Chlamydia suis *and tetracycline resistance genes in Italian wild boar (*Sus scrofa*)*

Antonietta Di Francesco[1*], Federico Morandi[2], Hanna Marti[3], Carmela Santagati[4] and Nicole Borel[3]

[1]*Department of Veterinary Medical Sciences, University of Bologna, Italy.*
[2]*Parco Nazionale dei Monti Sibillini, Visso, Macerata, Italy.*
[3]*Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland.*
[4]*Azienda AUSL, Bologna, Italy.*

** Corresponding author at: Department of Veterinary Medical Sciences, University of Bologna, Italy.*
E-mail: antoniet.difrancesco@unibo.it.

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Summary
The aim of this study was to investigate the occurrence of *Chlamydia suis* and tetracycline resistance determinants in conjunctival swabs of Italian wild boars, by PCR. Extracted DNA collected from 50 wild boars from Northern and Central Italy was examined by molecular methods. One sample (2%) from the Central Italy was positive for *C. suis*. Fragments of *tetR*(C) and *tetR*(C)-*tet*(C) resistance determinants were amplified from the same sample. Further molecular investigations suggested the attribution of these tetracycline resistance determinants to *C. suis*, such as the truncation of *tetR*(C) and absence of a intact invasion (*inv*)-like region. While tetracycline-resistant *C. suis* is very common in domestic pigs, its occurrence has not been reported in wild boar before. Wild boar might acquire tetracycline resistance determinants through direct or indirect contact with domestic pigs.

Chlamydia suis (order Chlamydiales, family *Chlamydiaceae*, genus *Chlamydia*) is the most common chlamydial species reported in pigs (Longbottom 2004), in which it has been associated with conjunctivitis, rhinitis, penumonia, enteritis and reproductive disorders. Moreover, subclinical forms are highly prevalent among domestic pigs making them more susceptible to other infections (Schautteet and Vanrompay 2011).

A stable tetracycline resistance phenotype has been detected in porcine *C. suis* strains worldwide (Lenart *et al.* 2001, Di Francesco *et al.* 2008, Borel *et al.* 2012, Schautteet *et al.* 2013, Donati *et al.* 2016, Wanninger *et al.* 2016). The resistance pattern has been associated with *tet*(C) genomic islands integrated into the chlamydial chromosome containing genes encoding a tetracycline efflux pump and a regulatory repressor (*tet*[C] and *tetR*[C], respectively), a *C. suis*-specific insertion element (ISCs*605*) and additional genes involved in plasmid replication and mobilization (Dugan *et al.* 2004).

Wild boar (*Sus scrofa*) has been suggested to represent a wildlife reservoir for the same *Chlamydiaceae* species as those detected in domestic pigs, including *C. suis* (Hotzel *et al.* 2004, Di Francesco *et al.* 2013). However, the occurrence of tetracycline resistant *C. suis* in wild boar has not been reported so far.

The aim of this study was to investigate the occurrence of *C. suis* and related tetracycline resistance determinants in two geographically different Italian wild boar populations.

In 2017, conjunctival swabs were collected from 50 wild boars in two Italian regions: 37 free-ranging animals were sampled alive for diagnostic investigations in a rural herd in Northern Italy and 13 wild boars were culled during a demographic control program taking place in a Regional Park located in Central Italy. The latter wild boars lived in the same geographical region where the presence of tetracycline resistant *C. suis* strains has previously been assessed in domestic pigs (Donati *et al.* 2016). DNA was extracted from the conjunctival samples using a commercial kit (DNeasy Kit Qiagen, Germany). The extracted genomic DNA was screened by a *Chlamydiaceae*-specific real-time polymerase chain reaction (rt-PCR) targeting a region of the 23S rRNA gene conserved among all *Chlamydiaceae* (Ehricht

et al. 2006). Samples with Ct values < 40 were considered positive and re-analysed by a rt-PCR assay targeting a *C. suis*-specific region 23S rRNA gene (Pantchev *et al.* 2010). DNA from *C. suis*-positive samples was used as a template for a 1,050-bp *omp*A gene fragment amplification (Sayada *et al.* 1995). Two additional PCR assays were performed on the *C. suis* positive samples in order to assess the presence and the features of *tet*(C) genomic island: one PCR amplified a 608 bp fragment including the *tetR*(C) region and another PCR amplified a 457 bp fragment of the *tetR*(C)-*tet*(C) region. The first PCR used the primer tetR-F (5'-TTGGGGCAACCATTTCTGGT-3') (Donati *et al.* 2016) and the primer CS38 (5'-CCAAGGGATGACGACGACTG-3') (Dugan *et al.* 2004). The second PCR was performed using the primer tetRC-F (5'-TGCGTCGAGCAACGCACGCT-3') (Donati *et al.* 2016) and the primer CS43 reverted (5'-CAAAGCGGTCGGACAGTGCT-3') (Dugan *et al.* 2004). Moreover, a PCR targeting a 900 bp fragment of the intact invasion (*inv*)-like region was applied using primers CS02 (5'-CGTTTCAGGAATACCCACTTCG-3') and CS106 (5'-ACACTTCAGGTTTTCGCCGTAG-3') according to Dugan *et al.* (2004). DNA of the Italian EU-21 tetracycline-resistant *C. suis* (Donati *et al.* 2016) and the Swiss 2-2 tetracycline-sensitive *C. suis* (Wanninger *et al.* 2016) field isolates were used as controls. All the amplicons were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and both DNA strands were sequenced (Bio-Fab Research, Rome, Italy). The sequences obtained were compared with the public sequences available in GenBank using the BLAST server from the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

All animals (n = 37) from the Northern Italy region were PCR-negative for *Chlamydiaceae*. One of the 13 (7.6%) wild boars from the Central Italy was positive to the *Chlamydiaceae*-specific rt-PCR. The amplicon was identified as *C. suis* by the specific 23S rRNA PCR. Sequencing of the *omp*A fragment revealed a nucleotide similarity of 88% with the EU-21 Italian tetracycline-resistant *C. suis* strain and 87% and 93% with the US R27 and R19 tetracycline [resistant *C. suis* strains described by Dugan and colleagues (Dugan *et al.* 2004)], respectively. *TetR*(C) and *tetR*(C)-*tet*(C) fragments were both amplified from the same wild boar sample, whereas no amplification was obtained using the intact *inv*-like primers, similarly to the results from EU-21 Italian tetracycline-resistant *C. suis* strain. Neither *tetR*(C) nor *tetR*(C)-*tet*(C) were detected in the Swiss tetracycline sensitive 2-2 *C. suis* strain, whereas a 900 bp amplicon was obtained using the intact *inv*-like primers. *TetR*(C) and *tetR*(C)-*tet*(C) sequences were identical to the corresponding sequences of EU-21 Italian *C. suis* strain, showing the truncation of *tetR*(C) and the presence of a sequence of eight nucleotides in the *tetR*(C)-*tet*(C) intergenic spacer, that was deleted in the US R19 and R27 tetracycline-resistant *C. suis* strains. The sequences obtained in this study were deposited in the GenBank database under accession numbers MH845047, MH893740 and MH893741.

In this study, PCR examination of conjunctival swabs collected from 50 wild boars in two Italian regions revealed only one (2%) *C. suis* positive sample. This is much lower than that reported in a previous Italian study where anti-chlamydial antibodies were detected in 110 of 173 wild boar blood samples (63.6%), with a specific reactivity to *C. suis* in 44 of 173 (25%) samples (Di Francesco *et al.* 2011). In a follow-up study (Di Francesco *et al.* 2013), 22 out of 44 wild boars (50%) were PCR-positive when tested for *Chlamydiaceae* and *Parachlamydiaceae* by PCR. Sequencing of the amplicons identified *C. suis* and *C. pecorum* in twelve (27%) and five (11%) samples, respectively. Both studies were undertaken in regions with high prevalence of domestic pig breeding with outdoor access suggesting possible spread of infection through direct and indirect contacts between domestic pigs and wild boar. In contrast, the low chlamydial prevalence found in this study might be attributed to limited contacts between wild boars and domestic pigs due to strict biosecurity measures or low numbers of rural pig herds. This is in line with results of a very recent study (Wahdan *et al.* 2020) investigating 292 wild boars with limited contacts with domestic pigs from Switzerland and Northern Italy resulting in a low *C. suis* prevalence (1.4%, 4/292).

Antimicrobial resistance (AMR) is one of the most important public health challenges of our time and its spread has been attributed to clinical or farming overuse of antimicrobials. Moreover, AMR has also been reported in the absence of antimicrobial treatments, such as in wildlife in which antimicrobial resistance has been increasingly investigated. Tetracyclines are one of the most classes of antimicrobial agents widely used in veterinary medicine and agriculture due to their broad spectrum of activity, low cost, oral administration, and few side effects. Due to their widespread and indiscriminate use, tetracycline resistance has become one of the most abundant antibiotic resistances among pathogenic and commensal microorganisms, also in wildlife. In wild boars, the presence of tetracycline resistant organisms, such as enterococci, *Salmonella* spp., *Campylobacter* spp., *E. coli, Staphylococcus aureus*, has been documented (Poeta *et al.* 2007, Zottola *et al.* 2013, Carbonero *et al.* 2014, Sousa *et al.* 2017).

In this study, a conjunctival DNA sample from a *C. suis* positive wild boar was investigated for the presence of tetracycline resistance determinants by PCR. In theory, the most rigorous scientific

approach for the evaluation of antibiotic resistance in chlamydiae consists of evaluating the behavior of bacterial isolates in cell culture in the presence and absence of the drug.

Here, a culture-independent approach was chosen as fresh samples were not available, a common problem when performing field studies in wildlife. Due to the lack of *C. suis* isolation, the attribution of the *tet*(C) gene to *C. suis* cannot be confirmed and thus, the genetic determinants of resistance might not be specific for *C. suis*, but might originate from other bacteria. However, the nucleotide sequence showed that *tetR*(C) in the positive wild boar sample was truncated, similarly to the tetracycline-resistant *C. suis* strains described by Dugan and colleagues (Dugan *et al.* 2004) and Donati and colleagues (Donati *et al.* 2016). In addition, the 900 bp fragment of the invasion (*inv*)-like region, which is intact and therefore amplified in tetracycline-sensitive *C. suis* strains, was not amplified in the wild boar DNA sample, similarly to the tetracycline resistant *C. suis* strains in which the (*inv*)-like region is interrupted due the insertion of the Tet-island.

Tetracycline-resistant *C. suis* strains are very common in domestic pigs. Domestic pig husbandry with outdoor access can be a source for transmission of *C. suis* and/or tetracycline resistance genes to wild boars by direct contact or fecal contamination of the environment. Further studies on *C. suis* isolates from wild boars are needed to gain comprehensive epidemiological data on tetracycline resistance gene transmission, in particular focussing on wild boar populations with confirmed contact to domestic pigs.

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