Identification of enteric *Helicobacter* in avian species

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Summary

The presence of enteric *Helicobacter* species was investigated in poultry (n = 130) and in pet and ornamental birds (n = 50) using a PCR sequencing method which permits the differentiation of many *Helicobacter* species derived from animal tissues. All samples were of Italian origin, except for 21 Guinea fowl from a French flock. About 80% of poultry (chickens, laying hens, Guinea fowl) were positive to *Helicobacter* DNA. *H. pullorum* was most frequently (62.1%) identified whereas *H. pylori* and 3 *H. sp. hamster B* strains were seen in only 3 cases each. Pet and ornamental birds were all negative. *H. canadensis* was found in all Guinea fowl from a French farm. This is the first report on the occurrence of this bacterium in poultry.

Keywords: *Helicobacter canadensis, Helicobacter pullorum,* PCR-DNA sequencing, avian species, Italy

Identifikation von Helicobacter im Darm verschiedener Vogelarten

Mit Hilfe von PCR, die eine genaue Charakterisierung verschiedener *Helicobacter*-Stämme erlaubt, wurde das Vorkommen dieses Bakteriums beim Geflügel (n = 130) wie auch bei Haus- und Ziervögeln (n = 50) untersucht. Mit Ausnahme von 21 Guinea Hühnern aus Frankreich stammten alle Proben aus Italien. Bei rund 80% des Geflügels (Mast- und Legehühner, Guinea Hühner) konnte *Helicobacter*-DNA gefunden werden, wobei *H. pullorum* am häufigsten (62.1%) und *H. pylori* sowie *H.* sp. *hamster B* nur in je 3 Fällen vertreten waren. Alle anderen Vogelarten waren *Helicobacter* negativ. Bei allen Guinea Hühnern aus Frankreich gelang erstmals der Nachweis von *H. canadensis.*

Schlüsselwörter: *Helicobacter canadensis, Helicobacter pullorum,* PCR-DNA Sequenzierung, Vogelarten, Italien

Introduction

Helicobacter are a group of bacteria that were at first included in the *Campylobacter* genus and have been reclassified as a separate genus in 1989 (Goodwin et al., 1989). Since then this group has rapidly expanded from the first two species, *H. pylori* and *H. mustelae,* to over 24 species which have been isolated from the gastrointestinal tract of humans and several animal species. In the last few years particular attention has been given to the enteric *Helicobacter* which have proven to cause inflammatory lesions and septicaemia in man. Since some of these bacteria have been found in domestic (*H. pullorum,* Burnens et al.,1996) and wild birds (*H. canadensis,* Waldenström et al., 2003), the issue of a zoonotic risk has been raised.

H. pullorum is an enterohepatic spirillum which was first described by Stanley et al. (1994) and was isolated from faeces of humans affected by gastroenteritis (Burnens et al., 1994; Steinbrueckner et al., 1997; Ceelen et al., 2005) and in the bile of humans affected by primary sclerosing cholangitis (Fox et al., 1998; Nilsson et al., 2000). In animals, *H. pullorum* has been isolated from the caecal contents and carcasses of slaughtered chickens (Burnens et al.,1996; Atabay et al., 1998; Ceelen et al., 2006).

H. canadensis was identified as a new species and described as an emerging pathogen by Fox in the year 2000. This organism was later isolated from patients with enteritis and patients with bacteraemia (Tee et al., 2001). So far in animals, it has only been found in wild geese (Waldenström et al., 2003) and recently in swine (Inglis et al., 2006). As a result of the increasing number of reports on the identification of *Helicobacter* recovered from bird and human specimens, the aim of the present study is to investigate the occurrence of enteric *Helicobacter* species in industrial farm poultry and pet and ornamental birds.

Animals, Material and Methods

Animals and specimen collection

One hundred and eighty birds, including 130 individuals obtained from industrial poultry farms (Group A) and 50 ornamental birds (Group B), were tested between 2004 and 2005 (Tab. 1). Group A consisted of samples randomly chosen from 4 categories of poultry, which had been slaughtered between the 8th and

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Table 1: Subdivision of samples in group A (farm poultry) and group B (pet and ornamental birds)

Group	Avian Species		No.
A	*Gallus domesticus*	Broiler chickens	39
		Leghorn chickens	30
		Laying hens	25
	Numida meleagris	Guinea fowl	36
B	*Agapornis, Ara, Cacatua*	parrots	33
	Pavo cristatus	Pea fowl	10
	Struthio camelus	Ostrich	4
	Ephippiorhynchus mycterius	Jabiru mycteria	2
	Casuarius casuarius	Cassowary	1

64th week of life, broilers and laying hens, respectively. The intestines were taken from a poultry slaughter immediately after evisceration.

Group B consisted of 33 dead parrots collected from 3 breeding centres and 17 ornamental birds from a bird park. The parrots were hosted in groups of about 20 individuals in aviaries whereas pet and ornamental birds lived in a private park, in a vast net bounded area where visitors could enter. These animals underwent a necropsy and intestines were collected. All samples examined were of Italian origin, except for 21 Guinea fowl birds from a French flock.

DNA extraction

Intestines were collected and individually packed in plastic bags, delivered to our laboratory and tested separately. The caeca were dissected, rinsed with sterile phosphate buffered saline solution (Sigma Chemical Co, St. Louis, MO) and cut up using sterile scissors. Approximately 25 mg of the caecum were collected in sterile vials and stored at −20°C until DNA extraction. Because of the lack of caeca in parrots, DNA was extracted from the duodenum. Genomic DNA was extracted using a commercial kit (DNeasy Tissue Kit, Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Enteric tissue extracts were first amplified with universal primers for the 16S rRNA gene to demonstrate the presence of amplifiable DNA (Gramley et al., 1999).

PCR and DNA sequencing

In order to detect the presence of *Helicobacter* in avian samples, PCR procedure was performed as described by Beckwith et al. (1997). Genus-specific primers (5'-TAT GAC GGG TAT CCG GC-3'; 5'-ATT CCA CCT ACC TCT CCC A-3') were designed from regions of the 16S rRNA gene, which is conserved among members of the *Helicobacter* genus. PCRs were prepared with 1 µmol/L each primer (Sigma-Genosys, Cambridge, UK), 200 µmol/L each dNTP (dATP, dCTP, dGTP, and dTTP, Amersham Biosciences, Europe, GmbH), 1× PCR buffer (10 µmol Tris-HCl, 1.5 mmol $MgCl_2$, 50mmol KCl), 5 µL of DNA template, 1.25 U/µL *Taq* polymerase (Qiagen) and distilled water in a total volume of 50 mL. Each experiment included a negative control (distilled water) and a positive control consisting of cultured *H. felis* DNA. The cycling profile consisted of a denaturation step at 94°C for 3 min, followed by 45 cycles of denaturation at 94°C for 2 s, primer annealing at 53°C for 2 s, and extension at 72°C for 30 s. All reactions were carried out using GeneAmp-PCR system 9700 (Applied Biosystems, Foster City, CA, USA).

A 375 bp product of the amplified samples was analyzed by electrophoresis (120V/208 mA) with 1× Tris-acetate EDTA electrophoresis buffer in 2.5% agarose (Sigma). The bands were visualized by UV trans-illuminator (Geldoc 2000; Biorad, Milano, Italy).

Amplicons were purified using QIAquick PCR purification kit (Qiagen). Sequencing of PCR products was performed using an ABI prism 310 Genetic Analyser (Applied Biosystems) and PCR-derived primers. Sequences were analysed with CHROMAS 2.0 software (Technelysium, Helensvale, Australia) and submitted to BLAST analysis (Altschul et al., 1997). The determined sequences were subsequently aligned using multisequence alignment program ClustalX (Thompson et al., 1997). Sequences obtained in this study along with some sequences of *Helicobacter* deposited in the NCBI GenBank database were used to construct phylogenetic trees (Figure 1). Genetic distances were computed by MEGA2 software (Kumar et al., 2001) and were used to construct a neighbor-joining tree with the Tamura-Nei two-parameter distance option (Tamura and Nei, 1993). Statistical confidence of the tree topology was assessed by bootstrap (Felsenstein, 1985).

Results

The presence of amplifiable DNA was observed in 174 out of 180 samples and six samples were negative (3 from Guinea fowl birds, 2 from broiler chickens, 1 from a hen), therefore they were excluded from further tests. A total of 100 out of 124 animals in Group A (80.6%) were positive for *Helicobacter* genus-PCR, whereas pet and ornamental birds in Group B (0%) were all negative. Sequencing of PCR products showed that 76 samples were positive for *H. pullorum* (similarity of 98%–100% with *H. pullorum* AJ876521) and 18 for *H. canadensis* (similarity of 98%–99% with *H. canadensis* AY323505). *H.* sp. *hamster B* (similarity of 99% with AF072333) and *H. pylori* (similarity of 97%–98% with *H. pylori* AY304558) were detected in 3 samples each.

In particular, *H. pullorum* was detected in 19 out of 37 broiler chickens (51.3%), 22 out of 24 laying hens (91.7%), 25 out of 30 leghorn chickens (83.3%), 11 out of 15 Italian Guinea fowl birds (73.3%). *H. canadensis* was found in all Guinea fowl from a French farm. Two *H. pylori* and 3 *H. sp. hamster B* were found in leghorn chickens and one *H. pylori* in an Italian Guinea fowl bird. *H. pullorum* and *H. canadensis* strains identified in this study fall within the group of closely related organisms. As shown in the phylogenetic study, *H. canadensis* strains isolated from Guinea fowls are identical to those from wild geese and have the same difference as human strains (Fig 1). *H. pullorum* resulted identical to both avian and human strains used to create a phylogenetic tree, in accordance with Fox et al. (2000).

Discussion

Detection of species such as *H. pullorum* and *H. canadensis* is becoming increasingly important since these organisms are recognised as human and/or animal pathogens. Their phenotypic identification is complex because of fastidious growth characteristics and atypical biochemical reactions of some strains (On, 1996). Furthermore, they share culture characteristics with *Campylobacter* species and might easily be mistaken for these bacteria (Atabay et al., 1998; Melito et al., 2000). Therefore, data regarding the presence of enteric *Helicobacter* in birds might be underestimated in favour of *Campylobacter* species. In this study, we report a simple PCR sequencing method, which allows the differentiation of many *Helicobacter* species directly from animal tissue. The protocol consists of one set of primers that amplifies a fragment within the region of 16S rRNA gene followed by sequencing of amplified products. This method is a useful assay to identify *Helicobacter* species in birds and should also be expanded to other animals.

From the sequences obtained, we identified 4 different species: *H. pullorum, H. canadensis, H. sp. hamster B* and *H. pylori*. Interestingly, this technique allows us to differentiate *H. pullorum* from *H. canadensis,* a closely related species. Phylogenetic analysis showed that *H. canadensis* identified in French Guinea fowl was homologous to strains isolated from wild birds (Waldenström et al., 2003), but slightly different from human strains.

H. pullorum was found in 77 poultry samples (62.1%). This percentage is higher than other studies (60%, 33.6%, 4%; 10, 11, 2 respectively), and shows that this bacterium is common in Italian poultry farms. Our study reveals a statistically significant difference ($p < 0.01$) between laying hens (91.7%) and broiler chickens (51.3%), probably because layers have a longer life and thus more likely to become infected.

Moreover, other helicobacters *(H. pylori, H. sp. hamster B)* were found in poultry, but such a low prevalence (6.0%) might simply be an index of environmental contamination by other animals or man.

No pets or ornamental birds were positive for *Helicobacter* spp. Since these birds live in a habitat that is completely different from poultry, it might be reasonable to presume that different housing systems may result in different levels of exposure to *Helicobacter.*

So far, in avian species *H. canadensis* has only been isolated in wild geese (Waldenström et al., 2003) and this is the first report on domestic birds (Guinea fowls).

Given the potential zoonotic of this bacterium (Fox, 2000; Waldenström et al., 2003), finding *H. canadensis* in farm birds might play a significant role in public health due to frequent human contact with poultry and the consumption of undercooked meat.

It will now be important to investigate the role of *H. canadensis* in human infection.

In conclusion, we observed a high prevalence of *H. pullorum* in poultry farms and, were table to detect for the first time, *H. canadensis* in domestic birds. These

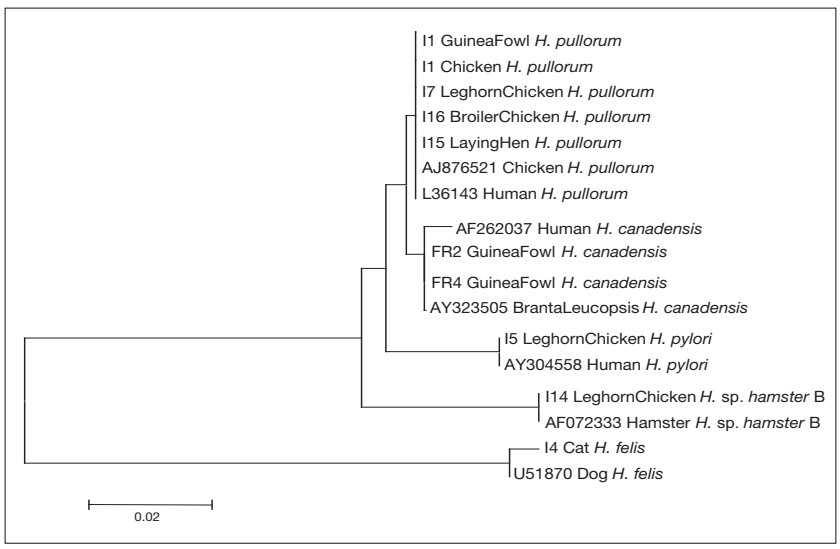

Figure 1: Phylogenetic tree of representative poultry isolates and selected GenBank entries of the most related Helicobacter species. The scale bar represents percentage of sequence divergence.

data suggest that farm poultry plays an important role as a reservoir for enteric helicobacters. However, further studies on the epidemiology and pathogenicity are needed before poultry may be considered as a significant source of human infection and therefore a public health challenge.

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Identification d'Helicobacters dans l'intestin de différents oiseaux

On a étudié la présence de diverses souches d'*Helicobacters* chez la volaille (m = 130) ainsi que chez des oiseaux de volière et d'ornement (m = 50) au moyen d'une PCR qui permet la caractérisation exacte des diverses souches. A l'exception de 21 pintades provenant de France, tous les échantillons venaient d'Italie. Chez environ 80% des volailles (poules pondeuse, poulets d'engraissement, pintades), de l'ADN d'*Helicobacters* a pu être mis en évidence et *H. pullorum* étant le plus fréquent (62.1%). *H. pylori* ainsi que *H.* sp. *hamster* B n'étaient présents que dans 3 cas. Toutes les autres espèces d'oiseaux étaient négatives quant à *Helicobacter.* Chez toutes les pintades françaises ont a pu mettre en évidence pour la première fois *H. canadensis.*

Identificazione di Helicobacter enterici in specie aviari Riassunto

Sono stati ricercati *Helicobacter* enterici in pollame d'allevamento (n = 130), uccelli d'affezione ed ornamentali (n = 50). E' stata utilizzata una metodica biomolecolare (PCR-sequencing) in grado di differenziare specie di *Helicobacter* direttamente da tessuti animali. Tutti i campioni erano di origine italiana, ad eccezione di 21 faraone che provenivano da un allevamento francese. Nell'80% del pollame esaminato (polli, galline ovaiole, faraone) è stato evidenziato DNA di *Helicobacter,* mentre gli uccelli d'affezione e ornamentali sono risultati negativi. In particolare abbiamo identificato *H. pullorum* nel 62.1% del pollame ed *H. canadensis* in tutte le faraone di provenienza francese. Si tratta della prima identificazione di *H. canadensis* in volatili di allevamento.

References

Altschul S. F., Madden T. L., Schaffer A. A., Zhang J. J., Zhang Z., Miller W., Lipman D. J.: Gapped BLAST and PSI-BLAST: a new generation of protein 3 database search programs. Nucleic Acids Res. 1997, 25: 3389–3402.

Atabay H. I., Corry J. E. L., On S. L.W.: Identification of unusual *Campylobacter*-like isolates from poultry products as *Helicobacter pullorum*. J. Appl. Microbiol. 1998, 84: 1017–1024.

Beckwith C. S., Franklin C. L., Hook R. R., Besch-Williford C. L. Jr., Ril L. K.: Fecal PCR assay for diagnosis of Helicobacter infection in laboratory rodents. J. Clin. Microbiol. 1997, 35: 1620–1623.

Burnens A. P., Stanley J., Morgenstern R., Nicolet J.: Gastroenteritis associated with *Helicobacter pullorum*. Lancet. 1994, 344: 1569–1570.

Burnens A. P., Stanley J., Nicolet J.: Possible association of *Helicobacter pullorum* with lesions of vibrionic hepatitis in poultry. In: Campylobacters, helicobacters and related organisms. Eds. Newell D. G., Ketley J. M., Feldman R. A., Plenum Press, New York, 1996.

Ceelen L. M., Decostere A., Verschraegen G., Ducatelle R., Haesebrouck, F.: Prevalence of Helicobacter pullorum among Patients with Gastrointestinal Disease and Clinically Healthy Persons. J. Clin. Microbiol. 2005, 43: 2984–2986.

Ceelen L. M., Decostere A., Van den Bulck K., On S. L. W., Baele M., Ducatelle R., Haesebrouck F.: Helicobacter pullorum in chickens, Belgium. Emerg. Infect. Dis. 2006, 12: 263–267.

Felsenstein J.: Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 1985, 39: 783–791.

Fox J. G., Chien C. C., Dewhirst F. E., Paster B. J., Shen Z., Melito P. L., Woodward D. L., Rodgers F. G.: Helicobacter canadensis sp. nov. isolated from humans with diarrhea as an example of an emerging pathogen. J. Clin. Microbiol. 2000, 38: 2546–2549.

Fox J. G., Dewhirst F. E., Shen Z., Feng Y., Taylor N. S., Paster B. J., Ericson R. L., Lau C. N., Correa P., Araya J. C., Roa I.: Hepatic Helicobacter species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. Gastroenterology. 1998, 114: 755–763.

Goodwin C. S., Armstrong J. A., Chilvers T., Peters M., Collins M. D., Sly L., Mcconnell W., Harper W. E. S.: Transfer of Campylobacter pylori and Campylobacter mustelae to Helicobacter gen. nov. as Helicobacter pylori comb. nov. and Helicobacter mustelae comb. nov., respectively. Int. J. Sys. Bacteriol. 1989, 39: 397–405.

Gramley W. A., Asghar A., Frierson H. F., Powell S. M.: Detection of Helicobacter pylori DNA in fecal samples from infected individuals. J. Clin. Microbiol. 1999, 37: 2236–2240.

Inglis G. D., McConville M., De Jong A.: Atypical *Helicobacter canadensis* strains associated with swine. Appl. Environ. Microbiol. 2006, 72: 4464–4471.

Kumar S., Tamura K., Jakobsen I. B., Nei M.: MEGA2: molecular evolutionary genetics analysis software. Bioinformatics. 2001, 17: 1244–1245.

Melito P. L., Woodward D. L., Bernard K. A., Price L., Khakhria R., Johnson W. M., Rodgers F. G.: Differentiation of clinical *Helicobacter pullorum* isolates from related *Helicobacter and Campylobacter* species. Helicobacter. 2000, 5: 142–147.

Nilsson H. O., Taneera J., Castedal M., Glatz E., Olsson R., Wadstrom T.: Identification of Helicobacter pylori and other Helicobacter species by PCR, hybridization, and partial DNA sequencing in human liver samples from patients with primary sclerosing cholangitis or primary biliary cirrhosis. J. Clin. Microbiol. 2000, 38: 1072–1076.

On S. L.: Identification methods for campylobacters, helicobacters, and related organisms. Clin. Microbiol. Rev. 1996, 3: 405–422.

Stanley J., Linton D., Burnens A. P., Dewhirst F. E., On S. L. W., Porter A., Owen R. J., Costas M.: Helicobacter pullorum sp. nov. – genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. Microbiol. 1994, 140: 3441–3449.

Steinbrueckner B., Haerter G., Pelz K., Weiner S., Rump J. A., Deissler W., Bereswill S., Kist M.: Isolation of Helicobacter pullorum from patients with enteritis. Scand. J. Infect. Dis. 1997, 29: 315–318.

Tamura K., Nei M.: Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondria DNA in Humans and Chimpanzees. Mol. Biol. Evol. 1993, 10: 512–526.

Tee W., Montgomery J., Dyall-Smith M.: Bacteremia caused by a Helicobacter pullorumLike Organism. Clin. Infect. Dis. 2001, 33: 1789–1791.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F., Higgins D. G.: The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 1997, 25: 4876–4882.

Waldenström J., On S. L. W., Ottvall R., Hasselquist D., Harrington C. S., Olsen B.: Avian Reservoirs and Zoonotic Potential of the Emerging Human Pathogen Helicobacter canadensis. Appl. Environ. Microbiol. 2003, 69: 7523–7526.

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