Veterinary Microbiology 166 (2013) 429–437

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Detection of *Helicobacter* species in the gastrointestinal tract of ringtail possum and koala: Possible influence of diet, on the gut microbiota

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ARTICLE INFO

Article history:
Received 7 October 2012
Received in revised form 21 May 2013
Accepted 19 June 2013

Keywords:
Ringtail possum
Koala
Helicobacter species
Phylogenetic study
Transmission electron microscopy

ABSTRACT

The presence of *Helicobacter* spp. was examined in the liver and in different regions of the gastrointestinal tract (GIT) including the stomach, 3 cm above ileum, ileum, caecum, colon and rectum of 10 ringtail possums (RTPs) and 3 koalas using a combination of microscopy, culture and PCR. *Helicobacter* was detected in the distal end of the GIT of 7 of 10 RTPs by direct PCR and in all (10/10) RTPs by nested PCR. Five 'S' shaped isolates with bipolar sheathed flagella were isolated from the lower bowel of 3 of the 10 RTPs. 16S rRNA sequence analysis of these 5 isolates confirmed them as potentially novel *Helicobacter* species. No *Helicobacter* species were cultured from the koalas, however *Helicobacter* DNA was detected, in the majority of liver and/or stomach samples of the three koalas and in the colonic region of one koala, using nested PCR. The 16S rRNA gene was sequenced directly from DNA extracted from the homogenised livers and mucus scrapings of the stomach from koala 1 and were confirmed to be *Helicobacter* species. Based on histopathological examination of sections from the liver and intestine no evidence of infection could be related to the presence of helicobacters in either the RTP or koala. Based on our results, it is possible that diet may influence the detection of *Helicobacter* species; however this required further investigation.

1. Introduction

One important component of the gastrointestinal ecosystem is the subset of microbiota that colonise the surface of the gastrointestinal mucosa of mammals. To be able to colonise the gastrointestinal tract (GIT) this complex community of organisms must be able to thrive in the environment and utilise nutritional conditions that exist there. Often these mucosa-associated microorganisms are actively motile, spiral-shaped organisms that differ significantly to the microorganisms found in the lumen of GIT (Lee, 1991). In addition the composition of these microorganisms has been reported to not only vary at different locations within the GIT but also among different animal species. The major bacterial genera that compose the actively motile, spiral-shaped mucosa-associated microbiota include *Borrelia*, *Spirillum*, *Helicobacter* and *Campylobacter* (Solnick and Schauer, 2001). To date members of the genus *Helicobacter* have been cultured from the GIT of humans as well as from a range of placental mammals and birds (Solnick and Schauer, 2001; Fox, 2002). In addition a recent study by our group has reported up to three morphologically different – comma, fusiform and spiral shaped *Helicobacter* species colonising mucus

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http://dx.doi.org/10.1016/j.vetmic.2013.06.026

layer of the lower bowel of an Australian marsupial, the common brushtail possum (*Trichosurus vulpecula*, BTP) (Coldham et al., 2011).

Only four tree-dwelling Australian marsupial herbivores that feed mainly on eucalypt leaves have been described, the common brushtail possum, the common ringtail possum (*Pseudocheirus peregrinus*, RTP), the koala (*Phascolarctos cinereus*) and the greater glider (*Petauroides volans*) (Kerle, 2001). All of these are caecum fermenters thus the majority of plant cell wall digestion takes place in a greatly expanded caecum (Hume, 1999).

The aim of the current study was to determine if, given the high abundance of tannins and phenols obtained from ingestion of eucalypt leaves in the RTP and koalas, *Helicobacter* spp., are present in the GIT and the liver of these marsupials. To answer this question the presence of *Helicobacter* spp., was determined in different regions of the GIT of RTPs and koalas using a combination of three methods: microscopy, culture and PCR. This approach is essential as no individual method is suitable for the detection of *Helicobacter* species, as firstly bacterial morphology (from fresh smear or silver stain section) can only serve as a guide for the detection of helicobacters, secondly some *Helicobacter* species are likely to be non-cultivable using current methodologies and thirdly *Helicobacter* genus specific PCR based on a selected primer set might not detect all *Helicobacter* species/strains, including unknown *Helicobacter* spp. that might present in Australian marsupials. Furthermore PCR inhibitors may be present in the DNA extracted from the tissue samples. To reduce any effect of inhibitors that could be present in the samples, nested PCR was performed in parallel to direct PCR. In addition, the colonisation location of *Helicobacter* spp. in the GIT was examined using Fluorescent *in situ* hybridisation (FISH) on tissue sections in which *Helicobacter* spp. had been detected by all three methods. To investigate signs of any pathology associated with *Helicobacter* spp. as has been observed in other animals histopathological analysis was conducted on all specimens.

The detection and isolation rates of these bacteria in the RTP were then compared with that in koalas to determine any possible impact of diet, feeding strategies or the type of digestive system on the colonisation of the GIT by *Helicobacter* spp. Currently only three of four tree-dwelling Australian marsupial herbivores have been studied the RTP and koala in this study and the BTP in a previous study by our group (Coldham et al., 2011). Given that the same methodologies were used in both studies we compared the results obtained in the RTP and koala with those previously reported in the BTP.

2. Materials and methods

2.1. Animal and collection of specimens

Liver and GIT specimens from 10 ringtail possums: 6 female and 2 male adults, 1 unspecified sex juvenile and 1 unspecified sex and age (named RTP 1–RTP 10) and 3 koalas: 1 male juvenile, 1 female adult and 1 male adult, (named koala 1 – koala 3) were collected from the Veterinary & Quarantine centre, Taronga Zoo, Sydney, Australia. All RTPs were non-caged animals while the koalas were zoo animals. These marsupials had been injured or were in ill health and had subsequently died or had been euthanised for compassionate reasons. For each animal, three samples of tissue were collected from each of the following sites: the liver, stomach, mid ileum, ileum at 3 cm above the caecum (3-ileum), caecum, colon and rectum. The first sample from a particular location was frozen at −70 °C for DNA extraction and PCR amplification. The second sample was frozen in Brain heart Infusion–Glycerol medium (BHIG) and kept at −70 °C until cultured. The third sample was fixed in formalin for histology.

2.2. Bacterial isolation and biochemical characterisation

Homogenised liver samples and scrapings of gastrointestinal mucus were cultured on horse blood agar plate (HBA-composition per 100 ml: sterile defibrinated horse blood 5 ml, Amphotericin 25 μg and blood agar base No. 2 (Oxoid) 3.5 g) and on campylobacter selective agar plates [CSA: 100 ml of HBA plus Skirrow's selective supplement (Polymyxin B (Sigma) 2.5 μg/ml, Vancomycin (Eli Lilly & Co., Australia) 10 μg/ml and Trimethoprim (Sigma) 5 μg/ml)]. Both the direct inoculation method and a selective filtration method (Robertson et al., 2001; Coldham et al., 2011) were used in this study.

The phenotypic characteristics of all isolates were determined using standardised methods recommended in an extensive identification scheme designed for *Campylobacter*, *Helicobacter* and related bacteria (On et al., 1996; Coldham et al., 2011). Nitrate reduction and gamma-glutamyl transferase activity were examined using the API-Campy Identification System (BioMe'rieux, Marcy-l' Etoile, France). Indoxyl acetate hydrolysis was examined using the disc method (Hodge et al., 1990). Alkaline phosphatase and hippurate hydrolysis was examined using Rosco diagnostic tablets (UTEC diagnostics, Denmark). Susceptibility to nalidixic acid (30 μg, NA 30, Oxoid), cephalothin (30 μg, KF 30, Oxoid) and metronidazole (5 μg, MTZ 5, Oxoid) was determined by disc diffusion on HBA plates. Strains were determined as sensitive if there was a zone of inhibition and resistant if there was no zone (Karmali et al., 1980; Coldham et al., 2011).

Freshly grown bacteria from HBA plates were negatively stained with 2% uranyl acetate on a colloidal/carbon grid (483 or 400 mesh), and were examined using transmission electron microscopy (H7000-Hitachi, Tokyo, Japan).

2.3. DNA extraction and PCR

DNA from the liver and GIT mucus scrapings of all 10 RTPs and 3 koalas was extracted using the phenol chloroform method. Briefly, DNA was extracted using proteinase K digestion, followed by phenol, phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform extraction and finally ethanol precipitation. DNA from bacterial cells was extracted using the puregene DNA isolation kit (Gentra systems, QIAGEN, Australia).

Helicobacter genus-specific PCR (direct PCR) was performed using primers H276f (5′-CTATGACGGGTATCCGGC-3′, *E. coli* position 276–293) and H676r (5′-