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Assessment of PCR-DGGE for the identification of diverse *Helicobacter* species, and application to faecal samples from zoo animals to determine *Helicobacter* prevalence

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Helicobacter species are fastidious bacterial pathogens that are difficult to culture by standard methods. A PCR-denaturing gradient gel electrophoresis (PCR-DGGE) technique for detection and identification of different *Helicobacter* species was developed and evaluated. The method involves PCR detection of *Helicobacter* DNA by genus-specific primers that target 16S rDNA and subsequent differentiation of *Helicobacter* PCR products by use of DGGE. Strains are identified by comparing mobilities of unknown samples to those determined for reference strains; sequence analysis can also be performed on purified amplicons. Sixteen DGGE profiles were derived from 44 type and reference strains of 20 *Helicobacter* species, indicating the potential of this approach for resolving infection of a single host by multiple *Helicobacter* species. Some more highly related species were not differentiated whereas in highly heterogeneous species, sequence divergence was observed and more than one PCR-DGGE profile was obtained. Application of the PCR-DGGE method to DNA extracted from faeces of zoo animals revealed the presence of *Helicobacter* DNA in 13 of 16 samples; a correlation was seen between the mobility of PCR products in DGGE analysis and DNA sequencing. In combination, this indicated that zoo animals are colonized by a wide range of different *Helicobacter* species; seven animals appeared to be colonized by multiple *Helicobacter* species. By this approach, presumptive identifications were made of *Helicobacter bilis* and *Helicobacter hepaticus* in a Nile crocodile, *Helicobacter cinaedi* in a baboon and a red panda, and *Helicobacter felis* in a wolf and a Taiwan beauty snake. All of these PCR products (~400 bp) showed 100 % sequence similarity to 16S rDNA sequences of the mentioned species. These results demonstrate the potential of PCR-DGGE-based analysis for identification of *Helicobacter* species in complex ecosystems, such as the gastrointestinal tract, and could contribute to a better understanding of the ecology of helicobacters and other pathogens with a complex aetiology.

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INTRODUCTION

Helicobacters are Gram-negative, usually spiral-shaped bacteria that colonize the gastrointestinal tract of man and many animals, including domestic species such as cats, dogs, pigs and poultry (Wadström & Hänninen, 1999; Choi *et al.*, 2001; On, 2001). At the time of writing, the genus *Helicobacter* contains 23 species with validly published names, including *Helicobacter nemestrinae* (Fox, 2002; On *et al.*, 2002), and is rapidly expanding: over 25 novel species have been described in the past 10 years. Of the extant species, *Helicobacter pylori* is considered to be a major cause of chronic gastritis and

Abbreviation: DGGE, denaturing gradient gel electrophoresis.

duodenal and peptic ulcers in man (Nomura *et al.*, 1994; Dunn *et al.*, 1997). Taxa such as *Helicobacter acinonychis*, *Helicobacter mustelae*, *Helicobacter bizzozeronii*, *Helicobacter felis* and '*Candidatus* H. suis' are associated with similar gastric diseases in various animal hosts, such as cheetahs, ferrets, cats, dogs and pigs (Fox, 1997). Animals used for biomedical research are commonly colonized by intestinal *Helicobacter* species (Fox, 1997; Goto *et al.*, 2000; Shen *et al.*, 2001) and there is an increasing body of evidence that shows that enteric and hepatic disease in humans and animals may be caused by taxa that include *Helicobacter canadensis*, *Helicobacter hepaticus* and *Helicobacter bilis* (On *et al.*, 2002). Infections that involve multiple *Helicobacter* species are also known (Jalava *et al.*, 1998).

The majority of *Helicobacter* species are difficult, or as yet impossible, to culture (Fox, 2002). It is therefore difficult to establish specific diagnostic methods for the increasing number of *Helicobacter* species. Establishment of serology-based diagnostic methods is not possible without bacterial culture; accurate diagnosis of mixed *Helicobacter* infections may require PCR-based assays that use a combination of *Helicobacter* species- and/or genus-specific primers and DNA sequencing. Species-specific PCR assays are difficult to validate for unculturable taxa and sequencing of cloned *Helicobacter* genus-specific PCR products is slow, expensive and not suitable for handling many samples. To improve our knowledge of the relatedness of *Helicobacter* species to various gastric, hepatic and enteric diseases, there is a need for new diagnostic methods to detect and identify *Helicobacter* species in clinical samples.

The 16S rRNA gene consists of highly conserved and highly variable regions (Gray *et al.*, 1984). One of the most efficient methods used to exploit 16S rDNA sequence divergence that is frequently observed in various species is denaturing gradient gel electrophoresis (DGGE) (Muyzer, 1999). DNA fragments of the same size but with different sequence compositions are separated in denaturing gradient gels that contain a linear gradient of DNA denaturants (urea and formamide); by this method, a single-base change in a given sequence can be resolved (Fischer & Lerman, 1983). Therefore, PCR-DGGE has great potential to identify closely related species based on 16S rRNA gene sequence divergence. However, comparatively few studies have applied PCR-DGGE for the identification and diagnosis of micro-organisms (de Oliveira *et al.*, 1999; Tannock *et al.*, 1999; Grehan *et al.*, 2002; Requena *et al.*, 2002). In this study, a PCR-DGGE technique was optimized for rapid identification of 20 *Helicobacter* species and used to study the intestinal *Helicobacter* flora of zoo animals.

METHODS

Bacterial strains and DNA extraction. Reference *Helicobacter* strains included in this study represented 20 species (Table 1). DNA was extracted with a QIAamp DNA kit (Qiagen) or an Easy-DNA kit (Invitrogen) according to the manufacturers' instructions and stored at −20 °C.

Zoo animals. Faecal samples of 16 different animals from Copenhagen Zoo, Denmark, were collected in sterile plastic tubes (50 ml) and stored at −20 °C. Details of the animals examined are given in Table 2. DNA was extracted from 200 mg frozen faeces by using a QIAamp Stool kit

Table 1. *Helicobacter* strains used in this study for construction and validation of PCR-DGGE assay

Helicobacter sp.	Strain designation
H. acinonychis	CCUG 29263^T
H. bilis	CCUG 38995^T, CCUG 41387
H. bizzozeronii	AF 53 (kindly provided by Dr L. P. Andersen, National University Hospital, Copenhagen, Denmark) and Hänninen isolate (kindly provided by Dr M.-L. Hänninen, University of Helsinki, Helsinki, Finland)
H. canis	CCUG 32756^T, CCUG 33835
H. cholecystus	CCUG 38167
H. cinaedi	CCUG 43521, R 5758, R 3026, ADN 0413, CCUG 33804
H. felis	CCUG 37850, CCUG 28540, CCUG 28539^T
H. fennelliae	CCUG 32184, CCUG 18820^T
H. ganmani	CCUG 43526^T, CCUG 43527
H. hepaticus	CCUG 44777, CCUG 33637^T
H. muridarum	CCUG 29262^T, Ferrero isolate (kindly provided by Dr Richard L. Ferrero, Pasteur Institute, Paris, France)
H. mustelae	NCTC 12198^T
H. pametensis	CCUG 29255^T, CCUG 29259
H. pullorum	NCTC 12825, NCTC 12827, CCUG 33837^T, CCUG 33839, CCUG 33840
H. pylori	CCUG 17875, CCUG 17874^T, 119/95, 007
'*H. rappini*'	CCUG 23435
H. rodentium	1707, 2060, 2178
H. salomonis	CCUG 37848, CCUG 37845^T
H. suncus	Goto isolate (kindly provided by Dr Kazuo Goto, Central Institute for Experimental Animals, Kawasaki, Kanagawa, Japan)
H. trogontum	MIT 955.369.9136

Table 2. Zoo animal species included in this study and results of PCR-DGGE and sequence similarity by using the BLAST algorithm

Species	DGGE results	Sequence similarity (accession number)
Pig deer (*Babyrousa babyrousa*)	Unidentified strain	408/408 (100 %) *Helicobacter* sp. (AF333340)
	H. bizzozeronii, *H. felis* or *H. salomonis*	415/420 (98 %) *H. cinaedi* (AF207739)
Nile crocodile (*Crocodylus niloticus*)	*H. hepaticus*, *H. cinaedi*	405/405 (100 %) *H. hepaticus* (L39122)
	H. bilis, *H. canis*, *H. ganmani* or *H. rodentium*	403/404 (99 %) *H. bilis* (U51873)
Sea lion (*Zalophus californus*)	*H. pylori* or *H. cinaedi*	420/423 (99 %) *Helicobacter* sp. (AF107494)
Ruffed lemur (*Varecia variegatus*)	*H. bizzozeronii*, *H. felis* or *H. salomonis*	420/425 (98 %) *H. cinaedi* (AF207739)
Cotton-top tamarin (*Saquinus oedipus*)	*H. bizzozeronii*, *H. felis* or *H. salomonis*	420/425 (98 %) *H. cinaedi* (AF207739)
	H. bizzozeronii or *H. salomonis*	413/422 (97 %) *H. ganmani* (AF000224)
Brown bear (*Ursus actus)*	*H. pylori*, *H. cinaedi*	400/405 (98 %) *Helicobacter* sp. (AF333339)
Wild boar (*Sus scrofa)*	Unidentified strain	392/398 (98 %) '*H. winghamensis*' (AF363063)
	H. bizzozeronii, *H. felis* or *H. salomonis*	391/399 (97 %) *H. cinaedi* (AF207739)
	H. bizzozeronii or *H. salomonis*	413/422 (97 %) *H. ganmani* (AF000224)
Polar bear (*Ursus maritimus)*	Unidentified strain	414/422 (98 %) *Helicobacter* sp. (AF333340)
	H. pylori or *H. cinaedi*	398/403 (98 %) *Helicobacter* sp. (AF333339)
Baboon (*Papio hamadryas*)	*H. bilis*, *H. canis*, *H. ganmani* or *H. rodentium*	408/408 (100 %) *Helicobacter* sp. (AF333339)
	H. fennelliae	407/407 (100 %) *H. cinaedi* strain (AF207739)
Siberian tiger (*Panthera tigris altaica*)	*H. pylori* or *H. cinaedi*	411/414 (99 %) *Helicobacter* sp. (AF107494)
Red panda (*Ailurus fulgens fulgens*)	*H. bilis*, *H. canis*, *H. ganmani* or *H. rodentium*	405/409 (99 %) '*H. rappini*' (AF286052)
	H. hepaticus, *H. cinaedi*	378/378 (100 %) *H. cinaedi* (AF396081)
Wolf (*Canis lupus*)	*H. bizzozeronii*, *H. felis* or *H. salomonis*	399/399 (100 %) *H. felis* (HFU51871)
Taiwan beauty snake (*Elaphe taenium freesei*)	*H. bizzozeronii*, *H. felis* or *H. salomonis*	378/378 (100 %) *H. felis* (HFU51871)

(Qiagen) according to the manufacturer's instructions. Extracted DNA was stored at −20 °C.

PCR mixture and incubation conditions. Amplification was carried out by using a GeneAmp PCR System 2700 thermocycler (Applied Biosystems) and *Helicobacter* genus-specific primers (Table 3), previously published by Goto *et al.* (2000). The reaction mixture for the first step (25 μl) contained 0·5 μM each primer (1F and 1R), 0·2 mM each dNTP (Pharmacia Biotech), 1× chelating buffer, 2·5 mM $MgCl_2$, 0·4 % (w/v) BSA, 1·25 U r*Tth* DNA polymerase (Applied Biosystems) and 5 μl extracted DNA. Amplification conditions for the first step were: 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; and finally, 72 °C for 5 min. The reaction mixture for the second step (25 μl) contained 0·5 μM each primer (1F with a GC-clamp and 2R), 0·2 mM each dNTP, 1× buffer II, 2·5 mM $MgCl_2$, 1·0 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 2 μl 10× diluted PCR product from the first step. Amplification conditions for the second step were: 95 °C for 10 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; and finally, 72 °C for 5 min. As a positive control, 0·1 ng *H. pylori* CCUG 17874^T DNA was added to the reaction mixture; 5 μl sterile, Millipore-filtered, deionized water was used as a negative control. The 0·47 kb PCR product was electrophoresed in 1·5 % agarose gel that contained ethidium bromide (Sambrook *et al.*, 1989) and visualized by the use of a GelFotoSystem (Techtum Lab).

DGGE. 16S rDNA sequences of different *Helicobacter* species were analysed by Melt94 (http://web.mit.edu/osp/www/melt.html) to assist in primer selection and to determine DGGE conditions. DNA extracted from reference *Helicobacter* strains and faeces of zoo animals was used as a template to amplify the V6–7 region of 16S rDNA by using primers

Table 3. *Helicobacter* genus-specific PCR primers, targeting 16S rDNA, that were used in this study

Primer*	Primer sequence	PCR product size (bp)
First PCR step:		~780
1F (254–271)	CTATGACGGGTATCCGGC	
1R (1018–1035)	CTCACGACACGAGCTGAC	
Second step:		~470
1F-GC	(GCGGCCGCCCGTCCCGCCGCCCCCGCCCCGCCGCGG CCGCCTATGACGGGTATCCGGC)	
2R (667–686)	TCGCCTTCGCAATGAGT TT	

*Primer positions are based on the sequence of *Escherichia coli* 16S rDNA.

1F-GC and 2R (Table 3). DGGE analysis of amplicons (15 μl) was performed on 9 % polyacrylamide (37·5 : 1 acrylamide/bisacrylamide) gels that contained a 15–30 % urea plus formamide gradient [100 % denaturing solution contains 7 M urea and 40 % (v/v) formamide]. Electrophoresis was performed in 0·5× TAE at 200 V at 60 °C for 4 h by using a DCode System for DGGE (Bio-Rad). Gels were stained with ethidium bromide (0·2 μg ml^{-1} in 0·5× TAE) for 15 min and visualized by using a GelFotoSystem (Techtum Lab).

16S rDNA sequence analysis. Separated DNA fragments were cut from DGGE gels with a scalpel and transferred to microcentrifuge tubes that contained 160 μl sterile, Millipore-filtered, deionized water. Tubes were centrifuged briefly and placed in a freezer at −80 °C for 1 h, then thawed at room temperature for 1 h and frozen again at −80 °C for 1 h. Samples were then thawed at 4 °C for 2 h. A sample of 2·0 μl was used as the template in a PCR mixture that contained primers 1F and 2R, using the same conditions as for the second step described above. *Helicobacter* genus-specific PCR products were purified from agarose gels by Ultrafree-DA centrifuge tubes (Millipore). Sequencing of both DNA strands was performed with an ABI 310 DNA sequencer (Applied Biosystems) by using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.0 (Applied Biosystems). Sequences were checked by using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The closest known relatives of the partial 16S rDNA sequences were determined by using BLASTN 2.2.1 (http://www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1997).

RESULTS

Development of DGGE for identification of *Helicobacter* species

The sensitivity of the semi-nested assay and agarose gel electrophoresis was 500 c.f.u. *H. pylori* (g spiked faeces)$^{-1}$ (data not shown). Multiple strains of each of 15 species were examined to assess the possible effect of infraspecific variance on the DGGE analysis. PCR products from strains of 10 of 15 species examined gave the same degree of mobility in DGGE as the type (or other reference) strain, i.e. no sequence divergence was detected. Such consistent results were obtained for strains of *Helicobacter muridarum* ($n = 2$), *Helicobacter canis* ($n = 2$), *Helicobacter pullorum* ($n = 5$), *H. pylori* ($n = 4$), *H. bilis* ($n = 2$), *Helicobacter ganmani* ($n = 2$), *Helicobacter rodentium* ($n = 3$) and *H. hepaticus* ($n = 2$). Species for which more than one profile was obtained included *H. felis* (two profiles from three strains), *Helicobacter cinaedi* (three profiles from five strains), *H. bizzozeronii* (two profiles from two strains), *Helicobacter fennelliae* (two profiles from two strains) and *H. salomonis* (two profiles from two strains).

In addition, some PCR products derived from strains of distinct taxa could not be distinguished on the basis of their mobility. Consequently, *H. bilis*, *H. canis*, *H. ganmani* and *H. rodentium*; *Helicobacter pametensis* and *H. pullorum*; *Helicobacter suncus* and *Helicobacter trogontum*; *H. cinaedi* (CCUG 33804) and *H. hepaticus*; *H. bizzozeronii* (AF 53) and *H. salomonis* (CCUG 37848); *H. felis* (CCUG 37850), *H. salomonis* (CCUG 37845^T) and *H. bizzozeronii* (Hänninen isolate) were not differentiated (Fig. 1).

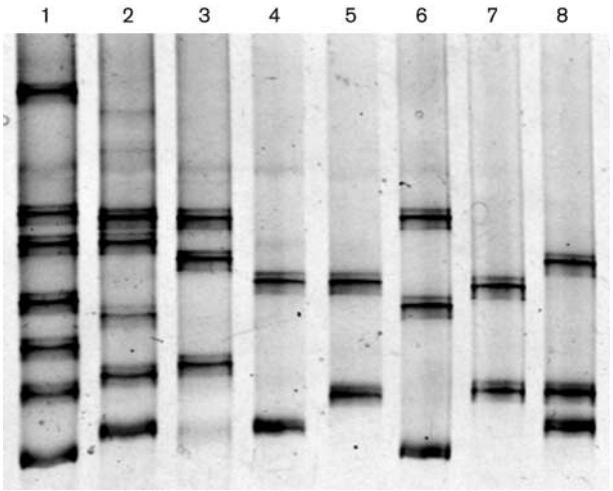

Fig. 1. Separation of PCR products amplified by genus-specific primers from different *Helicobacter* species in a 9 % polyacrylamide 15–30 % denaturing gradient gel (increasing gradient of denaturant from top to bottom). Lanes: 1, *H. muridarum* (CCUG 29262^T), *H. canis* (CCUG 32756^T), *H. pullorum* (NCTC 12825), *H. pylori* (CCUG 17875), *Helicobacter cholecystus* (CCUG 38167), *H. fennelliae* (CCUG 32184) and *H. bizzozeronii* (Hänninen isolate); 2, *H. bilis* (CCUG 38995^T), *H. pametensis* (CCUG 29255^T), *H. mustelae* (NCTC 12198^T), '*H. rappini*' (CCUG 23435) and *H. felis* (CCUG 37850); 3, *H. ganmani* (CCUG 43526^T), *H. suncus* (Goto isolate), *H. felis* (CCUG 28540) and *H. acinonychis* (CCUG 29263^T); 4, *H. cinaedi* (CCUG 43521) and *H. salomonis* (CCUG 37845^T); 5, *H. cinaedi* (R 5758) and *H. hepaticus* (CCUG 33637^T); 6, *H. rodentium* (R 1707), *H. cinaedi* (R 3026) and *H. salomonis* (CCUG 37848); 7, *H. cinaedi* (ADN 0413) and *H. cinaedi* (CCUG 33804); 8, *H. trogontum* (955.369.9136), *H. cinaedi* (CCUG 33804) and *H. bizzozeronii* (AF 53).

PCR-DGGE analysis and DNA sequence analysis of *Helicobacter* species of zoo animals

The semi-nested PCR assay and DGGE analysis detected *Helicobacter* DNA in 13 of 16 zoo animals tested. All distinct PCR products were sequenced and compared to those in GenBank by BLAST to verify the DGGE results. The results are summarized in Table 2; a wide range of helicobacters (principally associated with the lower intestinal tract) was found among the different animals examined. Multiple DNA fragments, an indication of colonization by more than one *Helicobacter* species, were present in the DGGE profiles of seven animals (Fig. 2).

DISCUSSION

Helicobacter species are widely distributed in the gastrointestinal tract of mammals, birds and other animals. An accurate assessment of their prevalence in various environments is important to improve our understanding of their role in gastric, enteric and hepatic disease and for evaluation of their potential as zoonotic agents. Substantial problems involved in isolation and identification of these bacteria

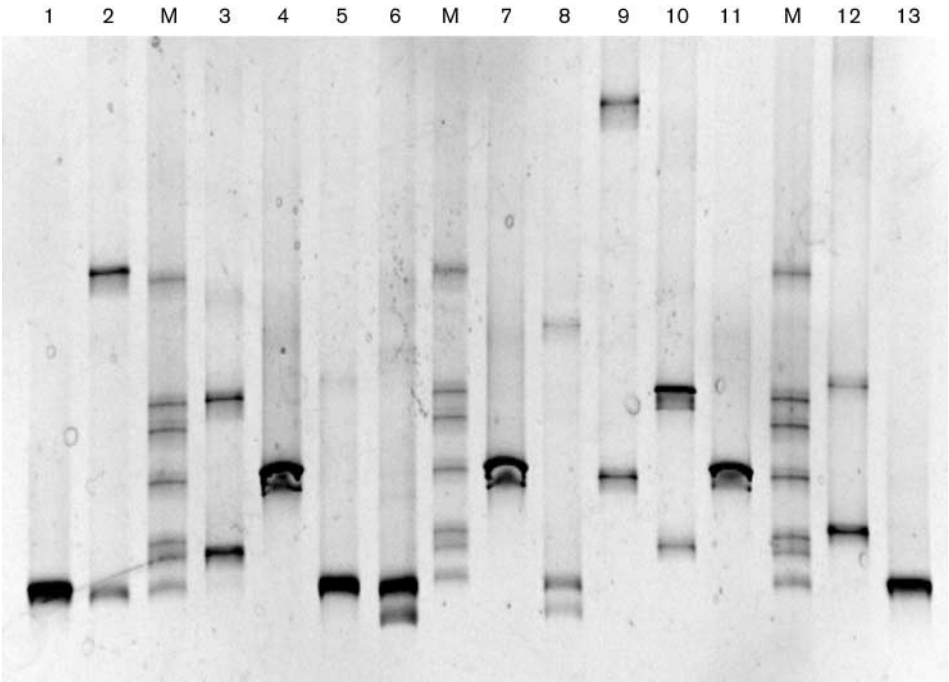

Fig. 2. PCR-DGGE analysis of PCR products, amplified by the semi-nested *Helicobacter* genus-specific PCR assay, of DNA isolated from faeces of 13 animals from Copenhagen Zoo. Lanes: 1, wolf; 2, pig deer; 3, Nile crocodile; 4, sea lion; 5, ruffed lemur; 6, cotton-top tamarin; 7, brown bear; 8, wild boar; 9, polar bear; 10, baboon; 11, tiger; 12, red panda; 13, Taiwan beauty snake. M, migration ladder [PCR products amplified from (top to bottom)]: *H. muridarum* (CCUG 29262^T), *H. bilis* (CCUG 38995^T), *H. pullorum* (NCTC 12825), *H. pylori* (CCUG 17875), '*H. rappini*' (CCUG 23435), *H. hepaticus* (CCUG 33637^T) and *H. bizzozeronii* (AF 53).

greatly hinder such studies. Clearly, the availability of a sensitive, culture-independent method that is capable of detecting a broad taxonomic range of helicobacters would be a valuable investigative tool in this regard.

PCR-DGGE is more frequently applied to evaluate the microbial diversity of complex environments. However, by altering the primer targets, the specificity of the method can be tailored to detect specific bacterial groups (de Oliveira *et al.*, 1999; Tannock *et al.*, 1999; Grehan *et al.*, 2002; Requena *et al.*, 2002) or even strains (Nielsen *et al.*, 2000). Although one study has described a PCR-DGGE method to identify *Helicobacter* species found in the rodent intestine (Grehan *et al.*, 2002), its restricted scope makes it of limited value for the investigation of the wider issues mentioned above. In our study, we developed and evaluated a PCR-DGGE approach that was intended for use in the identification and detection of a broader range of helicobacters that are associated with gastric, enteric or hepatic disease in a wide host range.

We selected 16S rDNA as the target because it contains both conserved and highly variable regions and gene sequences for almost all *Helicobacter* species are available in public databases. In our study, 16 DGGE profiles were derived from the reference strains (representing 20 extant species), indicating the potential of this approach for resolving multiple *Helicobacter* species that infect a single host (Jalava *et al.*, 1998). However, a number of species that are regarded as taxonomically highly related could not be differentiated and, in some species, sequence divergence was observed such that more than one PCR-DGGE profile was obtained. These observations correlate with our current knowledge of 16S rRNA gene sequence heterogeneity among helicobacters [reviewed by On (2001)] and illustrate some of the shortcomings of relying on this gene for species identification.

Results that were obtained when the method was subsequently applied to faecal samples from zoo animals illustrate some important features of this approach. Firstly, the results validate the use of a semi-nested PCR for direct examination of samples. This approach was selected instead of the more common one-step PCR assay to increase its specificity and sensitivity; nested PCR can be 10^4-fold more sensitive than one-step PCR (Stärk *et al.*, 1998). The PCR assay was further optimized by using: (i) a DNA polymerase that is less sensitive to PCR inhibitors (r*Tth* DNA polymerase); (ii) an amplification facilitator (BSA) in the first amplification step; and (iii) a hot-start enzyme (AmpliTaq Gold DNA polymerase) in the second amplification step. These three factors are known to improve the sensitivity and specificity of analytical PCR (Abu Al-Soud & Rådström, 2000, 2001). Detection of *Helicobacter* DNA in 13 of 16 faecal samples

illustrates the efficacy of our approach. Moreover, the advantages of the DGGE approach are illustrated by seven of the 13 PCR-positive samples, which yielded several 16S rDNA sequence types; this indicates that many animal hosts harbour more than one species of *Helicobacter* in their gastrointestinal tract, a finding that would be difficult to resolve by conventional culture methods.

Sequence analysis of distinct products suggested that several animal hosts (to our knowledge, hitherto unexamined for *Helicobacter* spp.) harbour potentially novel *Helicobacter* species. In addition, our results suggest that species such as *H. cinaedi*, *H. hepaticus*, *H. felis*, *H. bizzozeronii* and *H. canis* may have a greater zoonotic potential than is presently recognized, as these species appear to exhibit a broad host range. However, we emphasize that such results should be treated with caution, as species such as *H. cinaedi* and *H. trogontum* show substantial (4–5 %) infraspecific divergence in their 16S rRNA genes (Vandamme *et al.*, 2000; Hänninen *et al.*, 2003), leading to anomalous results in both DGGE and sequence analyses. Nevertheless, such extensive diversity was not detected in many species examined and, for these species, the method may function well as an accurate front-line identification tool, as with other bacteria. Further work on more strains is needed to fully elucidate the level of diversity in the 16S rRNA gene among helicobacters, to ascertain the security of identification by this approach. Improvements can be expected from extending both DGGE and sequence databases in tandem. The use of alternative gene targets to 16S rDNA could also prove beneficial, as suggested previously (On, 2001): the transaldolase gene has already been shown to be useful for PCR-DGGE-based discrimination of human bifidobacteria (Requena *et al.*, 2002).

In conclusion, our study has demonstrated that PCR-DGGE analysis has considerable potential for identification of *Helicobacter* species and investigation of multiple species infections in complex ecosystems such as the gastrointestinal tract.

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