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Difficulties in the molecular diagnosis of helicobacter rodent infections

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ABSTRACT

Molecular diagnostic methods using the polymerase chain reaction (PCR) are the gold standard in *Helicobacter* diagnostics. Most rely on the amplification of parts of the 16S rRNA gene sequence. Therefore, the validity and accuracy of results depends heavily on the PCR design and the time of its publication because new sequences are continually being submitted to databases. Here we report the presence of helicobacter in commercially bred mice supposedly free of this infection. Furthermore, three out of six different commercial laboratories performing helicobacter testing on the same spiked faecal samples failed to detect and identify *H. hepaticus*. We designed a simple generic PCR assay that amplifies a 261 bp amplicon spanning two of the seven variable regions in the 16S rRNA of helicobacter. Using this assay together with an established generic assay designed by Bohr [Bohr, U.R., Primus, A., Zagoura, A., Glasbrenner, B., Wex, T., Malfertheiner, P., 2002. A group-specific PCR assay for the detection of Helicobacteraceae in human gut. Helicobacter 7, 378–383] and then cloning and sequencing their products, we detected the *H. hepaticus* used in the study that three commercial laboratories failed to detect. We think these assays together could detect all the currently known species of helicobacter and hopefully the new ones as well. In addition, we have been able to identify different species of helicobacter and their relative proportions infecting a single animal. This information has also shown that some helicobacters may have a much broader host range than originally reported.

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1. Introduction

Since the identification of *Helicobacter hepaticus* in mice, many different PCR assays have been developed for the detection and identification of *Helicobacter* in rodents. This is due to the difficulty in growing the bacteria in culture. Most *Helicobacter* diagnostic PCRs target the 16S ribosomal RNA gene (Battles et al., 1995; Beckwith et al., 1997; Fermer et al., 2002; Ge et al., 2001; Grehan et al., 2002; Mahler et al., 1998; Nilsson et al., 2004; Riley et al., 1996; Shames et al., 1995). A current search of sequences for the 16S ribosomal RNA gene of *Helicobacter spp* in NCBI produces at least 513 different sequences (Table 1). This is a growing database, which constantly incorporates new sequences to the genus. Therefore, the validity and accuracy of results depends heavily on the PCR design and the time of its publication. Nowadays the genus contains 31 formally named species (http://www.bacterio.cict.fr/h/helicobacter.html). 14 are currently known to infect rodents, and nine of them have been found in mice (Fox and Whary, 2007).

In 2001, *H. hepaticus*-free mice received straight from commercial vendors tested positive in our laboratory for this organism by two different PCR assays (Mahler et al.,

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Table 1
Number of 16S sequences per *Helicobacter* species (NCBI)

Species	16S	Species	16S	Species	16S	Species	16S
H. pylori	134	*H. pullorum*	15	*H. pametensis*	1	*H. rodentium*	3
H. acinonychis	1	*H. canadensis*	17	*H. cholecystus*	2	*H. ganmani*	8
H. cetorum	4	*H. muricola*	1	*H. mesocricetorum*	2	*H. muridarum*	4
H. felis	17	*H. marmotae*	1	*H. mustelae*	5	*H. typhlonius*	1
H. bizzozeronii	4	*H. fennelliae*	3	*H. trogontum*	2	*H. bilis*	20
H. salomonis	3	*H. canis*	12	*Uncultured helicobacter*	116	*H. cinaedi*	14
H. aurati	1	*H. suncus*	2	*H. winghamensis*	7	*H. hepaticus*	9
H. sp.	117						

1998; Shames et al., 1995), culture and electron microscopy. This prompted an investigation into the cause of this discrepancy in health status.

Here, we report further inconsistencies between different commercial laboratories in identifying *H. hepaticus* on the same spiked faecal samples in 2007. We also highlight the deficiencies of two published assays (Mahler et al., 1998; Shames et al., 1995) used in our laboratory in 2001 by cloning and sequencing their products. We have also designed a simple generic PCR assay that amplifies a 261 bp amplicon, which was validated with and proved as effective as an already published 800 bp assay also used for generic helicobacter testing (Bohr et al., 2002).

Finally, by cloning and sequencing the products from several different PCR assays we have been able to identify the species of helicobacter and their relative proportions infecting a single animal and to show that some species of helicobacter have a much broader host range than originally reported. The new 261 bp assay also detected a naturally occurring infection by a single helicobacter strain in a mouse facility that some currently used assays failed to detect.

2. Materials and methods

2.1. *H. hepaticus* strain MIT 96-1809 controls

H. hepaticus strain MIT 96-1809 was grown on trypticase soy broth supplemented with 10% foetal calf serum at 37 °C. Genomic DNA from *H. hepaticus* strain MIT 96-1809 was extracted using the DNeasy Tissue Kit (QIAGEN, West Sussex, UK) and quantified by quantitative PCR. Dilutions of 10^7, 10^5 and 100 genomic DNA copies were prepared. Three triplicates of each dilution plus a negative sample were pippeted into eppendorfs to which a faecal pellet from Helicobacter negative animals was added. In 2007 ten samples were sent frozen at −20 °C from Centro Nacional de Biotecnología (CNB) in Madrid to each of six different commercial laboratories, two of them located in the US and four of them in Europe, plus to MRC-NIMR for PCR helicobacter detection. Diagnostic laboratories were named A, B, C, D, E and F.

2.2. Bacteria

The following NCTC/ATCC bacteria strains have been used in this study: *H. hepaticus* (ATCC 51448), *Helicobacter muridarum* (NCTC 12714), *Helicobacter cinaedi* (NCTC 11611), *Helicobacter fennelliae* (NCTC 11612), *Helicobacter canis* (NCTC 12220) *Helicobacter pylori* (NCTC 11637), *Helicobacter nemestrinae* (NCTC 12491), *Helicobacter acinonychis* (NCTC 12686), *Helicobacter mustelae* (NCTC12031), *Helicobacter bilis* (ATCC 51630), *Helicobacter rodentium* (NCTC 700285), *Staphylococcus aureus* (NCTC 10017), *Escherichia coli* (NCTC10002), *Yersinia enterocolitica* (NCTCC 10460), *Streptobacillus moniliformis* (NCTC 10651), *Salmonella typhimurium* (NCTC 12416), *Salmonella enteritidis* (NCTC 12694) *Pasteurella pneumotropica* (ATCC 35149), *Campylobacter jejuni* (NCTC 10983), *Campylobacter coli* (NCTC 11350), *Campylobacter fetus* (NCTC 10384) *Shigella flexneri* (NCTC 10512), *Shigella boydii* (NCTC 10024) *Shigella sonnei* (NCTC 10352). A laboratory isolate identified by biochemical characteristics and DNA sequencing was also used: *Actinobacillus muris* (EF597221).

All the bacteria except the helicobacter species were grown on Horse Blood agar (Oxoid Limited, Hampshire, UK) at 37 °C in an atmosphere of 5% CO_2. The helicobacter strains were cultured on *H. pylori* selective medium (Oxoid Limited, Hampshire, UK) in a MACS VA Variable Atmosphere Workstation (Don Whitley Scientific Limited, West Yorkshire, UK) with 88% Nitrogen, 2% Oxygen, 5% Hydrogen and 5% Carbon dioxide at 37 °C. *Helicobacter ganmani* was cultured anaerobically in a MACS VA Variable Atmosphere Workstation (Don Whitley Scientific Limited, West Yorkshire, UK) with a 10% Carbon dioxide, 10% hydrogen and 80% nitrogen gas mixture.

2.3. DNA extraction

DNA was extracted from bacteria using the Qiagen QIAamp Kit and from faecal samples using the Qiagen Stool Kit (QIAGEN, West Sussex, UK) according to the manufacturer's instructions. The DNA was dissolved in 200 μl of elution buffer (AE buffer, QIAGEN, West Sussex, UK) and stored at −70 °C. PCR products for cloning and sequencing were cleaned using the QIAquick kit (QIAGEN, West Sussex, UK) according to the manufacturer's instructions.

2.4. PCR assay

Table 2 shows the various primers used in this study. PCRs were carried out as follows: 50 ng of template was added to a PCR mix containing 2 mM dNTPs, 10 pmol of each primer, and 2.5 units of HotStart*Taq* DNA polymerase (QIAGEN, West Sussex, UK) in PCR buffer containing 15 mM $MgCl_2$. PCR for the *Helicobacter spp* assay and the *H. hepaticus* assay followed conditions defined previously (Mahler et al., 1998; Shames et al., 1995). PCR conditions for the 800 bp (Bohr et al., 2002) consisted of an initial

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