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Presence of *Helicobacter* and *Campylobacter* species in faecal samples from zoo mammals

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Abstract

Helicobacter and *Campylobacter* species (spp.) colonize the gastrointestinal tract of various domesticated animals. Apart from their pathogenic significance in animals, several species are of zoonotic importance as well. For most non-domesticated animal spp., however, little is known on the presence and importance of these agents. Therefore, we investigated the presence of *Helicobacter* and *Campylobacter* spp. in marine and terrestrial zoo mammals.

Faecal samples of various marine and terrestrial zoo mammals were collected from 6 different zoos in Belgium. These samples were tested for the presence of *Helicobacter* and *Campylobacter* spp. by isolation and direct demonstration of DNA using genus-specific PCRs, followed by sequencing of the obtained amplicons.

Helicobacter spp. were detected in 12 and *Campylobacter* spp. in 5 of the 22 faecal samples from marine mammals. In 4 of 5 dolphins, *H. cetorum* was demonstrated, a well-known pathogen associated with gastritis and gastric ulceration in marine mammals. *C. insulaenigrae* was isolated from 4 of 6 sea lions and from 1 of 11 seals. To our knowledge, this is the first description of the presence of *C. insulaenigrae* on the European mainland. *Helicobacter* spp. were detected in 5 and *Campylobacter* spp. (mainly *C. jejuni* subsp. *jejuni* and *C. coli*) in 9 of the 26 faecal samples from terrestrial mammals. Potential novel enterohepatic *Helicobacter* spp. were detected in both marine and terrestrial zoo mammals.

For the first time, the presence of several known and unknown *Campylobacter* and *Helicobacter* spp. was demonstrated in the gastrointestinal tract of various marine and terrestrial zoo mammals. Further investigation is needed on the pathogenic and zoonotic importance of these species.

Keywords: *Helicobacter* sp.; *Campylobacter* sp.; zoo mammals; prevalence; 16S rRNA

Introduction

Helicobacter (*H.*) and *Campylobacter* (*C.*) species (spp.) are Gram-negative, microaerophilic micro-organisms with a typical spiral- or comma-shaped morphology. These agents colonize the gastrointestinal tract of many domestic animal spp. and have been associated with the development of gastrointestinal and hepatic diseases in their animal hosts. In addition, some of these agents are of zoonotic importance (Haesebrouck et al., 2009; Fitzgerald, 2015). For most non-domesticated animal species, however, little is known on the presence of *Helicobacter* and *Campylobacter* spp. in the gastrointestinal tract. These animals might serve as a reservoir for the transmission of pathogenic micro-organisms to humans, especially when close relationships with humans have been established, for instance in zoos (Flahou et al., 2017). Identification of such agents in these animals may thus be important in the prediction and prevention of emerging infectious diseases in humans (Saunders et al., 2017).

Therefore, the aim of the present study was to investigate the presence of *Helicobacter* and *Campylobacter* spp. in the gastrointestinal tract of marine and terrestrial zoo mammals.

Material and Methods

Fresh faecal droppings were collected in 6 different zoos in Belgium from various marine and terrestrial mammals, namely 5 dolphins, 11 seals, 6 sea lions, 3 elephants, 2 tigers, 1 mixed sample of 2 lions, 3 mixed samples from 11 chimpanzees, 2 gorillas,

1 mixed sample of 2 siamangs, 1 mixed sample of 2 jaguars, 1 amur leopard, 2 hippopotamuses, 3 spectacled bears, 1 mixed sample of 3 dromedaries, 1 giraffe, 1 giant red panda, 2 lemurs, 1 rhinoceros and 1 hyena.

Two hundred mg was weighed from each faecal sample and homogenized in 400 µl enriched medium containing 7.5 g D(+)-Glucose monohydrate® (Sigma-Aldrich, Saint Louis, Missouri, USA); 25 ml Brain heart broth® (Bio-Rad, Hercules, California, USA) and 75 ml HyClone™ Donor Equine Serum® (Life technologies, Carlsbad, California, USA). After homogenization, the modified filter technique of Steele and McDermott was used (Ceelen et al., 2006). In brief, a sterile cellulose acetate membrane filter (0.45 µm) was applied onto the surface of 2 different agar plates. The first plate contained Brain Heart Infusion (BHI) Agar® (Bio-Rad, Hercules, California, USA) supplemented with 10% horse blood, 20 µg/ml amphothericin B (Sigma-Aldrich, Saint Louis, Missouri, USA) and Vitox supplement (Oxoid, Basingstoke, UK) and was used for *Helicobacter* spp. isolation. The second plate contained Tryptone Soy Agar® (Oxoid, Basingstoke, UK) supplemented with 5% sheep blood, 20 µg/ml amphothericin B and Vitox supplement and was used for *Campylobacter* spp. isolation. After absorption of the filter in the agar, 300 µl of the homogenized faecal material was placed on top of the filter. The plates were incubated for 1h at 37°C under microaerobic conditions (5% H_2, 5% CO_2, 5%O_2 and 85% N_2), after which the filter was removed. The remaining filtrate was then streaked with a loop on the agar. Thereafter, the plates were incubated for a minimum of 3 days under microaerobic conditions at 37°C. Small, greyish-white colonies, indicative for presence of *Helicobacter* and/or *Campylobacter* spp., were selected and purified on Columbia agar plates® (Oxoid, Basingstoke, UK). DNA was extracted from the colonies using PrepMan Ultra Sample Preparation Reagent® (Life Technologies, Carlsbad, California) according to the manufacturer's

instructions. The near complete *16S rRNA* gene was amplified with *αβ-NOT* and *ωMB* primers and sequenced by GATC Biotech, Supremerun sequencing® (Constance, Germany).

Additionally, DNA was extracted directly from 200 mg of the faecal samples using the QIAamp DNA Stool Mini Kit® (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All these DNA samples were tested with *Campylobacter* genus specific and *Helicobacter* genus specific PCRs (Hermans et al., 2012; Flahou et al., 2014). These PCRs amplify a ~816 bp (*Campylobacter*) and ~389 bp (*Helicobacter*) fragment of the *16S rRNA* gene.

All sequences obtained from the *16S rRNA* gene fragments of isolated bacteria and from the amplicons after PCR analysis of faecal samples were analysed with Vector NTI® (Life Technologies, Carlsbad, California) and compared with those in the NCBI database using the BLAST search tool (Flahou et al., 2014).

To further analyse the sequences that could not be assigned to a *Helicobacter* species present in the NCBI database, phylogenetic analysis was performed. A multiple alignment of the *16S rRNA* gene sequences from all known *Helicobacter* spp. and the unidentified sequences was generated using MUSCLE® (EMBL-EBI, Cambridge, United Kingdom) with Gblocks as alignment curation. A phylogenetic tree was then created using PhyML® (ATGC, Montpellier, France) with the maximum likelihood method and a bootstrap value of 1000 to estimate the robustness of the topology of the tree. Finally, the 16S rRNA tree was visualized using TreeDyn® (GEMI Bioinformatics, Montpellier, France).

The GenBank/EMBL/DDBJ accession numbers of the partial *16S rRNA* gene sequences of the unidentified *Helicobacter* spp. isolated from 3/11 seals, 5/6 sea lions, 1/3 elephants, 1/2 hippopotamuses, 2/3 spectacled bears, 1/1 rhinoceros and 1 mixed

sample of 2 lions are LT960591.1, LT960592.1, LT960593.1, LT960594.1, LT960595.1, LT960596.1, LT960597.1, LT960598.1, LT960599.1, LT960600.1, LT960601.1, LT960602.1, LT960603.1 and LT960604.1, respectively.

Results

Helicobacter DNA was demonstrated by PCR in 12 of 22 (12/22) samples from the marine mammals and in 6/26 samples from terrestrial mammals. *H. cetorum* DNA was present in faecal samples from 4/5 dolphins, whereas amplicons with a sequence that did not match the sequence of known *Helicobacter* spp. in the NCBI database were obtained from faecal samples of 3/11 seals, 5/6 sea lions, 1/3 elephants, 1/2 hippopotamuses, 2/3 spectacled bears, 1/1 rhinoceros and 1 mixed sample of 2 lions. Phylogenetic analysis demonstrated that these putative unknown *Helicobacter* spp. clustered together with a 16S rRNA partial sequence of 99-100% homology. The cluster formed a distinct lineage in the genus *Helicobacter,* supported by bootstrap values of 99-100% (Figure 1). Only the 4 sea lions' strains, obtained from 2 different zoos (2 from one zoo and the 2 others from another zoo), showed 100% similarity (Figure 1). *H. bilis* (U18766), *H. canis* (L13464), *H. anseris* (DQ415545) and *H. hepaticus* (U07574) were identified as the known *Helicobacter* spp. with the highest sequence similarities, showing values of 98%, 97.7%, 97.7%, 97.7% and 96.9%, respectively. Unfortunately, from none of the samples *Helicobacter* spp. could be isolated.

The prevalence of *Campylobacter* spp. was 5/22 in marine animals and 9/26 in terrestrial animals. *C. insulaenigrae* was isolated from 1/11 seals and 3/6 sea lions, while *C. jejuni* subsp. *jejuni* was isolated from 1/3 elephants, 1/3 spectacled bears, and 1/2 lemurs. *C. coli* was isolated from 1/3 mixed samples from 11 chimpanzees and from

1/1 mixed sample from 3 dromedaries. Additionally, DNA of the following species was directly demonstrated in faecal samples by PCR, while the bacterium could not be isolated. *C. insulaenigrae* DNA was demonstrated to be present in 1/6 sea lions and *C. jejuni* subsp. *jejuni* DNA in 1/3 mixed samples from 11 chimpanzees and 1/1 mixed sample from 3 dromedaries. The sample from 3 dromedaries and the giraffe also contained *C. lanienae* DNA.

Discussion

Only faecal samples were collected from the different animal species. Such samples are useful for detection of *Helicobacter* and *Campylobacter* spp. colonizing the large intestine, but may be less suitable for detection of *Helicobacter* spp. residing in the stomach, as it has been shown that these species are not always shed in the faeces (Haesebrouck et al., 2009). In addition, *Helicobacter* spp. DNA may be degraded during passage in the intestinal tract resulting in false negative results. Therefore, the results with regard to gastric *Helicobacter* spp. are most likely an underestimation of their real presence in marine and terrestrial zoo mammals.

In the majority of the dolphins, *H. cetorum* was detected. Previous studies have also demonstrated the presence of *H. cetorum* in cetaceans and pinnipeds worldwide and have associated *H. cetorum* infection with the development of gastritis and gastric ulcers in marine mammals (Harper et al., 2003; Goldman et al., 2011; McLaughlin et al., 2011; Davison et al., 2014). In the present study, no data were available on the presence of gastritis. Nevertheless, infection with *Helicobacter* spp. in humans and animals do not always result in pronounced disease signs and lesions (Haesebrouck et al., 2009). The importance of *H. cetorum* in dolphins needs thus to be further investigated.

166 *C. insulaenigrae* was isolated from the faeces of sea lions and seals. Although
167 similar results have been described in North America, South America and Scotland
168 (Foster et al., 2004; Stoddard et al., 2007; González et al., 2011), this is, to our
169 knowledge, the first report of detection of *C. insulaenigrae* on the European mainland.
170 This bacterial species has been associated with septicemia and diarrhea in an
171 immunocompromised human patient (Chua et al., 2007). Its pathogenic potential in
172 marine mammals, however, remains unknown and needs to be further investigated.
173 Interestingly, the presence of several, putative unknown *Helicobacter* spp. was
174 demonstrated in both marine and terrestrial zoo mammals. These species most likely
175 belong to the group of enterohepatic helicobacters, since *H. bilis*, *H. canis*, *H. anseris*
176 and *H. hepaticus* were identified as known enterohepatic *Helicobacter* spp. with the
177 highest sequence similarities. Phylogenetic analysis showed a clustering of these
178 unknown species with formation of a distinct lineage in the genus *Helicobacter,*
179 indicating that they represent putative novel enterohepatic *Helicobacter* spp.. Only in
180 strains obtained from sea lions an identical sequence was detected, which might
181 indicate that these represent the same *Helicobacter* sp.. However, since only part of the
182 *16S rRNA* gene was sequenced and because comparison of *16S rRNA* gene sequences
183 is not always sufficient for differentiation between different *Helicobacter* species, no
184 definite conclusion at species level can be drawn from this study (Vandamme et al.,
185 2000). Unfortunately, no isolates were obtained from these putative novel *Helicobacter*
186 spp., impairing further phenotypic and phylogenetic characterization.
187 Several terrestrial zoo mammals appeared to be colonised with *C. jejuni* subsp.
188 *jejuni* or *C. coli*. These species are a major cause of gastroenteritis in humans worldwide
189 and recent surveys have revealed the presence of a ‘generalist’ genotype that may move
190 between host species (Weis et al., 2016), confirming thus its zoonotic potential.

Although the majority of these surveys mainly focused on birds, livestock and primates, an outbreak of campylobacteriosis has been associated with raccoon contact, suggesting that wildlife may act as zoonotic vectors as well (Saunders et al., 2017). Humans, and in particular people working in zoos, should be made aware of the presence of these pathogens and should be encouraged to take hygienic measures in order to prevent infection.

In conclusion, we demonstrated the presence of several known and unknown *Helicobacter* and *Campylobacter* spp. in marine and terrestrial zoo mammals. These findings necessitate further research for their pathogenic potential to wildlife as well as their zoonotic potential.

Declarations

Conflict of interest statement

Declaration of interest: none.

Authors' contributions

FH, AS, BF, CL and CDW participated in the design of the study. CL and CDW carried out the experiments, analysed the data and drafted the manuscript. FH, AS, BF, CL and CDW coordinated the study and participated in the design of the study, analysis of the data and drafting of the manuscript. All authors read and approved the final manuscript.

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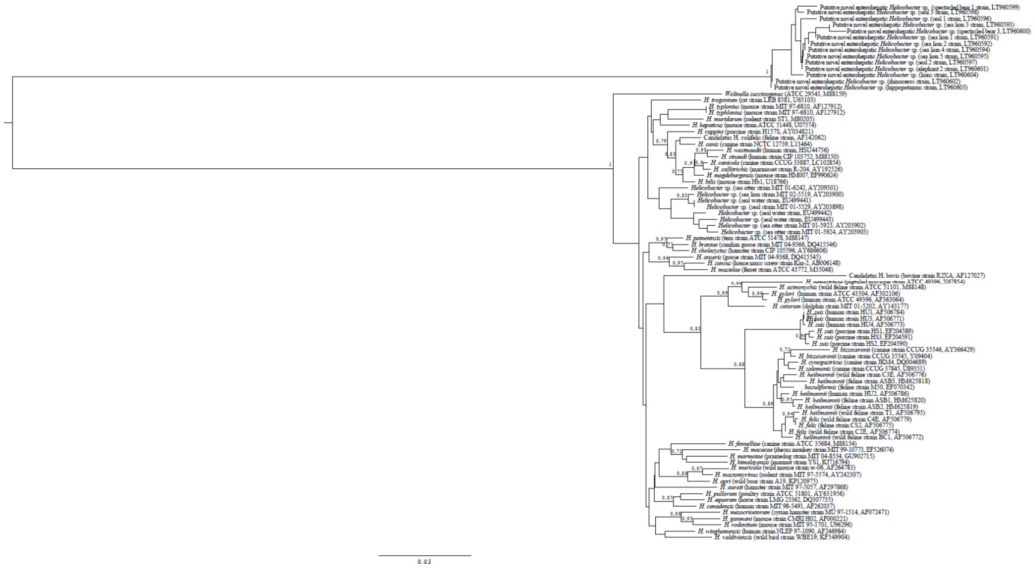

Fig. 1: Phylogenetic tree based on 16S rRNA sequences and maximum likelihood method shows the genetic relationships between *Helicobacter* spp. and the 14 putative novel enterohepatic *Helicobacter* spp. The scale-bar represents 3% differences in nucleotide sequences; bootstrap values (≥0.7) of 1000 replicates are displayed next to the corresponding branch and GenBank accession numbers are included.