

Screening for Chlamydiaceae in Eurasian lynx (*Lynx lynx*) reveals a case of ocular infection

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Abstract

Bacteria from the family *Chlamydiaceae* can cause asymptomatic, persistent infections in humans and animals, but they may also lead to clinical syndromes of varying severity. The actual impact of these infections on the overall health of wildlife populations remains unclear. In the context of conservation translocations, identifying asymptomatic carriers may be particularly important to prevent pathogen introduction into vulnerable or genetically bottlenecked populations. The aim of this study was to investigate ocular *Chlamydiaceae* infection in free-ranging Eurasian lynx (*Lynx lynx*). Ocular swabs were collected from 34 free-ranging Eurasian lynx originating from Romania (n = 21), Slovakia (n = 7), and Croatia (n = 6), representing both the Carpathian and Dinaric populations. *Chlamydiaceae* DNA was detected using quantitative PCR targeting the 23S rRNA gene, while species identification was performed by amplification and sequencing of the *pmp9* gene. *Chlamydiaceae* DNA was detected in one sample (2.9%), and sequence analysis confirmed the presence of *C. felis*. The positive sample originated from a clinically healthy adult male lynx captured in Romania for conservation translocation. Mild unilateral blepharospasm and seromucous discharge that developed four days after capture were misinterpreted as a consequence of handling and were not treated. Screening for other major feline pathogens yielded negative results. This is the second documented case of *C. felis*-associated ocular infection in Eurasian lynx. Although the observed prevalence was low, the findings emphasize the value of pathogen surveillance during conservation translocations and support the inclusion of testing for *Chlamydia* spp. in health-screening protocols for Eurasian lynx.

Introduction

The order Chlamydiales (phylum Chlamydiae, class Chlamydiia) comprises Gram-negative bacteria with a two-phase obligate intracellular life cycle. These organisms depend on a eukaryotic host cell for replication, which occurs within an inclusion inside the host cell, and for dispersal following host cell lysis. The host range of bacteria within the order Chlamydiales includes over 400 animal species, predominantly wildlife (amphibians, reptiles, birds, mammals, and marsupials) (Burnard et al. 2016; Burnard and Polkinghorne 2016). To date, the order comprises nine families, of which *Chlamydiaceae* is the most important pathogenic family within the phylum. This family includes two genera - *Chlamydia* (with 18 species, four of which have Candidatus rank) and *Chlamydiifrater*, which comprises two species (Borel and Sachse 2023).

Chlamydia species, which show varying degrees of host specificity, cause a wide range of clinical symptoms in humans and animals, affecting ocular, pulmonary, genital, articular, and intestinal tissues. The most commonly detected species in wildlife include several traditional veterinary pathogens, *C. pecorum*, *C. abortus* and *C. psittaci*, as well as the human respiratory pathogen, *C. pneumoniae*. To date, most scientific attention has focused on *C. psittaci*, with wild birds regarded as important reservoirs (Krawiec et al. 2015). However, there are indications that current research has only begun to uncover the true diversity of chlamydial species (Taylor-Brown et al. 2015; Marti and Jelocnik 2022). Besides taxonomy, an important area for further investigation is whether wildlife acts as a reservoir and what

risks exist for zoonotic and/or cross-species transmission (Hoffmann et al. 2015). Additionally, the actual impact of these infections on the overall health of wildlife populations, particularly those that are threatened or endangered, remains unclear (Burnard and Polkinghorne 2016; Marti and Jelocnik 2022).

One of the taxonomic groups of wild animals for which data on the presence of *Chlamydia* are scarce is the European wild Felidae, including the European wildcat (*Felis sylvestris*), the Iberian lynx (*Lynx pardinus*), and the Eurasian lynx (*Lynx lynx*). Studies on the European wildcat in Spain detected antibodies against chlamydia (Millán and Rodríguez 2009) and the presence of *C. felis* DNA in mucosal swabs (Candela et al. 2019). In Scotland, a study of freeliving cats (domestic cats and hybrids between domestic and wildcats) detected *C. felis* DNA in 2.61% of examined mucosal swabs (Alves et al. 2023). A serological study of 26 Iberian lynx individuals in Spain detected antibodies in two animals, indicating exposure without confirmation of clinical disease (Millán et al. 2009). Similarly, a serological study of five Eurasian lynx from Russia yielded negative results (Naidenko et al. 2019). The only evidence of a clinical chlamydiosis in European wild Felidae originates from Switzerland, where an adult female Eurasian lynx showed signs of conjunctivitis (Marti et al. 2019). As this individual was part of a reintroduction programme and given the increasing number of lynx reintroduction projects conducted in recent years and planned for the future (Mueller et al. 2022; Krofel et al. 2025), this case raised the question of the potential role of *Chlamydia* spp. in lynx health management and conservation. Conservation translocations involve movement of animals between populations; therefore, pathogen screening represents an important component of risk mitigation. Identification of asymptomatic carriers may be particularly important in preventing pathogen introduction into vulnerable or genetically bottlenecked lynx populations (Hartley and Sainsbury 2017).

DNA amplification is considered the state-of-the-art method for diagnosing chlamydial infections in animals and humans due to its sensitivity and specificity (Borel et al. 2018). However, sampling presents a major challenge for Eurasian lynx health surveillance, as is the case for many wildlife species. Eurasian lynx occupy large home ranges, most European populations occur at low densities, are threatened by multiple factors, and are legally protected (Breitenmoser and Breitenmoser-Würsten 2024). These factors significantly hinder the capture of live animals and the detection of fresh carcasses (Sindičić et al. 2016). Consequently, limited data are available in the scientific literature on the prevalence of infectious and non-infectious diseases in Eurasian lynx, as well as on causes of mortality (Behnke and Walzer 2019). The aim of this study was to investigate ocular *Chlamydiaceae* infection in free-ranging Eurasian lynx (*Lynx lynx*). Samples were collected from three countries (Romania, Slovakia, and Croatia) and two distinct populations (autochthonous Carpathian and reintroduced Dinaric population), providing valuable insight into both clinical signs and the prevalence of ocular chlamydiosis in Eurasian lynx.

Materials and methods

The study was conducted on 34 ocular swab samples collected from Eurasian lynx in Romania (n = 21), Slovakia (n = 7), and Croatia (n = 6). All sampled animals were adults, except for three yearlings; in total, 28 males and six females were sampled. Samples were collected between 2018 and 2025 from both live

animals and fresh carcasses. In Slovakia and Romania, lynx were captured for translocation to Croatia and Slovenia (LIFE Lynx project “Saving the Dinaric and South-Eastern Alpine Lynx Population from Extinction”, LIFE16 NAT/SI/000634) and to Germany (project “Lynx Thuringia – Connecting Lynx Populations across Europe”). In Croatia, samples were collected from three live animals captured to monitor the success of reintroduction within the LIFE Lynx project, and from three fresh carcasses of lynx found dead in the wild. All procedures involving animals were performed by authorized veterinarians and experienced wildlife professionals in accordance with national legislation and permits issued by the competent authorities.

Lynx were captured using cage traps and subsequently chemically immobilized by intramuscular injection of medetomidine (Domitor, 1 mg/mL, Orion-Corporation, Espoo, Finland) and 4.9 mg/kg ketamine hydrochloride (Ketasol, 100 mg/mL, Graeb AG, Bern, Switzerland); in some cases, atipamezole (Antisedan, 5 mg/mL, Orion-Corporation, Espoo, Finland) was administered as a reversal agent. All animals underwent a general clinical examination, including assessment of body condition, rectal temperature, and heart and respiratory rate. Oxygen saturation was measured when equipment was available. Body weight and morphometric measurements were recorded, age estimation was performed according to Marti and Ryser-Degiorgis (2018), and blood and fecal samples were collected. Conjunctival, oropharyngeal, and rectal swabs were collected using sterile cotton swabs and placed in sterile tubes. Samples were kept cooled in portable refrigerators until transported to the laboratory and stored at – 20°C until further processing. As part of the health screening protocol implemented within the translocation project, PCR assays were performed on all captured animals to detect feline immunodeficiency virus (FIV), feline leukaemia virus (FeLV), canine parvovirus type 2 (CPV-2), feline panleukopenia virus (FPLV), feline herpesvirus type 1 (FHV-1), feline coronavirus (FCoV) and *Mycoplasma felis* (Sindičić, unpublished data).

For the identification of the family *Chlamydiaceae* DNA was extracted from ocular swabs using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. Extracted DNA was stored at – 20°C. Amplification of the 23S rRNA gene was performed using quantitative real-time polymerase chain reaction (qPCR) and primers (F: 5'-CTGAAACCAGTAGCTTATAAGCGGT-3'; R: 5'-ACCTCGCCGTTTAACTTAACTCC-3') (Ehrlich et al. 2006). Each reaction mixture contained 10 µl of 2× Universal Mastermix (Promega, Madison, WI, USA), 0.5 µl of each primer (25 µM), 2 µl of probe (1 µM), 1.8 µl of nuclease-free water, 0.2 µl of ROX dye, and 5 µl of DNA, giving a total reaction volume of 20 µl. Each sample, as well as positive and negative controls, was analyzed in duplicate. Samples were analyzed using an Mx3005P instrument (Stratagene, USA) under the following cycling conditions: initial activation at 95°C for 15 s, followed by 50 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 60 s. Ct values ≤ 38 were considered positive for amplification of *Chlamydiaceae*, and positive samples were further analyzed for species identification.

Identification of *C. felis* was carried out by amplification of the *pmp9* gene using conventional PCR and primers (F: 5'-GCGATTCATGTAGCAGCAAA-3'; R: 5'-GTCCAGCTTCCTTGATACCC-3') (Harley et al. 2007). A reaction mixture containing 25 µl GoTaq Green Master Mix (Promega, Madison, WI, USA), 2 µl of each

primer (10 µM), 16 µl of nuclease-free water, and 5 µl of DNA was prepared. A 2720 Thermal Cycler (Applied Biosystems, USA) was used according to the protocol described by Harley et al. (2007) - initial activation at 95°C for 15 s, followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s, and elongation at 72°C for 60 s, with a final extension step at 72°C for 15 min. For visualization of the obtained PCR products, a 1.5% agarose gel (Sigma-Aldrich Co., St. Louis, MO, USA) stained with Midori Green Advance (Nippon Genetics Europe GmbH, Düren, Germany) was prepared. The size of the PCR amplicons of the *pmp9* gene was determined using a BenchTop 100 bp DNA ladder (Promega, Madison, WI, USA). Electrophoresis was performed at 70 V for 55 min, and the expected product size was 632 bp. Isolation and purification of PCR products from the agarose gel were performed using the ReliaPrep DNA Clean-Up and Concentration System (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA concentration and purity were assessed using a BioDrop spectrophotometer (BioDrop Ltd., Cambridge, United Kingdom), after which samples were sequenced by the Sanger method at Macrogen Inc. (Amsterdam, The Netherlands).

Raw sequences were edited and used to create a consensus sequence through BioEdit Sequence Alignment Editor version 7.0.5.3 (Hall 1999). Subsequently, the online Basic Local Alignment Search Tool (BLAST) was used to search and analyze nucleotide sequences deposited in the GenBank database of the National Center for Biotechnology Information (NCBI) (Altschul et al. 1990).

Serum from the animal that tested positive for *C. felis* was further tested for antibodies against FHV-1 (service provided by Laboklin GMBH & CO.KG, Germany).

Results

No clinical signs of disease or symptoms of ocular infection were observed in the examined animals. Using qPCR targeting the 23S rRNA gene, *Chlamydiaceae* DNA was detected in one of the 34 analyzed (2.9%) ocular swab samples (Ct value 34.57). Subsequent amplification of the *pmp9* gene by conventional PCR and sequence analysis confirmed that the obtained sequence belonged to *Chlamydia felis*. The *pmp9* gene sequence showed the highest similarity (99.6%) and 100% identity with sequences deposited in GenBank under accession numbers AP006861.1 (Azuma et al. 2006) and DQ652581.1 (Harley et al. 2007), which were isolated from domestic cats in Japan and the United Kingdom.

The positive sample originated from a 2-year-old male Eurasian lynx (named RO012 Kras), captured in Romania on 19 January 2023. At the time of capture, the animal weighed 23 kg and no symptoms of disease were detected by veterinary examination. The lynx tested negative for FIV, FeLV, CPV-2, FPLV, FHV-1, FCoV and respiratory *Mycoplasma felis*. Haematology and blood chemistry did not reveal abnormalities compared to apparently healthy, free-ranging lynx from Dinaric and Carpathian populations (Belić et al., unpublished). Following capture, the lynx was transported to a quarantine facility. Four days later (23 January 2023), partial eyelid closure and increased ocular moisture in the left eye were observed, without conjunctival or corneal redness (Fig. 1). As the symptoms were mild and did not progress, the quarantine staff interpreted them as eye irritation due to manipulation during capture, and

no further tests or treatment were performed. On 20 March 2023 the animal was chemically immobilized for a pre-transport clinical examination and for fitting a telemetry collar to monitor activity and movement. Good overall health was confirmed; thus, the animal was transported to Croatia and released into Plitvice Lakes National Park on 24 March 2023. After release, the lynx established a home range in the Gorski Kotar region, where it was monitored using a telemetry collar and camera traps. The last photographic record of Kras was obtained in June 2024, while the final GPS telemetry collar position was recorded on 16 January 2025, confirming that the animal survived at least 22 months after translocation to Croatia (Hočevár et al. 2024).

After PCR analysis detected *C. felis* in the eye swab sample, we inspected the photographs taken before capture (Fig. 1.A), at capture (Fig. 1B) and in the quarantine enclosure (Fig. 1C). Changes visible in the photo from the quarantine (Fig. 1C) indicate blepharospasm and seromucous discharge from the left eye. The serum sample tested negative for antibodies against FHV-1.

Discussion

Bacteria from the family *Chlamydiaceae* can cause asymptomatic, persistent infections, but they may also lead to clinical syndromes of varying severity, suggesting that strain and/or host-related factors play a major role in disease outcome (Speck et al. 2012; Jelocnik et al. 2014). Members of *Chlamydiaceae* have been associated with ocular disease in wild animals; however, knowledge about the frequency and diversity of chlamydial ocular infections in wildlife remains limited with available data mostly originating from studies on ungulates. *Chlamydia* spp. have been linked to infectious keratoconjunctivitis (IKC) in several ungulate species, including bighorn sheep (*Ovis canadensis*) (Meagher et al. 1992; Jansen et al. 2016); red deer (*Cervus elaphus*) (Zaragoza et al. 1998); reindeer (*Rangifer tarandus*) (Sánchez Romano et al. 2019;); Alpine chamois (*Rupicapra r. rupicapra*) (Arnal et al. 2013); and Pyrenean chamois (*Rupicapra pyrenaica*) (Dias-Alves et al. 2021). But also, studies in ungulates have detected *C. pecorum* in the eyes of asymptomatic animals (Holzwarth et al. 2011a, b; Dias-Alves et al. 2021), highlighting the importance of active health surveillance in wildlife.

In domestic cats (*Felis catus*), the most common microorganisms involved in conjunctivitis are *C. felis* and FHV-1, often with primary FHV-1 infection and secondary bacterial infection (Ramsey 2000). Clinical differentiation between *C. felis* related conjunctivitis and FHV-1 related conjunctivitis can be problematic (Andrew 2001), therefore, we additionally tested for FHV-1 antibodies, which were negative, confirming the aetiological role of *C. felis*. In domestic cats, *C. felis* has a predilection for conjunctival epithelial cells, causing acute and chronic conjunctivitis, with zoonotic potential (Borel and Sachse 2023). Conjunctivitis usually begins unilaterally but often spreads to the other eye and is characterized by conjunctival chemosis, blepharospasm, ocular discharge, and hyperaemia of the nictitating membrane. Spontaneous recovery is possible, but most untreated cats develop chronic conjunctivitis with ocular signs persisting for 22–45 days. Ocular shedding may last up to 60 days, but intermittent shedding for up to 8 months has also been observed in experimental cats, suggesting an asymptomatic carrier state (Sykes 2005; Borel and Sachse 2023). In our study, the animal that tested positive showed no ocular symptoms at the

time of capture when the swabs were taken and very mild symptoms (blepharospasm and chemosis) developed four days later. The case of conjunctivitis described in the Swiss lynx exhibited more severe clinical signs, including marked hyperaemia, pronounced chemosis, and yellowish, cloudy mucopurulent ocular discharge, without visible damage to ocular structures (Marti et al. 2019). In both cases, the source of infection could not be confirmed; however, infection probably occurred prior to capture, given that the incubation period ranges from three to ten days (Aiello and Moses 2016). Marti et al. (2019) suggested that infection in their case resulted from contact with an infected domestic cat or European wildcat, as two scratches were observed on the muzzle. Chlamydiosis is transmitted through direct contact, with ocular secretions playing a key role (Seki et al. 2016). Direct contact between lynx is rare and typically occurs only between animals of opposite sexes before and during the breeding season (Breitenmoser and Breitenmoser-Würsten 2024). The male in this study was captured shortly before the mating season, so it may have been infected through contact with a female, or possibly during a conflict with another male. However, as lynx prey on live animals, including both wild and domestic cats (Jobin et al. 2000), contact with such species also represents a potential source of infection.

Apart from ovine and avian chlamydiosis, *Chlamydia spp.* are often considered pathogens of little significance and, accordingly, detailed diagnostic investigations are not performed (Borel et al. 2018). The fact that the only two reported cases of ocular chlamydiosis in Eurasian lynx have occurred in animals captured for reintroduction projects highlights the importance of thorough health screening and quarantine procedures for animals involved in such programs. It also underscores the added value of biological samples collected during these projects when they are incorporated into active, targeted surveillance of specific diseases and pathogens. Capture, transport, and quarantine represent substantial physiological stressors and may contribute to recrudescence or clinical manifestation of latent infections. As persistent *C. felis* infection has been described in asymptomatic domestic cats (Wills et al. 1987), stress-associated activation of subclinical chlamydial infection may have contributed to disease expression in the present case.

Although the low apparent prevalence observed in this study should be interpreted cautiously due to the limited sample size, we do not expect a high prevalence or rapid spread of infectious conjunctivitis in Eurasian lynx, as they are solitary animals and direct contact occurs primarily during the mating season. However, in the context of conservation translocations surveillance remains essential because of the risk of introducing new pathogen species or strains into a new habitat, and because the survival of every translocated individual is critical to programme success. Severe conjunctivitis can impair vision and reduce hunting success, thereby compromising the successful adaptation of translocated individuals. In addition, the circumstantial evidence suggesting that *C. felis* may cause abortion (Gruffydd-Jones et al. 2009) should not be overlooked.

We therefore recommend that eye swabs be collected and tested for the presence of chlamydia in Eurasian lynx involved in translocation. A similar recommendation was made in the disease risk analysis for the reintroduction programme of the Amur leopard (*Panthera pardus orientalis*), in which *C. felis* was identified as a high priority hazard when originating from source (Lewis et al. 2020). Routine

pathogen surveillance integrated into conservation translocations can provide valuable baseline epidemiological data while simultaneously reducing the risk of inadvertent pathogen dissemination among fragmented lynx populations.

Declarations

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Data Availability Not applicable.

Ethics approval Animal trapping and handling were conducted in accordance with the European Commission Humane Trapping Standards (Council Regulation (EEC) No 3254/91) and the IUCN *Guidelines for Reintroductions and Other Conservation Translocations* (2013). All activities were carried out under permits and approvals issued by the competent national authorities. No human subjects were involved in this research.

Competing interest The authors declare no competing interests.

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Author contribution Magda Sindičić and Tomislav Gomerčić conceptualized the study; Magda Sindičić, Tomislav Gomerčić, Mihai Pop and Jakub Kubala secured funding; Tomislav Gomerčić, Jakub Kubala, Teodora Sin, Andrea Gazzola, Jakub Kubala, Branislav Tám, Rudol Kropil and Ira Topličanec captured lynx and collected samples; Liča Lozica performed laboratory analysis; Valentina Plichta performed an ophthalmological assessment based on photographs, Magda Sindičić wrote the original draft. All authors reviewed, edited and approved the final manuscript.

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Figures



Figure 1

Photographs of a male Eurasian lynx (R0012 Kras) captured in Romania, which tested positive for ocular *C. felis* a) Camera trap photograph taken on 8 January 2023, 11 days before capture b) Photograph from a box trap at the time of capture on 19 January 2023 c) Photograph taken during quarantine on 24 January 2023 d) Photograph at the release site in Plitvice Lakes National Park on 24 March 2023