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Citation: Swennes, Alton G., et al., "Helicobacter canis colonization in sheep: a zoonotic link." *Helicobacter* 19, 1 (February 2014): p.65-68 doi:10.1111/hel.12097 ©2014 Author(s)

As Published: 10.1111/HEL.12097

Publisher: Wiley

Persistent URL: <https://hdl.handle.net/1721.1/124798>

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

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Published in final edited form as:

Helicobacter. 2014 February ; 19(1): 65–68. doi:10.1111/hel.12097.

***Helicobacter canis* colonization in sheep: a zoonotic link**

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Abstract

Helicobacter canis has been associated with hepatobiliary and gastrointestinal disease in dogs, cats, and humans. Here we report *H. canis* isolation from sheep feces confirmed by restriction fragment length polymorphism, biochemical profiles, and 16S rRNA sequence analysis. This study identifies sheep as *H. canis* reservoirs potentially important in zoonotic or foodborne transmission.

Keywords

zoonoses; sheep diseases; *Helicobacter* infections

INTRODUCTION

Helicobacter canis was originally isolated from a child with gastroenteritis (1). Its identification in dogs suggested that pets were reservoirs facilitating zoonotic transmission (2). Subsequently, *H. canis* was isolated from a dog with hepatitis (3), a colony of Bengal cats with endemic diarrhea (4), and healthy cats (5). In these cases, *H. canis*' role in hepatic and intestinal disease was given further plausibility by extensive prior experimental enterohepatic *Helicobacter* use in mouse inflammation and neoplasia models (6–8). *H. canis* has since been cultured from bacteremic humans (9–12). It has also been identified in a duodenal biopsy from a Crohn's disease patient (13) and in a liver biopsy from an autoimmune hepatitis patient (14). Most of these reports state that the patient had dog or cat ownership history, and all of these authors hypothesized zoonotic transmission. While previously identified in dogs, cats, and humans, *H. canis* has not been known to naturally infect other species. Here we report *H. canis* isolation from sheep feces, expanding its host range and raising important questions regarding potential avenues for zoonotic or foodborne transmission.

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MATERIALS AND METHODS

Bacterial culture

Fecal samples were collected from 22 sheep in sterile *Brucella* broth containing 20% glycerol. These sheep were from a single open flock of Dorsets, Hampshires, and Dorset-Hampshire crosses used in teaching and research. The cohort's average age was 4 (range of 1 – 10) and consisted of 21 predominantly multiparous ewes and 1 ram. Collection was approved by the Committee on Animal Care of the Massachusetts Institute of Technology. Samples were plated on 5% sheep blood agar (Thermo Fisher Scientific, Lenexa, KS) and CVA (Cefoperazone-Vancomycin-Amphotericin B) agar (BD, Franklin Lakes, NJ), and cultured at 37°C under microaerobic conditions in vented jars containing N₂, H₂, and CO₂ (80:10:10).

Isolate characterization

Helicobacter-positive samples were identified by colony morphology, phase contrast microscopy, Gram-negative staining, and *Helicobacter* genus-specific 16S rRNA PCR (15). Isolate species identity and clonality were confirmed by RFLP and REP-PCR (15,16). Biochemical testing was performed using the Remel RapID NH kit (Thermo Fisher Scientific, Lenexa, KS). DNA was extracted from pure cultures for 16S rRNA sequencing and a neighbor joining phylogenetic tree was constructed based on sequence similarity (17). All isolates were evaluated for HeLa cell cytotoxicity as previously described, with *H. hepaticus* strain 3B1 as a positive control (18).

RESULTS

Fecal culture yielded mixed bacterial populations that made separation of *Helicobacter*-associated colony morphologies technically difficult. Despite this, 4 isolates, namely MIT 12-7708, MIT 12-7709, MIT 12-7728, and MIT 12-7730 were recovered. RFLP showed *H. canis*-typical banding patterns when their 1200 bp genus-specific 16S rRNA PCR products were digested with the AluI and HhaI restriction enzymes, although 2 distinct AluI patterns were observed as previously reported (Figure 1A) (15). *H. canis* strains NCTC 12740 (human-origin) (1), NCTC 12739 (dog-origin) (2), MIT 98-0152 (cat-origin) (4), and MIT 99-7633 (rhesus macaque-origin) were analyzed simultaneously for comparison.

Sheep-origin *H. canis* isolates shared the same banding pattern by REP-PCR, indicating clonality, but were distinct from the control strains tested (Figure 1B). All sheep-origin isolates were catalase, urease, and γ -glutamyl transpeptidase-negative, oxidase-positive, and did not reduce nitrate to nitrite. Strains from other species shared the same biochemical profile, except that non-sheep strains were γ -glutamyl transpeptidase-positive. Because a previously reported *H. canis* strain was shown to produce cytolethal distending toxin, all isolates were evaluated for *in vitro* cytotoxicity. The sheep-origin isolates did not induce cellular changes consistent with cytotoxicity. 16S rRNA sequencing and BLASTn analysis confirmed that the 3 sheep-origin isolates tested shared 99% identity with *H. canis*. A neighbor joining phylogenetic tree was constructed based on sequence similarity (Figure 1C). Sheep-origin *H. canis* isolates clustered with *H. canis* strains from other species, but were distinct from other enterohepatic *Helicobacter* species (EHS) previously isolated from sheep.

DISCUSSION

In addition to *H. canis*, sheep have been shown to harbor EHS, namely *H. bilis* (Flexispira taxon 2) and *H. troglodytes* (Flexispira taxa 4 and 5) (19–21). Two of these sheep-origin

strains were associated with fetal hepatic necrosis and late-term abortion, a phenomenon that was later experimentally reproduced (21,22,23). *H. canis* has not been associated with a specific ovine disease syndrome, though interestingly it has been isolated from a dog's liver with active hepatitis (3). While no definitive connection has been established, the flock studied here has had several mummified and late-term dead fetuses born to ewes delivering multiple lambs. Also, the flock has historic exposure to dogs and cats.

This study identifies sheep as a new and potentially important *H. canis* reservoir host that could promote direct zoonotic transmission or transmission via dogs or cats. Interestingly, a similar dynamic has been proposed to explain the high *H. pylori* prevalence in individuals with direct or indirect sheep or sheep dog exposure. Several prior reports showed 98% *H. pylori* prevalence in Sardinian (24) and Polish (25) shepherds by CagA ELISA and ¹³C urea breath test. Sheep contact also disproportionately increased *H. pylori* prevalence odds in Columbian children when measured by ¹³C urea breath test (26). These prior studies established sheep as a potential *H. pylori* reservoir and have fueled speculation that sheep may be a natural *H. pylori* host species. This report demonstrates that sheep are colonized with *H. canis*, suggesting that they function as reservoirs for the organism. Whether *H. canis* persists in the sheep intestine and is responsible for any disease process requires further study. Sheep may promote zoonotic *H. canis* transmission either directly or via dogs and cats. Foodborne transmission from eating undercooked lamb contaminated by *H. canis* is also a possibility. Interspecies transmission of EHS merits continued study.

Acknowledgments

This work was supported by NIH T32 RR007036, NIH R01 OD011141, NIH P01 CA028842, and NIH P30 ES02109.

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Figure 1.

(A) RFLP and (B) REP-PCR profiles of sheep-origin *Helicobacter canis* isolates MIT 12-7708, MIT 12-7709, MIT 12-7728, and MIT 12-7730 with reference strains NCTC 12740 (human-origin), NCTC 12739 (dog-origin), MIT 98-0152 (cat-origin), and MIT 99-7633 (rhesus macaque-origin) included for comparison. (C) Phylogenetic tree of *H. canis* isolates and other *Helicobacter* species based on 16S rRNA sequence. Scale bar = 5% nucleotide sequence difference. GenBank accession numbers are shown in brackets.