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Potential wildlife reservoir for *Parachlamydia* in red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*)

NADINE REGENSCHEIT¹, NATHALIE HOLZWARTH¹, GILBERT GREUB²,
SEBASTIEN AEBY², ANDREAS POSPISCHIL¹, NICOLE BOREL¹

¹Institute of Veterinary Pathology, University of Zürich, Vetsuisse Faculty Zürich,
Switzerland

²Center for Research on Intracellular Bacteria, Institute of Microbiology, University
Hospital Center and University of Lausanne, Switzerland

Corresponding author:

Nicole Borel, DVM, FVH, Dipl. ECVP

Institute of Veterinary Pathology, Vetsuisse Faculty

University of Zurich, Winterthurerstrasse 268

CH-8057 Zurich, Switzerland

Phone: +41-44-635-8576

Fax: +41-44-635-8934

Email: n.borel@access.uzh.ch

Abstract

The wildlife population represents an important reservoir for emerging pathogens and transboundary livestock diseases. Despite this, there is a lack of knowledge on the occurrence of infectious diseases in wild animal populations, i.e. *Chlamydia* and *Chlamydia*-like organisms such as *Parachlamydia*. During hunting season 2008, 863 samples, including blood, eye swabs, organs and fecal samples, out of 99 red deer and 64 roe deer were collected in the eastern Swiss Alps. They were tested by *Chlamydia (C.) abortus* – specific ELISA, real-time PCR, ArrayTube Microarray, 16S rRNA PCR, sequencing and immunohistochemistry for *Chlamydiaceae* and *Parachlamydia*, respectively. The seroprevalence for *C. abortus* was 1.4% by ELISA, the prevalence for *Chlamydiaceae* 2.5% by real-time PCR. The identified chlamydial species were *C. pecorum* (n=1) in a fecal sample and a non-classified DNA sequence with 94% homology to *C. muridarum* in fecal samples (n=2) and blood samples (n=1) of two different roe deer. The detection of this non-classified DNA sequence of the family *Chlamydiaceae* needs further investigation. We conclude that *Chlamydiaceae* do not play an important role in the investigated deer population in the eastern mountainous part of Switzerland. In contrast, the prevalence for *Parachlamydia sp.* was 30% by real-time PCR in eye swabs, feces, liver and lung. It was possible to detect *Parachlamydia* in macrophages and pneumocytes in two lungs of red deer by immunohistochemistry. To our knowledge, this is the first description of *Parachlamydia sp.* in a wild ruminant species. Possible mode of transmission via fecal excretion or eye secretions would imply a risk of spreading the bacteria on mountainous pastures. Thus red and roe deer may play a role as a reservoir or transmission host for *Parachlamydia* for domestic ruminants.

Keywords: ArrayTube Microarray, *Chlamydiaceae*, ELISA, *Parachlamydia*, red deer, real-time PCR, roe deer

1. Introduction

Ovine enzootic abortion (OEA) caused by *Chlamydia (C.) abortus* with its worldwide spread (Europe, North America and Africa) is a major cause of (fetal) lamb loss and abortion in sheep and goat. There is a possible zoonotic risk for humans (illness in pregnancy and abortion) having contact to chlamydial abortion material (i.e. hunters, gamekeepers, butchers, veterinarians, farmers) (Longbottom and Coulter, 2003). In 2002, *C. abortus* was reported as the most common infectious abortigenic agent in small domestic ruminants in Switzerland (Chanton-Greutmann et al., 2002). As domestic and wild ruminants are grazing on the same alpine pastures, interactions between them are likely, favoring transmission of infectious agents (Ryser-Degiorgis et al., 2002, 2009). Other animal chlamydioses, i.e. *C. pecorum* is described as the possible most prevalent chlamydial species of clinically healthy domestic ruminants (Mohamad and Rodolakis, 2010). *Parachlamydia (P.) acanthamoebae* was discovered as a new *Chlamydia*-like organism (Greub and Raoult, 2002) and is described as an emerging agent of pneumonia in humans (Greub, 2009). Abortions in sheep, goat and cattle due to *P. acanthamoebae* have been recently reported in Switzerland and Scotland (Borel et al., 2006, 2007, Ruhl et al., 2008, 2009, Deuchande et al., 2010). Affected cows showed late-term abortion with purulent to necrotizing placentitis. The parachlamydial presence was demonstrated within the

lesions by immunohistochemistry. However data for *Parachlamydia* in wild ruminants are lacking so far.

Recent investigations on chlamydial infections in Swiss Alpine ibex (*Capra i. ibex*) (Holzwarth et al., 2010, Marreros et al., in press) and Alpine chamois (*Rupicapra r. rupicapra*) (Holzwarth et al., 2011), reported a low prevalence for *Chlamydiaceae* in general and particularly a low seroprevalence for *C. abortus*. However, other wild ruminant families, such as the *Cervidae* have not been investigated. Therefore, two species, the red deer (*Cervus elaphus*) and the roe deer (*Capreolus capreolus*) were tested in the context of this study. The aims of the investigations were to elucidate the prevalence of *Chlamydia* and *Parachlamydia* in cervids and to analyze their possible role as a reservoir or transmission host for domestic ruminants and possible zoonotic risk for humans having contact to them in the eastern mountainous part in Switzerland.

2. Materials

The calculated population in Grisons was 14'000 for red deer and 15'500 for roe deer in the year 2008. During the hunting season of 2008 (September to December), 4'384 red deer and 3'274 roe deer were hunted by gamekeepers and hunters (annual report hunting 2008, hunting and fishing service, canton of Grisons).

All investigated samples were collected in the geographical region Surselva (including side valleys) in the canton of Grisons in the eastern part of the Swiss Alps (46°36 to 46°4 N, 8°42 to 9°20 E) during the above mentioned hunting season of 2008. They originated from the two free-living species of the family *Cervidae* in

Switzerland (n=163): Roe deer (*Capreolus capreolus*) (n=64) and red deer (*Cervus elaphus*) (n=99). These deer were sampled out of a healthy population in a natural habitat. For each hunted animal the signalement (species, sex, age), body condition and the coordinates of death were recorded by the gamekeepers and hunters. In total, 863 samples out of 163 hunted deer were collected. They included sera (n=146), EDTA blood (n=147), eye swabs from both eyes (n=306), organ samples (n=204) and fecal samples (n=60). The investigated organs were liver, lung and kidney.

3. Methods

3.1 DNA extraction

3.1.1 Eye swabs and EDTA blood

The eye swabs (n=306) were pretreated over night (using a thermomixer, 55°C, 550 rpm) with 200 µl of an in-house developed lysis buffer. First a stock solution was prepared consisting of 8 ml of EDTA (0.5 M, pH 8), 4 ml of Tween50% and 20 ml of Tris/HCl (1 M, pH 8). By diluting the stock solution 1:10, the working solution was achieved. 196 µl of the working solution was mixed with 4 µl Proteinase K (recombinant, PCR Grade, 20 mg/ml, Roche Diagnostics GmbH, Mannheim, Germany) to get the final lysis buffer. After incubation with the final lysis buffer, the eye swabs were centrifuged three times one minute at 14000 rpm. The pretreatment of the EDTA blood (n=147) consisted of a one to four dilution with HBBS (GIBCO®, Invitrogen AG, Basel, Switzerland). 200 µl of each pretreated eye swab and EDTA blood sample was used for further automatically DNA extraction by the MagNA Pure®

LC System (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions.

3.1.2 Organ and fecal samples

The organ samples (n=204) and fecal samples (n=60) were cut with a sterilized blade into small pieces of about 10mg. Extraction was then performed by using a commercially available DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

3.2 Chlamydia

3.2.1 Real-time PCR assay for Chlamydiaceae

All extracted samples (n=717) (eye swabs: n=306, EDTA blood: n=147, organ samples: n=204 and fecal samples: n=60) were examined on an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA) applying the 23S-based *Chlamydiaceae* family-specific real-time PCR as described recently (Ehrlich et al., 2006). This real-time PCR amplifies a 111-bp product, specific for members of the family *Chlamydiaceae*. A final volume of 25µl for each tested sample was achieved by adding 2.5µl of extracted DNA to 12.5µl of 2X TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and a final concentration of 5pmol/l of each primer (Ch23S-F 5'-CTGAAACCAGTAGCTTATAAGCGGT-3', Ch23S-R 5'-ACCTCGCCGTTTAACTTAACTCC-3'), and probe Ch23S-p (5'-FAM-CTCATCATGCAAAAGGCACGCCG-TAMRA-3'), (Microsynth, Balgach, Switzerland). An internal amplification control producing of a 177-bp PCR product was added. The control consisted of the following primers EGFP-1-F (5'-

GACCACTACCAGCAGAACAC-3'), EGFP-10-R (5'-CTTGTACAGCTCGTCCATGC-3') and probe EGFP-HEX (5'-AGCACCCAGTCCGCCCTGAGCA-BHQ-1-3'). The cycling conditions were 10 min at 95°C for initial denaturation, followed by 45 cycles of denaturation for 3 sec at 94°C and 30 sec at 60°C with an automatically calculated cycle threshold value. A cycle threshold (Ct value) of ≤ 38.00 was considered as positive. A Ct value of > 38.00 was interpreted as questionable and real-time PCR was repeated.

3.2.2 Chlamydial species identification by ArrayTube Microarray

All positive and questionable samples by real-time PCR for *Chlamydiaceae* were further investigated by ArrayTube Microarray (AT) (Alere™, Jena, Germany), as described previously (Borel et al. 2008).

3.2.3 16S rRNA PCR and sequencing

Samples positive by real-time PCR for *Chlamydiaceae* but negative by AT were investigated by a PCR method targeting the 16S rRNA gene (Everett et al. 1999, modified). DNA sequencing was performed as described previously (Borel et al., 2006). The obtained 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>).

3.2.4 Serology for C. abortus

The serum samples were tested with two commercially available antibody-detecting ELISA assays, both specific for *C. abortus*:

(i) Pourquier[®] ELISA *Chlamydophila Abortus* (Institute Pourquier, Montpellier, France): The test is based on the recombinant antigen POMP (Recombinant Polymorphic Outer Membrane Protein, 80-90 kDa) and was performed according to the manufacturer`s instructions. For our roe deer and red deer sera, we used the validation values for sheep and goats. The final values were determined as ratio between the corrected optical density (OD) 450 of the sample (S) and the mean corrected OD of positive control (P), expressed as S/P%. Sera with S/P%-values equal to or lower than 50% were interpreted as negative. A S/P% value between 50% and 60% was considered as doubtful and a S/P% value higher or equal than 60% was classified as positive for antibodies against *C. abortus*.

(ii) ID Screen[®] *Chlamydia abortus* ELISA (ID Vet Innovative Diagnostics, Montpellier, France): The test is MOMP-based (Major Outer Membrane Protein) and was performed according to the manufacturer`s instructions. Sera with S/P%-values equal to or lower than 50% were interpreted as negative, sera with an S/P% between 50% and 60% were considered as doubtful and sera with an S/P% greater or equal to 60% were classified positive for antibodies against *C. abortus*

3.3 *Parachlamydia*

3.3.1 Real-time PCR assay for *Parachlamydia* sp.

The possible presence of *Parachlamydia* sp. in eye swabs (n=306), fecal samples (n=60) and organ samples (n=204) was examined by a recently described real-time PCR assay (Casson et al. 2008a) with a few modifications. The real-time PCR amplifies a 103-bp product of the 16S rRNA gene specific for *Parachlamydia* sp.. 0.1

µM of probe PacS (5'-teta-chloro-6-carboxyfluorescein-TTCCACATGTAGCGGTGAAATGCGTAGATATG-BHQ-1-3'), and 0.2 µM of each primer PacF (5'-CTCAACTCCAGAACAGCATTT-3') and PacR (5'-CTCAGCGTCAGGAATAAGC-3') were mixed with iTaq Supermix (Bio-Rad, Rheinach, Switzerland) and 5 µl of DNA sample. The samples were examined on an ABI 7500 instrument (Applied Biosystems). The cycling conditions were 3 min at 95°C for initial denaturation, followed by 45 cycles of denaturation for 15 s at 95°C and 1 min at 60°C. All samples were tested at least in duplicate. A cycle Ct value of ≤ 38.00 was considered as positive. A Ct value of > 38.00 was interpreted as questionable and the real-time PCR was repeated.

3.4 Immunohistochemistry for *Chlamydiaceae* and *Parachlamydia* sp.

Formalin-fixed and paraffin-embedded tissue samples from real-time PCR positive or questionable organs (liver, lung, and kidney) were investigated by immunohistochemistry (IHC) for (i) *Chlamydiaceae* and (ii) *Parachlamydia* sp.. For both reactions, a detection kit (Dako ChemMate, Dako, Glostrup, Denmark) was used for detection according to the manufacturer's instructions. The paraffin-embedded tissue slides were deparaffinated in xylene for 15 minutes and then rehydrated by graded ethanol through water followed by PBS-Tween.

(i) To detect *Chlamydiaceae* immunohistochemically, a mouse monoclonal antibody directed against the chlamydial lipopolysaccharide (LPS; Clone ACI-P, Progen, Heidelberg, Germany) was applied. The sections were pretreated for 10 minutes with Proteinase K (Dako) and then incubated with the primary antibody diluted 1:200 in antibody diluent (Dako) for one hour at room temperature (RT). A

blocking solution was used for 5 minutes at RT to inhibit the endogenous peroxidase. After that, the slides were incubated each for 10 minutes at RT with the link-antibody (secondary antibody) followed by HRP-conjugated streptavidin and developed in 3-amino, 9-ethyl-carbazole (AEC) substrate solution (Dako) for 10 minutes. Finally, hematoxylin was used for counterstaining. A negative control of each section was included using only the antibody diluent instead of the primary antibody. For positive control, there was intestinal tissue of gnotobiotic piglets available (experimentally infected with porcine *Chlamydia suis*, Guscetti et al. 2009).

(ii) To detect *Parachlamydia sp.* immunohistochemically, a polyclonal mouse antibody was used as described previously (Casson et al., 2007, Ruhl et al., 2009). The sections were pretreated for 20 minutes in citrate buffer for antigen retrieval (pH 6.0, 98°C). A blocking solution was added for 5 minutes at RT to inhibit the endogenous peroxidase. The slides were incubated with the primary antibody diluted 1:1000 in antibody diluent (Dako) for one hour at RT. Then the slides were incubated each for 10 minutes at RT with the link-antibody followed by HRP-conjugated streptavidin and developed in 3-amino, 9-ethyl-carbazole (AEC) substrate solution (Dako) for ten minutes. Finally hematoxylin was used for counterstaining. A negative control of each section was included using only the antibody diluent instead of the primary antibody. Cell pellets of experimentally infected amoeba with *P. acanthamoebae* were used as positive control (Borel et al., 2009).

4. Results

4.1 Chlamydia

Details of cases positive for *Chlamydiaceae* are shown in Table 1a (n=4) and for positive and questionable cases in *C. abortus* specific serology in Table 1b (n=4).

4.1.1 Real-time PCR assay for *Chlamydiaceae*

In summary, five out of 717 investigated samples (0.7%) were positive for *Chlamydiaceae* by real-time PCR. Positive samples were found in EDTA blood of roe deer (n=1) and in fecal samples of roe deer (n=2) and red deer (n=2). The positive EDTA blood and one positive fecal sample were from the same animal (roe deer no. 1). Thus four out of 163 investigated red and roe deer were at least positive in one sample (2.5%). All other samples of EDTA blood (n=146), eye swabs from both eyes (n=306), organ samples (n=204) and fecal samples (n=56) were tested negative.

4.1.2 Species identification by Array Tube (AT) Microarray

The five samples positive for *Chlamydiaceae* by real-time PCR were investigated by AT Microarray, but the chlamydial species identification was not possible.

4.1.3 16S rRNA PCR and sequencing

The sequencing of one fecal sample (red deer no. 3) revealed 100% homology to *C. pecorum*. The 16S rRNA product of EDTA blood (n=1) and feces (n=2) (roe deer no. 1 and 2) were 100% identical to each other, but did not align completely to any known sequence on BLAST GenBank. The highest homology was 94% to *C. muridarum* followed by 93% to *C. trachomatis* and *C. suis*, respectively.

The sequence of one fecal sample (red deer no. 4) could not be analyzed due to poor DNA quality.

4.1.4 Serology for *C. abortus*

Two out of 146 tested sera were positive (1.4%) and another two sera were interpreted as questionable by the ID Screen[®] ELISA. None was positive by the Pourquier[®] ELISA.

4.2 *Parachlamydia*

Details of cases positive for *Parachlamydia sp.* are shown in Table 2a (red deer, n=25) and in Table 2b (roe deer, n=23).

4.2.1 Real-time PCR assay for *Parachlamydia sp.*

570 samples including eye swabs from both eyes (n=306), fecal samples (n=60) and organ samples (n=204) out of 163 red and roe deer were investigated by real-time PCR for *Parachlamydia sp.* Therefrom, 68 samples originating from 48 red and roe deer (red deer, n=25; roe deer, n=23) were positive. In total, 49 eye swabs, 15 fecal samples and four organ samples (liver, n=1; lung, n=3) had a positive ct value in duplicate. 19 red and roe deer (red deer, n=9; roe deer, n=10) were concurrently positive at least in two different samples. Thus out of 163 investigated red and roe deer 48 were positive resulting in a prevalence of 30%. Of PCR-positive organ samples originating from fresh-frozen liver (n=1) and lung (n=3), the corresponding formalin-fixed and paraffin-embedded tissue were further investigated by IHC for *Parachlamydia sp.* All other samples of eye swabs from both eyes (n=257), fecal samples (n=45) and organ samples (n=200) were tested negative.

4.2.2 Immunohistochemistry for *Parachlamydia sp.*

IHC for *Parachlamydia sp.* was performed on four organ samples (liver, n=1; lung,

n=3) of three red deer. Thereof two lungs (red deer no. 9 and 10) were positive by IHC, whereas the liver and the third lung (red deer no. 8) were negative. Granular positive IHC labeling in the lung was recognized intracytoplasmic in bronchial epithelial cells (Figure 1, red deer no. 10), alveolar macrophages (Figure 2, red deer no. 9) and pneumocytes type II.

5. Discussion

The aim of the study was to elucidate the prevalence of *Parachlamydia* and *Chlamydia* in hunted red and roe deer and their possible role as a reservoir or transmission host for domestic ruminants and humans. Therefore, samples were collected from red deer (n=99) and roe deer (n=64) during hunting season in autumn 2008.

5.1 *Chlamydia*

Four out of 163 (2.5%) investigated red and roe deer were positive for *Chlamydiaceae* by real-time PCR. Fecal samples (n=4) had most frequently positive results, followed by EDTA blood (n=1). One fecal sample revealed clearly *C. pecorum* while the other three and the blood sample were classified within the family of *Chlamydiaceae*, most closely related to *C. muridarum*. The low prevalence for *Chlamydiaceae* (2.5%) is in concordance with previous investigations on other wild ruminant species in the same mountainous area in Switzerland, where seven out of 412 Alpine ibex (1.7%) and three out of 79 Alpine chamois (3.8%) were positive for *Chlamydiaceae* (Holzwarth et al., 2010, 2011). In both previous studies, *C. pecorum*, *C. pneumoniae* and mixed infections of *C. abortus* and *C. pecorum* were detected.

Interestingly, they were exclusively found in eye swabs, whereas in the present study, all eye swabs of deer were negative. In contrast to ibex and chamois, *C. abortus* DNA was not found in any deer sample. The finding of *C. pecorum* in feces of a red deer was not a particular finding, as it is known that domestic ruminants can harbor *C. pecorum* in their intestine (Longbottom and Coulter, 2003), possibly contaminating pastures and being an infection source for wild ruminants. Feces and blood samples of two roe deer showed exactly the same 16S rRNA sequences most closely related (94%) to *C. muridarum*. These roe deer were sampled in different valleys, on different days by different hunters, implying the occurrence of a possible yet unclassified chlamydial species in roe deer at two different locations in Switzerland. An attempt to cultivate this new species in embryonated chicken eggs was not successful (data not shown) and needs further investigation. The clinical impact of such non-classified *Chlamydiaceae* species is not yet known. Similar results have been obtained in samples from wild and domestic birds in different European countries (Laroucau et al., 2009, Zweifel et al., 2009, Christerson et al., 2010).

A very low seroprevalence for *C. abortus* (0.7% in the ID Screen[®] ELISA and 0% in the Pourquier[®] ELISA) was found in the investigated red and roe deer. This is in concordance to the above-mentioned previous studies in Alpine ibex and chamois, where the same methods revealed prevalences for *Chlamydiaceae* of 1.5% and 1.4%, respectively (Holzwarth et al, 2010, 2011). In contrast, various studies in deer in the US and Europe revealed higher seroprevalences ranging from 6.4% up to 88.4% by complement fixation test (CFT) (Debbie, 1967, Giovannini et al., 1988, Cubero-Pablo et al., 2000). However, the CFT (based on LPS) is known to have an inferior sensitivity and specificity leading to possible cross-reaction to *C. pecorum*

(Sachse et al., 2009). Red and roe deer sera in this study were analyzed by two commercially available ELISA based on the major outer membrane protein (MOMP) and polymorphic outer membrane protein (POMP), respectively. In a recent Spanish study, several wild ungulates were tested by two “in house” blocking ELISA tests, one *Chlamydiaceae* LPS antigen- and the other *C. abortus* POMP antigen-based (Salinas et al., 2009). In contrast to our study, high seroprevalences of 16.7% in red deer, 20% in roe deer and 24.4% in fallow deer were detected by the POMP-based ELISA. Wilson et al. (2009) described the Pourquier[®] ELISA (POMP) as specific for *C. abortus* with low cross-reactions to *C. pecorum* but with a relatively low sensitivity, especially when applied on hemolytic field sera. Vretou et al. (2007) suggested that this is either due to the used recombinant POMP fragments or possibly due to the assigned high cut-off of 60% for small ruminants. In our study, an adaption of the cut-off to 40% would not have changed the results as all sera showed an S/P% less than 20% (data not shown). To conclude, either the investigated deer population in this study is free of chlamydial infections or the Pourquier[®] ELISA is not sensitive enough to detect *C. abortus* in hemolytic field sera. To date, the second applied ID Screen[®] ELISA, based on the MOMP, is not evaluated or compared to other serological test to our knowledge. Other MOMP- based serological tests as the competitive ELISA (cELISA) yielded 98.1% specificity and 77.7% sensitivity, respectively (Vretou et al., 2007). In our study, more sera appeared positive by the ID Screen[®] ELISA than by the Pourquier[®] ELISA, leading to the assumption that the ID Screen[®] ELISA is probably more sensitive than the Pourquier[®] ELISA on field sera.

5.2 *Parachlamydia*

To our knowledge, this is the first description of *Parachlamydia* in a wild ruminant

species. In bovine abortion material, the prevalence for *Parachlamydia* in the same mountainous area in Switzerland was 18.3% by real-time PCR (Ruhl et al., 2009). In contrast, another study of goat and sheep abortion material in the same area revealed a low prevalence of 0.9% when using the same real-time PCR for *Parachlamydia* (Ruhl et al., 2008). The prevalence in red and roe deer in the present study was somehow unexpectedly high with 30% positive animals by real-time PCR. Around one third (19 out of 48 animals) of the deer had a positive result in at least two samples. The most frequently positive samples were eye swabs (n=49), followed by fecal samples (n=15) and few lung (n=3) and liver (n=1) samples. Macroscopic changes in these positive animals were not recorded by the hunters. However, thorough post-mortem examination of the carcass was not possible under field conditions. Thus, the question whether *Parachlamydia* is of pathogenic potential in wild ruminants as suggested in domestic ruminants, or if they are only asymptomatic carriers of *Parachlamydia*, remains yet unresolved. An asymptomatic carrier status of *Parachlamydia* could imply a risk for spreading the bacteria in the environment i.e. as source of infection for domestic ruminants on shared summer pastures in the mountains. Possible fecal excretion of *Parachlamydia* is supported by its detection in fecal samples (n=15) of both, red and roe deer. Infection of wild ruminants with *Parachlamydia* could have been possible by drinking water containing *Parachlamydia* as recently described in a bovine herd in Scotland (Wheelhouse et al., 2011). In this study, 22.5% of the detected 16S rRNA sequences out of the water of cattle drinking troughs were identical to the found parachlamydial 16S rRNA gene out of a placental sample of an abortion from the same farm in Scotland.

We detected *Parachlamydia* positive eye swabs (n=49) in red and roe deer. As no obvious eye lesions were recorded by the hunters, we assume there is no

association between clinical ocular disease and the detection of parachlamydial DNA in the eyes of red and roe deer. Similarly, no clear association between *Chlamydia*-like organisms and ocular disease in sheep was reported by Polkinghorne et al., (2009). However, a possible mode of transmission by eye secretions was assumed by Gerber et al., (2007) where *C. abortus* was found in eye swabs of naturally infected Swiss sheep. Thus the eye excretion is another possible source of transmission between wild and domestic ruminants.

The real-time PCR positive lung (n=3) and liver (n=1) in red deer indicates a systemic infection of *Parachlamydia* in those animals. One red deer (no. 8) was even positive by real-time PCR in both, lung and liver. Positive real-time PCR results were confirmed by IHC in two lungs (red deer no. 9 and 10). The used polyclonal mouse anti-*Parachlamydia* antibody was established, tested and shown to be specific in previous studies (Casson et al., 2008a, Borel et al., 2009). Therewith *Parachlamydia* has been detected in bovine abortion material in Switzerland and Scotland, respectively (Borel et al., 2007, Deuchande et al., 2010), in sheep and goat abortion material in Switzerland (Ruhl et al., 2008) and in mice experimentally infected with *Parachlamydia* (Casson et al., 2008b). In the IHC positive lungs of red deer, the labeling was mainly intracytoplasmic in bronchial epithelial cells, pneumocytes type II and alveolar macrophages. In concordance, earlier in vitro studies showed that *P. acanthamoebae* is able to enter, grow and persist within human pneumocytes, lung fibroblasts (Casson et al., 2006) and enters and multiplies within human macrophages (Greub et al., 2003). Positive IHC results in the lungs of red deer were comparable to those of mice experimentally infected with *Parachlamydia* were the same antibody was used (Casson et al., 2008b).

Histologically, the real-time PCR positive lung samples (red deer no. 8, 9 and 10)

showed in no. 9 and no. 10 a mild multifocal subacute eosinophilic and lymphoplasmacellular peribronchiolitis with a mild to moderate BALM-hyperplasia and in red deer no. 8 and in 10 a moderate hypertrophy of the media of lung arterioles and arteries. In all lungs, a mild to moderate amount of different development stages of lungworm larvae and eggs could be seen (data not shown). This is in contrast to the lesions of purulent and/or interstitial pneumonia in mice experimentally infected with *Parachlamydia* (Casson et al., 2008b). A *Parachlamydia* positive lung of a sheep fetus of a late term abortion showed an interstitial pneumonia (Ruhl et al., 2008) different to the lung lesions in red deer. Therefore the lung histology in our red deer is most likely related to lungworms. Mixed infections with lungworms and *Parachlamydia* were observed in red deer (n=3). Another mixed infection of *Parachlamydia* and *Chlamydiaceae* has been described in a lung with interstitial pneumonia of an aborted sheep fetus (Ruhl et al., 2008). The real-time PCR positive liver (red deer no. 8) showed moderate multifocal chronic lymphoplasmacellular hepatitis with bile duct proliferation (data not shown). These lesions are comparable to those described in an aborted sheep fetus positive for *Parachlamydia* by real-time PCR (Ruhl et al., 2008). However, IHC of both livers were negative.

6. Conclusion

This study reports the first evidence of *Parachlamydia* in wild ruminants by real-time PCR in eye swabs, fecal samples and organs of free-living roe deer and red deer. The very high prevalence of 30% leads to the assumption that deer is a possible wildlife reservoir for *Parachlamydia*. The very low prevalence for *Chlamydiaceae*

(2.5%) of the investigated red and roe deer is in agreement with the few positive serological results (0.7% seroprevalence for *C. abortus* by ID Screen[®] ELISA).

Therefore, we conclude that *Chlamydiaceae* do not play an important role neither in the investigated deer population, nor as a reservoir or transmission host for domestic ruminants in the eastern mountainous part of Switzerland. Further investigations on *Parachlamydia* are needed to elucidate the zoonotic potential, the pathogenicity, the prevalence and distribution in wild ruminants and the mode of transmission from wild ruminants to domestic ruminants and vice versa.

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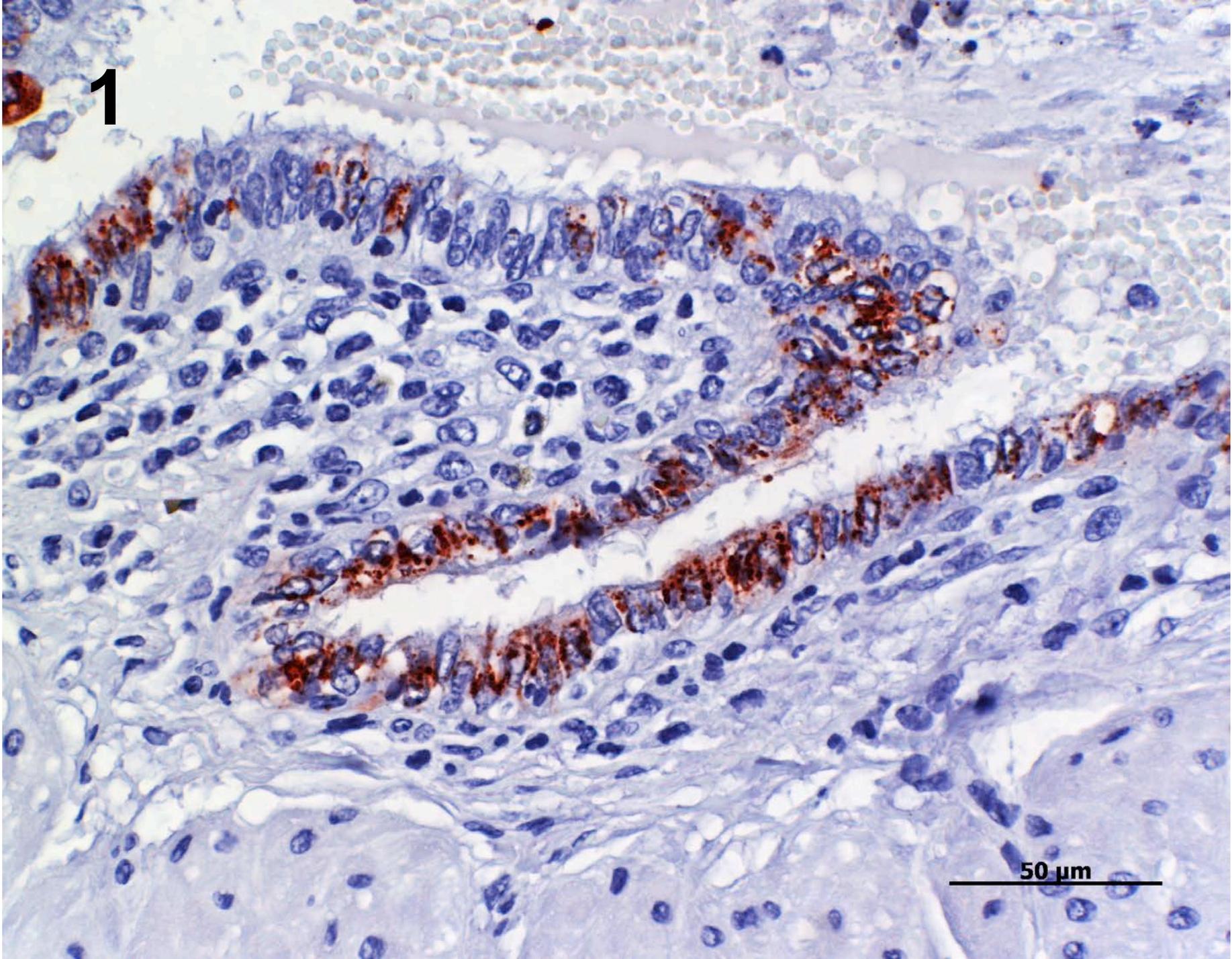
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Figures

Fig. 1. Lung; red deer, case no. 10. Immunohistochemistry with the anti-*Parachlamydia* antibody. Presence of positive granular reaction intracytoplasmic in bronchiolar epithelium cells. AEC/oxidase method, hematoxylin counterstain.

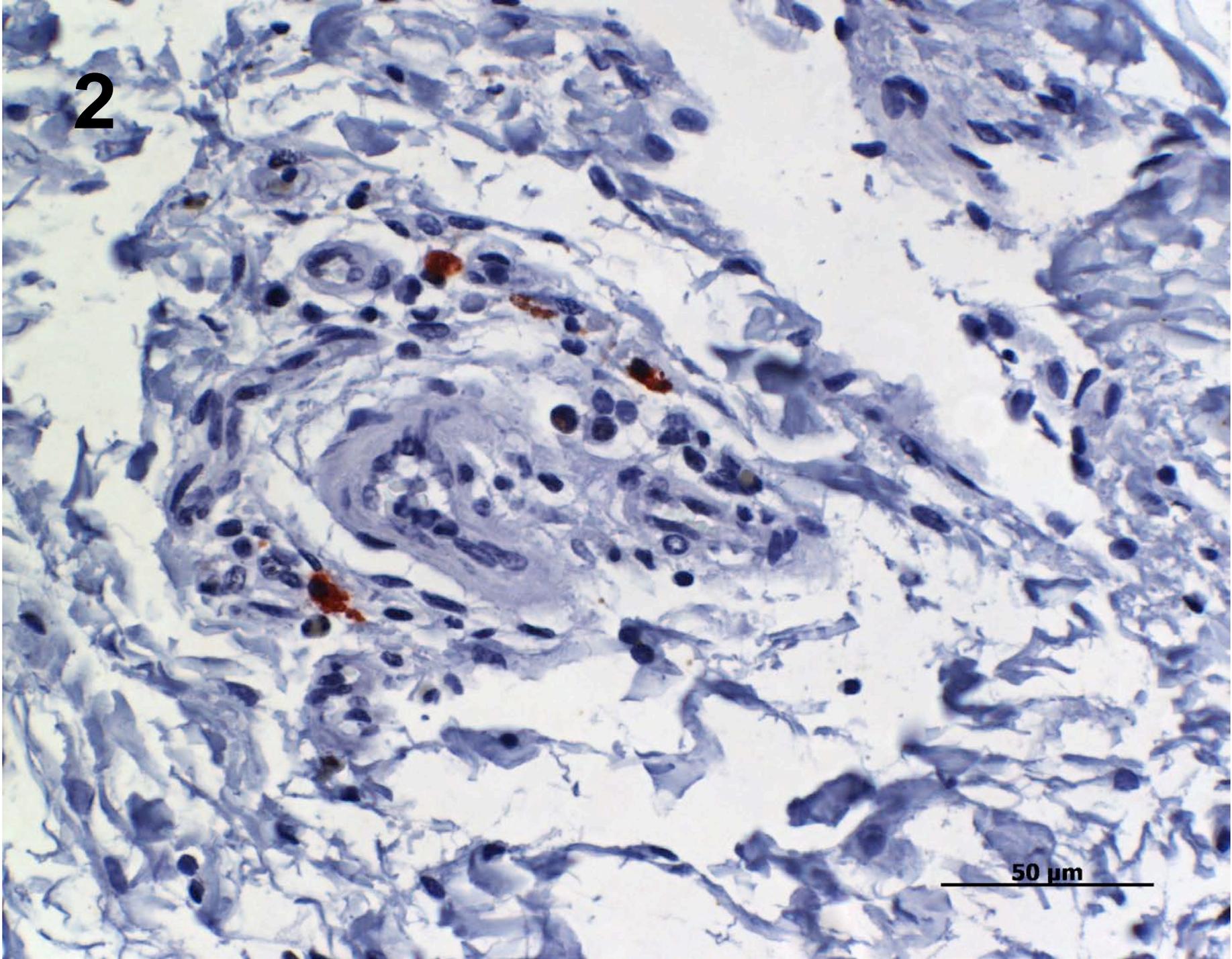
Fig. 2. Lung; red deer, case no. 9. Immunohistochemistry with the anti-*Parachlamydia* antibody. Presence of positive granular reaction intracytoplasmic in alveolar macrophages. AEC/oxidase method, hematoxylin counterstain.

1



50 μ m

2



50 μ m

Table 1a. Details of cases positive for *Chlamydiaceae* by real-time PCR and further analysis (n=4)

Case No.	Species	Material	Real-time PCR for <i>Chlamydiaceae</i> (ø ct value)	ArrayTube Microarray	16S rRNA PCR	Homology (%)
1	Roe deer	EDTA blood	pos (32.1)	neg	<i>C. muridarum</i>	94
		feces	pos (29.3)	neg	<i>C. muridarum</i>	94
2	Roe deer	feces	pos (26.9)	neg	<i>C. muridarum</i>	94
3	Red deer	feces	pos (33.3)	neg	<i>C. pecorum</i>	100
4	Red deer	feces	pos (34.8)	neg	neg	-

pos positive

neg negative

Table 1b. Details of cases positive or questionable in *C. abortus* specific serology (n=4)

Case No.	Species	ELISA ID Screen (% Value)	ELISA Pourquier (% Value)
2	Roe deer	pos (281)	neg
5	Roe deer	pos (155)	neg
6	Roe deer	quest (56)	neg
7	Roe deer	quest (55)	neg

pos positive

neg negative

quest questionable

Table 2a. Details of red deer (*Cervus elaphus*) positive samples for *Parachlamydia* sp. by real-time PCR (n=25)

Case No.	Material	Real-time PCR	IHC
		<i>Parachlamydia</i> sp. (\emptyset ct value)	<i>Parachlamydia</i> sp.
8	liver	pos (37.1)	neg
	lung	pos (34.5)	neg
9	lung	pos (37.5)	pos
10	lung	pos (37.7)	pos
	eye swab	pos (37.0)	nd
11	eye swab	pos (35.4)	nd
12	eye swab	pos (36.6)	nd
13	eye swab	pos (36.7)	nd
14	eye swab	pos (37.2)	nd
15	eye swab	pos (37.2)	nd
16	eye swab	pos (37.2)	nd
17	eye swab	pos (37.4)	nd
18	eye swab	pos (37.4)	nd
19	eye swab	pos (37.6)	nd
20	eye swab	pos (37.7)	nd
21	eye swab	pos (37.8)	nd
22	feces	pos (35.3)	nd
23	feces	pos (36.3)	nd
24	feces	pos (36.7)	nd
25	feces	pos (37.2)	nd

26	eye swab	pos (34.8)	nd
	feces	pos (37.9)	nd
27	eye swab	pos (37.6)	nd
	feces	pos (36.1)	nd
28	eye swabs ¹	pos (23.5, 36.5)	nd
29	eye swabs ¹	pos (34.5, 36.5)	nd
30	eye swabs ¹	pos (35.5, 37.4)	nd
31	eye swabs ¹	pos (36.7, 36.2)	nd
32	eye swabs ¹	pos (36.4, 36.8)	nd

¹ both eyes positive

pos positive

neg negative

nd not done

Table 2b. Details of roe deer (*Capreolus capreolus*) positive samples for *Parachlamydia sp.* by real-time PCR (n=23)

Case No.	Material	Real-time PCR
		<i>Parachlamydia sp.</i> (ø ct value)
33	feces	pos (35.8)
34	feces	pos (37.1)
35	feces	pos (37.5)
36	feces	pos (37.9)
37	eye swab	pos (35.3)
38	eye swab	pos (36.2)
39	eye swab	pos (36.6)
40	eye swab	pos (37.1)
41	eye swab	pos (37.3)
42	eye swab	pos (37.4)
43	eye swab	pos (37.5)
44	eye swab	pos (37.8)
45	eye swab	pos (37.9)
46	eye swabs ¹	pos (36.1, 36.3)
47	eye swabs ¹	pos (36.2, 36.9)
48	eye swabs ¹	pos (37.3, 37.6)
49	eye swabs ¹	pos (37.4, 37.6)
50	eye swabs ¹	pos (37.6, 38.0)
51	eye swabs ¹	pos (35.8, 36.2)
	feces	pos (37.6)

52	eye swab	pos (36.0)
	feces	pos (36.6)
53	eye swab	pos (37.6)
	feces	pos (36.4)
54	eye swab	pos (36.9)
	feces	pos (38.0)
55	eye swab	pos (37.8)
	feces	pos (37.7)

¹ both eyes positive

pos positive

neg negative