydia hartmannellae* strain A1Hsp	98.4	NR_025037	-		
Fleas (*S. tripectinata*)	AST1	0.38	*Parachlamydiaceae*	Uncultured *Chlamydiales* organism clone SBYX_4984	96.3	JN489825	*Candidatus* Protochlamydia sp. cvE14	91.1	FJ976
Fleas (*S. tripectinata*)	AST2	5.19	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone VS30007	95.2	HQ721231	*Candidatus* Protochlamydia amoebophila strain UWE25	94.1	JQ34
Fleas (*Nosopsyllus* spp)	AN1	1.24	*Parachlamydiaceae*	*Parachlamydiaceae* bacterium CRIB38	100	EU683886	*Candidatus* Metachlamydia lacustris strain CHSL	93. 4	GQ22
Fleas (*Nosopsyllus* spp)*	AN2-1	1.9	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone VS30007	95.1	HQ721231	*Candidatus* Protochlamydia amoebophila strain UWE25	94	JQ34

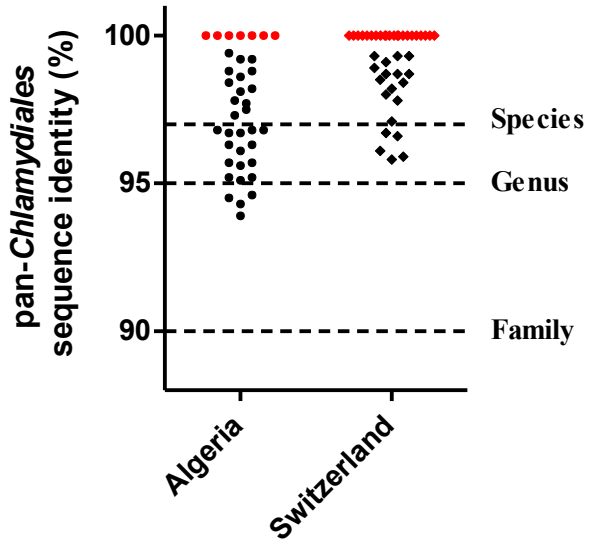
	AN2-2		Unclassified *Chlamydiales*	Uncultured *Chlamydiales bacterium* clone HE210050	99.4	HQ721227	*Chlamydia psittaci*	87.1	AB28
Fleas (*Nosopsyllus* spp)	AN3	3.49	ND (seq. failure)	-					
Fleas (*X. cheopis*)	AXC1	0.4	*Parachlamydiaceae*	Uncultured *Chlamydiales* bacterium clone VS30007	94.5	HQ721231	*Candidatus* Protochlamydia amoebophila strain UWE25	93.3	JQ34
Fleas (*C. felis*)	ACF1	0.9	*Parachlamydiaceae*	*Parachlamydiaceae* bacterium CRIB38	100	EU683886	*Candidatus* Metachlamydia lacustris strain CHSL	98.4	GQ22

6

7 * Different sequencing results were obtained from single wells of one pan-*Chlamydiales* qPCR performed in quadruplicate. ND (seq. failure):

8 Not determined due to sequencing failure.

Supplementary Figure 1.



Supplementary Figure1. Pan-*Chlamydiales* sequences identities. A total of 79 sequences of pan-*Chlamydiales* positive qPCR were obtained and plotted according to the Best Blast identity with closest previously sequenced *Chlamydiales* species or candidate species 16S rDNA. Despite a partial read length of about 200bp, the percent identity can be used to provide some insight of the taxonomic classification of the detected *Chlamydiales* DNA at the species level (97%), at the genus level (95%) and at the family level (90%) according to cut-offs proposed by Everett *et al.* (Everett, et al., 1999). Red dots and squares correspond to sequences showing a 100% identity with *Chlamydiales* DNA sequences previously submitted to NCBI databases.