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. In totale, 22 (50%) cinghiali selvatici erano PCR positivi. 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 congiuntivalle contenente paraclamidie è stato evidenziato DNA di amebe. 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.

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

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Keywords
Chlamydiaceae, Italy, Nested PCR, Parachlamydiaceae, Wild boar.

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. Ameoba 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 si occupa della presenza di Chlamydiaceae e Parachlamydiaceae nelle congiuntive di 44 cinghiali selvatici. I tamponi sono stati prelevati durante un programma di controllo demografico in un parco regionale dell'Italia del Nord. In totale, 22 (50%) cinghiali selvatici hanno mostrato positività alla PCR per Chlamydiaceae. Sequenziamento dei segmenti amplificati ha evidenziato la presenza di Chlamydia suis in 12 campioni e di Chlamydia pecorum in 5 campioni. Per un campione positivo alla PCR, non è stato possibile determinare la sequenza nucleotidica. In 4 tamponi congiuntivali, 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 congiuntivalle sono state riscontrate anche amebe. Questo studio conferma 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.
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 km²) 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: 16SIFG (5’-CGCGCTGGATGAGGCAT-3’) (9) - CL1 (5’-CGGCGTGGATGAGGCAT-3’) and 16SIGR (5’-TCGTCCACGTTGGCC-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 lymphonodes (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 castellani 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**

Chlamydiaceae 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...
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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