Evidence for Chlamydiaceae *and* Parachlamydiaceae *in a wild boar (*Sus scrofa*) population in Italy*

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Summary
Conjunctival swabs from 44 free-living wild boars culled during a demographic control programme applied in a Regional Park located in the Northern Italy were examined by 16S rRNA encoding gene nested PCR. In total, 22 (50%) wild boars were PCR positive. Sequencing of the amplicons identified *Chlamydia suis* and *Chlamydia pecorum* in 12 and 5 samples, respectively. For one sample found PCR positive, the nucleotide sequence could not be determined. Four conjunctival samples showed ≥ 92% sequence similarities to 16S rRNA sequences from *Chlamydia*-like organisms, as did large intestine, uterus, and vaginal swabs from the same four animals. Amoeba DNA was found in one *Chlamydia*-like organism positive conjunctival swab. To our knowledge, this is the first detection of members of the *Parachlamydiaceae* family in wild boars, confirming a large animal host range for *Chlamydia*-like organisms.

Chlamydiaceae *e* Parachlamydiaceae *riscontrate in una popolazione di cinghiali selvatici in Italia*

Parole chiave
Chlamydiaceae, Cinghiali selvatici, Italia, Nested PCR, *Parachlamydiaceae*.

Riassunto
Il presente studio riporta i dati riguardanti tamponi congiuntivali di 44 cinghiali selvatici. I tamponi sono stati prelevati nell'ambito di un programma di controllo demografico in un parco regionale dell'Italia del Nord. Essi sono stati esaminati tramite una nested PCR riguardante il gene che codifica il 16S rRNA. I risultati della PCR sono stati positivi per 22 (50%) cinghiali selvatici. Il sequenziamento dei segmenti amplificati ha evidenziato la presenza di *Chlamydia suis* in 12 campioni e di *Chlamydia pecorum* in 5 campioni. Non è stato possibile determinare la sequenza nucleotidica di uno dei campioni PCR-positivi. In 4 tamponi congiuntivali, ed anche in tamponi prelevati da intestino, utero e vagina degli stessi animali, sono state riscontrate analogie con sequenze di 16S rRNA di paraclamidie. In un tampone congiuntivale contenente paraclamidie è stato evidenziato DNA di ameba. Questo studio riporta per la prima volta la presenza di organismi della famiglia *Parachlamydiaceae* in cinghiali selvatici a conferma della diffusione di questi organismi in numerose specie animali.

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Introduction

Parachlamydiaceae are gram-negative obligate intracellular bacteria, showing 80-90% sequence homology of rRNA genes with *Chlamydiaceae* (9). *Parachlamydiaceae* naturally infect free-living amoebae (5), but these *Chlamydia*-like organisms are also able to enter and multiply within human macrophages (6), pneumocytes and lung fibroblasts (3). Among *Parachlamydia*, *Neochlamydia* and *Protochlamydia* genera belonging to the *Parachlamydiaceae* family, members of the genus *Parachlamydia*, and especially *Parachlamydia acanthamoebae*, have been investigated for a potential pathogenic role in humans and animals.

In humans, *P. acanthamoebae* is considered an emerging agent of lower respiratory tract infections, which may cause bronchitis, bronchiolitis, community-acquired pneumonia and aspiration pneumonia. In addition, *P. acanthamoebae* has been linked to atherosclerosis, uveitis, urogenital infection and miscarriage. Water and free-living amoebae have mostly been suggested to be the source of the human infections (reviewed by 7).

Regarding the occurrence in animals, there is evidence that *Parachlamydia* might represent a new abortigenic agent in cattle and small ruminants (1, 16), suggesting a potential zoonotic risk from ruminant abortion material. DNA of *Chlamydia*-like organisms has also been detected in cervical swabs and genital tracts of sows, in semen of boars, and in conjunctival samples of koalas, cats, guinea pigs, pigs, and sheep (reviewed by 2). The DNA detection of *Chlamydia*-like organisms in symptomatic and asymptomatic animals indicates exposure to these bacteria but their pathogenicity remains unclear. Detailed studies relating to the occurrence of *Chlamydia*-like organisms in wildlife populations are lacking, nevertheless a recent report (14) described *Parachlamydia* spp. detection in conjunctival swabs, faeces and internal organs of wild ruminants.

Wild boar (*Sus scrofa*) has been suggested to represent a wildlife reservoir for the same *Chlamydiaceae* species detected in domestic pigs, including *Chlamydia suis*, *Chlamydia psittaci*, *Chlamydia abortus* and *Chlamydia pecorum* (8, 10, 17), but no data are currently available about the occurrence of *Chlamydia*-like organisms in wild boar. In the present study, we report the molecular detection of *Chlamydiaceae* and *Parachlamydiaceae* in a wild boar population in Italy.

Materials and methods

Conjunctival swabs from 44 free-living wild boars (21 females and 23 males), 24 of which were aged under 10 months, were collected from February to September 2011 from animals culled during a demographic control program applied in a Regional Park (Gessi Bolognesi e Calanchi dell'Abbadessa, 48.15 km^2) located in the Emilia-Romagna region of Northern Italy. The samples were examined for *Chlamydiaceae* by 16S rRNA encoding gene nested PCR.

DNA was extracted using a commercial DNA Blood and Tissue Kit (QIAGEN). One extraction control constituting only kit reagents was also tested. The primary PCR amplifying a 369 bp region was performed using the following forward and reverse oligonucleotide primers: 16SIGF (5'-CGGCGTGGATGAGGCAT-3') (9) - CL1 (5'-GCGTCGCTTCGTCAGACTT-3'). Cycling conditions were as follows: 5 min of denaturation at 95° C and 30 cycles each consisting of denaturation at 94° C for 1 min, annealing at 56° C for 1 min and extension at 72° C for 1 min. A final elongation step of 5 min at 72° C completed the reaction.

In the secondary PCR amplification, a 243 bp fragment, primers CL2 (5'-TTAGTGGCGGAAGGGTTAG-3') - 16SIGR (5'-TCAGTCCCAGTGTTGGC-3') (9), were used: 1 µl of product from the first PCR step was added to a final volume of 50 µl. PCR conditions were as described above and 20 cycles were carried out. The extraction control and a distilled water negative control were included in both PCR. The amplified products were visualized after electrophoresis in 2% agarose gel by ethidium bromide staining under UV light. The secondary PCR products were purified by using a QIAquick PCR purification kit (QIAGEN) and both strands were sequenced (Bio-Fab Research, Italy). The nucleotide sequences were compared with those available in GenBank by using the BLAST server from the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi.). Preliminary tests were performed on the Italian *C. pecorum* PV5268 isolate obtained from a bovine cervical swab and the Italian *C. suis* MS04 isolate obtained from a pig conjunctival swab, both characterised by molecular analysis, and *C. abortus* S26/3 and *C. psittaci* 6BC reference strains, to assess if the nested PCR and following sequencing were able to detect and differentiate these *Chlamydia* species.

Results

In total, 22/44 (50%) wild boars, 11 females and 11 males, were positive by 16S rRNA nested PCR. The chlamydial prevalence detected in wild boars aged under 10 months was higher than that found in animals aged over 10 months (62.5% vs 35%). Twelve out of the 44 (27%) animals tested were positive for *C. suis* (≥ 99% sequence similarity to GenBank entry AY661797.1) and 5/44 (11%) for *C. pecorum* (≥ 98% sequence similarity to GenBank entry HQ457465.1).

For one animal found PCR positive, the nucleotide sequence could not be determined because of a mixed signal, probably caused by multiple infections.

Four out of the 44 (9%) conjunctival samples showed varying sequence similarities (92-98%) to 16S rRNA entries from *Chlamydia*-like organisms reported in the GenBank database. In order to evaluate the systemic diffusion of these *Chlamydia*-related bacteria, tissue samples of lung (L), pulmonary limphonodes (LN), small intestine (SI), large intestine (LI), liver (LV), uterus (U) and vaginal swab (VS) collected from the four wild boars, were subjected to the DNA extraction and nested PCR, as described above. The results of the nested PCR and species identification by nucleotide sequencing are presented in Table I.

As *Parachlamydiaceae* occur as endosymbionts in protozoa, specifically *Acanthamoeba* spp., DNA extracted from these PCR-positive samples and water samples taken from stagnant puddles were also screened with an *Acanthamoeba* specific 18S rDNA gene PCR for the presence of *Acanthamoeba* species, according to Schroeder *et al.* (18). As a positive control, *Acanthamoeba castellanii* ATCC 50739 was used. Only one conjunctival swab reacted positive, showing 99% sequence similarity to *Acanthamoeba* sp. CRIB-25 DNA (GenBank entry EU273827.1).

Discussion

Chlamydial DNA was detected in 22/44 (50%) animals. This chlamydial prevalence is consistent with data by Hotzel *et al.* (10), confirming the wild boar as a *Chlamydia* wildlife reservoir. Sequencing of the 16S rRNA PCR products identified *C. suis* and *C. pecorum* in 12 and 5 samples, respectively. The prevalence of *C. suis* was in line with the results of a previous seroepidemiologic study performed in Italy (8), showing a specific antibody reactivity to *C. suis* in 44/173 (25%) sera from wild boars hunter-killed in three Italian regions; the higher antibody prevalence was found in an area with high outdoor domestic pig breeding, suggesting an epidemiologic role of domestic pig infection for wildlife infection. This suggestion was confirmed by the present data, since the wild boars sampled in this study were from areas where free-ranging husbandry of domestic pigs was present. The finding of a *Chlamydia* species usually infecting ruminants, *C. pecorum*, is not surprising, considering previous reports (12, 13) and suggests an extended host range of individual *Chlamydia* species.

Interestingly, 4 wild boars showed PCR positivity for *Chlamydia*-like organisms. We observed a low degree of systemic infection, according to Regenscheit *et al.* (2012). The PCR positivity of wild boar genital tract and conjunctival swabs is consistent with previous reports in other animal species, as well as the PCR positivity of the large intestine and the recent detection of *Chlamydia*-like organisms in faecal samples of wild ruminants (14). All 4 wild boars positive for *Chlamydia*-like organisms showed PCR positivity to one or one more *Chlamydia* species, supporting the suggestion that *Chlamydia*-like organisms could be a part of a multifactorial disease (11).

Amoeba DNA was found in only 1 *Chlamydia*-like organism-positive sample. The persistence of *Chlamydia*-related bacteria in the absence of an amoebal host has been presumed from other authors (4, 15), as well as suggested by the ability of these bacteria to multiply in different mammalian cells.

Conclusions

To our knowledge, this is the first detection of members of the *Parachlamydiaceae* family in wild boars, confirming a large animal host range for *Chlamydia*-like organisms. Although the pathogenicity of these *Chlamydia*-like bacteria

Table I. Chlamydiaceae *and* Chlamydia*-like organisms detected by 16S rRNA gene nested PCR and sequencing in wild boar samples.*

Case number	Samples (% homology)							
	L	LN	SI	LI	LV	U	VS	CS
2741 f	–	*C. suis*[1] (99%)	*C. psittaci*[2] (99%)	CRIB38[3] (93%)	na	CRIB38[3] (93%)	CRIB38[3] (95%)	CRIB38[3] (96%)
2851 m	*C. psittaci*[4] 99%	na	*C. psittaci*[4] (99%)	*P. acanthamoebae*[5] (93%)	na	/	/	*P. acanthamoebae*[5] (92%)
2994 m	*C. suis*[1] (99%)	*C. suis*[1] (97%)	na	*C. suis*[1] (99%)	–	/	/	USC9[6] (98%)
34514 m	–	*C. pecorum*[7] (99%)	*C. pecorum*[7] (94%)	CRIB38[3] (94%)	*C. pecorum*[7] (99%)	/	/	CRIB38[3] (98%)

SL = lung; LN = pulmonary lymphonode; SI = small intestine; LI = large intestine; LV = liver; U = uterus; VS = vaginal swab; CS = conjunctival swab; na = not available.
f = female; m = male. GenBank accession numbers of sequences: [1]AY661797.1, [2]CP002807.1, [3]EU683886.1, [4]AB001809.1, [5]JN051144.1, [6]FJ160741.1, [7]HQ457465.1

is still unclear, growing evidence suggests that *Parachlamydia* spp. may play a role in respiratory tract infections and ocular diseases in humans. The molecular detection of organisms belonging to the *Parachlamydiaceae* family in wild boar could be an additional component of the zoonotic potential of this animal species known to be receptive to zoonotic agents such as *C. psittaci* and *C. abortus.* In view of potential exposure of hunters and other persons handling carcasses and raw game meat, the occurrence of *Chlamydia*-related bacteria in wild boars should be further investigated.

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