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Authors: Mörner, Torsten, Bröjer, Caroline, Ryser-Degiorgis, Marie-Pierre, Gavier-Widén, Dolores, Nilsson, Hans-Olof, et al.

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Detection of Gastric *Helicobacter* Species in Free-ranging Lynx (*Lynx lynx*) and Red Foxes (*Vulpes vulpes*) in Sweden

Torsten Mörner,[1] Caroline Bröjer,[1] Marie-Pierre Ryser-Degiorgis,[1,2] Dolores Gavier-Widén,[1,4] Hans-Olof Nilsson,[3] and Torkel Wadström[3] [1]Department of Wildlife, Fish and Environment, National Veterinary Institute, SE-751 89 Uppsala, Sweden; [2]Centre for Fish and Wildlife Health, Institute of Animal Pathology, Länggassstr. 122, Postfach, Berne, Switzerland; [3]Department of Laboratory Medicine, Division of Medical Microbiology, Lund University, Sölvegatan 23, SE-223 62 Lund, Sweden; [4]Corresponding author (email: dolores@sva.se)

ABSTRACT: Specimens of gastric mucosa and liver of 25 free-ranging Eurasian lynx (*Lynx lynx*), and four red foxes (*Vulpes vulpes*) shot in Sweden during 1999–2000, were investigated for the presence of *Helicobacter* species. Histopathology, bacteriologic culture and urease test, *Helicobacter* genus-specific 16S rDNA PCR analysis, and DNA sequence analysis were applied. Numerous *Helicobacter*-like organisms were observed histologically in the gastric mucosa of one fox. *Helicobacter* spp. were detected in the stomach by PCR analysis in 17 (68%) of the lynx and in three (75%) of the foxes. Seven of the positive lynx were also positive in the urease test. PCR fragments, amplified from lynx and foxes, were sequenced and compared with those of known *Helicobacter* species. PCR products from lynx were closely related (≥98% homology) to *H. heilmannii*, and PCR fragments from foxes demonstrated close homology to *H. heilmannii* and *H. salomonis*. No *Helicobacter* spp. or *Helicobacter*-like organisms could be cultured. The PCR analysis of the liver was negative for all animals. The pathologic significance of the presence of *Helicobacter* spp. in the stomach of free-ranging lynx and foxes remains uncertain.

Key words: Bacteria, gastric mucosa, *Helicobacter*, lynx, PCR, red fox, *Vulpes*.

In 1983, unidentified curved bacilli, later identified as *Helicobacter pylori*, were isolated from the gastric mucosa of humans with chronic active gastritis (Warren and Marshall, 1983). *Helicobacter pylori* is now recognized as the major cause of chronic gastritis in man (Blaser and Atherton, 2004). Other gastric and so-called enteric *Helicobacter* spp. have been identified as pathogens in domestic, laboratory, and zoo animals. More than 20 *Helicobacter* spp. have been described to date (Nilsson et al., 2005). In free-ranging wild animals, *Helicobacter* spp. have been demonstrated in red foxes (*Vulpes vulpes*), grey foxes (*Urocyon cinereoargenteus*), bobcats (*Lynx rufus*), and rodents (Erginsoy et al., 2004; Goto et al., 2004; Hamir et al., 2004). *Helicobacter* infections in some animals may be subclinical, whereas some enterohepatic *Helicobacter* spp. cause severe diseases such as chronic hepatitis, hepatic neoplasia, and cholangiofibrosis in rodents (Whary and Fox, 2004).

Helicobacters are helical, curved, gram-negative, fastidious bacteria which require serum-rich or blood-supplemented media and micro-aerobic culture conditions (Andersen and Wadström, 2001). The difficulties encountered, when attempting to culture *Helicobacter* spp. from nondomestic carnivores, are illustrated by several reports: *Helicobacter*-like organisms are often observed, but not easily isolated (Kinsel et al., 1998). Little information is available on the occurrence of *Helicobacter* and its pathogenicity in wild animals in Sweden. The aim of the present study was to examine the presence of *Helicobacter* spp. in lynx (*Lynx lynx*) and red foxes in Sweden.

The study involved 25 free-ranging lynx and four red foxes shot in several counties in Sweden. The lynx were apparently healthy animals shot in the field and submitted to the National Veterinary Institute (NVI) as part of a health-monitoring program. There were 15 males, nine females, and one for which the sex was not recorded. Their age, estimated by dentition, varied between <1 yr old to approximately 6 yr old. The foxes were shot during regular hunting

and submitted to NVI because of skin lesions caused by *Sarcoptes scabiei*. There were one male, two females, and one for which the sex was not recorded. The location of the animals ranged between 58°21′ to 65°15′N and 12°38′ to 18°49′W. All animals were collected during 1999 and 2000.

A necropsy was performed 24 hr to 72 hr after death. Specimens of stomach (two from each animal, obtained from random areas of the stomach) and liver (one sample from each) were fixed in 10% neutral buffered formalin, routinely paraffin embedded, sectioned at 5 μm, and stained with hematoxylin-eosin and by the Warthin-Starry procedure for visualization of *Helicobacter*-like organisms (HLO).

For culture, 50–100 mg of gastric mucosa and liver were either directly smeared onto, or homogenized and spread on, brucella blood or GAB-Camp agar (Soltesz et al., 1992). Cultures were maintained for 7 days at 37 C in standard culture jars under micro-aerophilic conditions (8% v/v CO_2, 6% v/v O_2, and N_2) using Anaerocult® C gas generating envelopes (Merck, Darmstadt, Germany). From the same specimen, 5–10 mg was analyzed by a rapid urease test (RUT). Samples were placed in tubes containing urea/phenol red agar and incubated for 30 min at 37 C. Samples were regarded as positive when a rapid (10–15 min) color reaction was observed.

For PCR analysis, DNA was extracted using a method of detergent and heat treatment, and ion-exchange purification, as previously described (Nilsson et al., 2000). The upper phase containing the DNA was analyzed with *Helicobacter* genus-specific primers (Fox et al., 1998). The forward (5′-GCTATGACGGGTAT CC-3′), and the reverse primer (5′-GATTTT ACCCCTACACCA-3′) amplify approximately 400 base pairs (bp) of the 16S rRNA gene. The PCR mixture and temperature cycling have been previously described (Nilsson et al., 2000). The PCR was performed in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, California, USA), and amplified products were analyzed in 1.5% agarose gels.

For DNA sequencing, *Helicobacter* genus-specific PCR products were purified from agarose gels by Ultrafree DA centrifuge tubes (Millipore, Billerica, Massachusetts, USA), according to instructions. Sequence analysis was performed with an Applied Biosystems DNA sequencer (ABI 310) (Applied Biosystems) by the protocols of the manufacturer, using their ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit. Both DNA strands were sequenced. Sequence similarity comparisons were carried out using the BLASTn algorithm at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov).

The gastric mucosa from 17 of 25 (68%) lynx, and three of four (75%) red foxes, was positive for *Helicobacter* spp. by PCR analysis. Polymerase chain reaction-positive stomach tissues from seven of the lynx were also urease positive. All liver tissue samples were negative by both PCR and RUT. Neither helicobacter nor HLO were cultured from gastric or liver tissues from any animal.

None of the investigated lynx or foxes showed macroscopic lesions in the stomach or liver. Helical, coiled, corkscrew-like organisms closely resembling *Helicobacter* spp. were observed in one fox in a section of gastric mucosa stained with Warthin-Starry stain. These HLO were present as single organisms or as aggregates on the surface epithelium and in the glandular lumina. A mild diffuse infiltration of lymphocytes and plasma cells was observed in the lamina propria of the gastric mucosa of both positive and negative lynx. No other histologic changes, or the presence of HLO, were observed in any of the animals.

The 400-bp PCR fragments were sequenced and compared with known *Helicobacter* 16S rDNA sequences using the

BLASTn algorithm at NCBI. The PCR products ($n=3$) amplified from foxes showed >98% homology to "*Candidatus* Helicobacter heilmannii" (AF058768) or to *H. salomonis* (HSU89351). Sequenced PCR fragments ($n=2$) from lynx demonstrated 98% similarity to an uncultured *Helicobacter* species detected in captive cheetahs (AY634577) and to "*Candidatus* H. heilmannii" strain C3E (AF506776). Phylogenetic analysis (data not shown) demonstrated that the *Helicobacter* 16S rDNA sequences, from both fox and lynx, grouped into a cluster including "*Candidatus* H. heilmannii," *H. salomonis*, *H. felis*, and *H. bizzozeronii*.

Even though this study involved a small number of animals, the high proportion of lynx and foxes in which *Helicobacter* spp. were detected suggests that gastric colonization with these bacteria probably occurs with high frequency in Sweden. The present study is, to our knowledge, the first report of *Helicobacter* spp. in Eurasian lynx, and also the first PCR identification of these bacteria in free-ranging red foxes in Sweden.

Helicobacter spp. has been associated with gastritis in animals (Whary and Fox, 2004). In the present study, it was not clear if the lymphoid cell infiltration in the mucosa of the stomach in the lynx was more severe in PCR-positive animals or if the inflammatory process was related to helicobacter colonization. Similar observations have been reported in other species. In a North American zoo, Kinsel et al. (1998) demonstrated HLO in bobcat (*Felis rufus*), Canada lynx (*Felis lynx canadensis*), and other felids. Only mild inflammatory reactions were reported in the gastric mucosa of these animals. Erginsoy et al. (2004) identified HLO in the gastric mucosa of red foxes but found no correlation with inflammatory reaction. The histologic examination conducted in the present study had several limitations, such as various degrees of tissue autolysis; small number of samples per animal; and random sampling of different regions of the gastric wall, which did not allow drawing conclusions on the pathologic significance of the findings. Further studies, specially designed to investigate the pathogenic implications of *Helicobacter* spp. colonization, would be needed to clarify its significance.

The PCR analysis is more sensitive for *Helicobacter* spp. detection than many other methods. Norris et al. (1999) demonstrated, by DNA sequence analysis, that a group of nonculturable *Helicobacter* spp. closely related to *H. felis* and "*H. heilmannii*" colonized gastric and intestinal mucosa of domestic cats. The 16S rDNA sequences from the red foxes and lynx included in this study also had close homology to "*Candidatus* H. heilmannii." These results suggest that a group of related *Helicobacter* spp., with 16S rDNA similarity to "*Candidatus* H. heilmannii," colonize the gastrointestinal tract of both wild and domestic cats. "*Helicobacter heilmannii*"-like organisms have proved very difficult to isolate (Whary and Fox, 2004), which might explain the absence of bacterial growth in this study.

The role of domestic and wild felids in the possible spread of gastric and perhaps bile-tolerant intestinal species of helicobacter, such as the novel *H. ganmani* isolated from mouse intestines (Robertson et al., 2001) and detected by PCR in human liver tissue with chronic inflammation (Tolia et al., 2004), should be further explored.

In conclusion, the present study shows that *Helicobacter* spp. occur, with probably high frequency, in free-ranging lynx and red foxes in Sweden. The role of *Helicobacter* spp. as a possible cause of disease, or as a commensal, could not be established.

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