Description of *Helicobacter ibis* sp. nov. isolated from faecal droppings of black-faced ibis (*Theristicus melanopis*)

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The following gene sequences have been deposited in GenBank/EMBL/DDBJ: 16S rRNA gene sequences of strains A82^T and WB-40 (ON950425 and ON950426); and *hsp60* gene sequences of strains A82^T and WB-40 (OQ116579 and OQ116580). The accession numbers for the draft genome sequence data of strains *H. ibis* A82^T and WB-40 are JAQHXR000000000 and JAQHXS000000000, respectively.

Abstract

As part of a larger study on *Epsilonproteobacteria* carried by wild birds in the city of Valdivia (southern Chile), two curved rod-shaped Gram-stain-negative strains (A82^T and WB-40) were recovered from faecal samples and subjected to a taxonomic study. Results of a genus-specific PCR showed that these isolates belonged to the genus *Helicobacter*. Further identification by 16S rRNA and *hsp60* (60 kDa heat-shock protein) gene sequence analysis revealed that they formed a separate phylogenetic clade, different from other known *Helicobacter* species with "*H. burdigaliensis"* CNRCH 2005/566H^T and *H. valdiviensis* WBE14^T being the most closely related species. This was confirmed by core-genome phylogeny as well as digital DNA-DNA hybridization (dDDH) and average nucleotide identity (ANI) analyses between the genome of strains A82^T and WB-40 and the rest of *Helicobacter* species. The draft genome sequence of A82^T and WB-40, obtained by Illumina NextSeq 2000 sequencing, consisted of ∼1.6 Mb with a G+C content of 31.9-32.0 mol%.

The results obtained from the phylogenetic and genomic characterisation, together with their different morphological and biochemical features, revealed that these two strains represent a novel species, for which we propose the name *Helicobacter ibis* sp. nov. with A82^T (=LMG 32718^T= CCCT 22.04^T) as the type strain.

Introduction

The genus *Helicobacter* contains 51 validly published species names (https://lpsn.dsmz.de/genus/helicobacter) which can be divided into two main groups: gastric (GH) and enterohepatic *Helicobacter* (EHH) species. The latter includes more than 60 % of the species of the genus, and in recent years the EHH group has been linked

to human diseases such as acute gastroenteritis, inflammatory bowel and hepatobiliar diseases, as well as extra-intestinal infections [1].

The EHH species have been isolated from a wide range of wild and domestic animals [2]. However, the pathogenic significance of these bacteria is uncertain as they have been detected both in healthy animals and in those with symptoms of intestinal or liver disease [2]. Wild birds are an important reservoir for EHH, with *H. pametensis* [3], *H. anseris*, *H. brantae* [4], *H. canadensis* [5], and *H. valdiviensis* [6] having been isolated from faecal samples of these hosts. Additionally, very recently two new species from birds were described in Turkey, "*H. anatolicus*" and *H. kayseriensis* [7]. Some of these species have also been linked to human infection. Indeed *H. canadensis* was originally isolated from diarrheic faeces [8], and *H. valdiviensis* has also been detected in human diarrheal stool specimens, alongside systemic infection [9]. Evidently, wild bird droppings can be a source of spreading EHH and other campylobacteria, as they contaminate parks, lakes, and urban areas, which could pose a risk to human health [10]. In the framework of a study developed in the city of Valdivia (southern Chile), on the prevalence and diversity of *Epsilonproteobacteria* in wild bird dropping samples, two bacterial strains (A82^T and WB-40) were subjected to phylogenetic analysis using the 16S rRNA and *hsp60* (60 kDa heat-shock protein) gene sequences, and were suspected of belonging to a not-yet described *Helicobacter* species. Whole genome-based analyses and phenotypic characterisation were consequently performed to determine their taxonomic positions. Based on our results, we propose and describe these strains as representing a novel species within the genus *Helicobacter*.

Isolation and identification

Strains A82^T and WB-40 were isolated in November 2020 and January 2022, respectively. The samples analysed were from faecal droppings excreted by birds in public urban green spaces in Valdivia. Faecal samples were collected using sterile cotton-tipped swabs that were immediately placed in 9 ml of Bolton broth (Oxoid). Incubation was performed at 37 °C for 48 h under micro-aerobic conditions (Anaerocult® C, Merck Millipore). After enrichment, 100 µL of incubated broth was inoculated on a modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid) plate, and incubated at 42 °C. Meanwhile 400 µL of broth was placed on the surface of a Millipore membrane filter (pore size 0.45 µm) on Columbia Blood Agar Base (Oxoid) supplemented with 5 % sheep blood (CBA) and was allowed to filter passively under room conditions for approx. 30 min. The filter was later removed with sterile forceps and discarded, while the plate was incubated at 37 °C for 48 h under the above-mentioned conditions. In cases where no growth was observed, incubation was extended for an additional 5–7 days [6]. Finally, along with several known *Campylobacter* and *Helicobacter* species, three isolates suspected of belonging to a new *Helicobacter* species were obtained from the 270 wild bird faecal droppings analysed. These isolates were genotyping by Enterobacterial repetitive intergenic consensus PCR [11] resulting in two strains with different genotypes (A82^T isolated on CBA and mCCDA, and WB-40 isolated only on CBA) (data not shown). In an attempt to identify the origin of the samples, faecal DNA was extracted using the Stool DNA Kit (Omega Biotek) and the mitochondrial COI (cytochrome c oxidase subunit 1) gene was amplified and sequenced using the universal primers BirdF1 and BirdR1, and alternative reverse primer BirdR2 [12]. Only one of the two bird faecal samples from which the strains under study were isolated generated a good quality

sequence (GenBank accession number OP236466), corresponding to strain WB-40. A BLASTN search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) identified the bird sequence as belonging to the black-faced ibis (*Theristicus melanopis*) with a 100 % identity score. It is one of the most abundant bird species observed at the sampling site.

Bacterial DNA was extracted from A82^T and WB-40 colonies using the Wizard® Genomic DNA Purification kit (Promega), in accordance with the manufacturer's instructions. Genus-identification was carried out by *Helicobacter* genus-specific PCR [13]. For identification at species level, the 16S rRNA and *hsp60* genes were amplified and sequenced as described by Vandamme *et al*. [14] and Hill *et al*. [15], respectively. DNA sequences were then compared directly with the NCBI non-redundant nucleotide database using BLASTN, and top hit sequences were retrieved for further comparisons. Sequence alignment was performed with ClustalW [16] and phylogenetic trees were reconstructed using the neighbour-joining method [17] as implemented using MEGA X software [18], with Kimura's two-parameter evolutionary distance model [19]. The stability of the groupings was estimated by bootstrap analysis based on 500 re-samplings. The phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) clearly showed that strains A82^T and WB-40 can be affiliated to the genus *Helicobacter*, most closely related to "*H. burdigaliensis*" CNRCH 2005/566H^T (97.10 % similarity) and *H. valdiviensis* WBE14^T (97.02 % similarity). Given that the two new isolates were closely related, forming a single cluster, it was hypothesized that they may represent a single species. This taxonomic situation of the novel strains was also confirmed by the *hsp60* -based phylogenetic tree (Fig. 2).

It is important to highlight the taxonomic similarity of the new taxon (putatively comprised of strains A82^T and WB-40) to *H. valdiviensis*, considering that both species were isolated in the same geographic area and from the same type of reservoir.

Genome analysis

Genomic DNA of strains A82^T and WB-40 was extracted using an Easy-DNA™ gDNA Purification Kit (Invitrogen), and further quantified by spectrophotometry (NanoQuant – Infinite M200, Tecan) and fluorometry (Qubit 3.0 fluorometer). Genomic libraries were prepared using the Illumina DNA Prep kit and IDT 10 bp UDI indices, and then sequenced on an Illumina NextSeq 2000 device at the Microbial Genomics Sequencing Center (MIGS, Pittsburgh, PA, USA). Reads were quality checked with FastQC v.0.11.9 [20], filtered with Trimmomatic v.0.39 [21], and finally assembled using SPAdes v3.13.1 [22] (option --careful). Assembly quality was assessed using Quast v5.0.2 [23]. Completeness and contamination of the genomes was estimated using CheckM v1.0.6 at default settings [24]. The genome sequences were annotated using Prokka v1.14.6 [25]. Sequence reads and assemblies were submitted to the NCBI database as BioProject PRJNA913774 with SRA accession numbers SRX18781885 and SRX18781886, and GenBank accession numbers JAQHXR000000000 and JAQHXS000000000 for strains A82^T and WB-40, respectively. The draft genome sequences of strain A82^T and WB-40 were approx. 1,593,022 bp and 1,638,157 bp long, respectively, with a DNA G+C content of 31.9 mol% and 32.0 mol%, respectively, which is in line with values reported for the genus *Helicobacter* [26]. The chromosome of the type strain A82^T is predicted to contain approx. 1,655 coding sequences (CDS).

Available genome sequences from all type strains of members of the genus *Helicobacter* were recovered from public databases and further used to carry out a phylogenomic analysis. Orthologous genes were determined using an all-versus-all BLASTP comparison among the translated CDS features of the annotated genomes under study, as previously described [27]. Translated single-copy core gene sequences were then individually aligned with Muscle v.3.8.31 [28] (options -maxiters 1 -diags) and concatenated into a super-protein alignment, which was further utilized to infer the phylogenomic tree by means of the approximately maximum-likelihood algorithm, as implemented in FastTreeMP v.2.1.8 [29]. Branch support values were estimated using the Shimodaira-Hasegawa test [30]. The new strains A82^T and WB-40 formed a separate lineage, different from all other *Helicobacter* spp. but closely related to "*H. burdigaliensis*" and *H. valdiviensis* (Fig. 3), confirming the results obtained from the initial single marker-based phylogenetic analyses (Figs. 1 and 2).

Overall Genome Relatedness Indexes (OGRI) were computed for the genomes of isolates A82^T and WB-40 and those of the type strains of the *Helicobacter* species. Specifically, Orthologous Average Nucleotide Identity (OrthoANI) was determined using the OrthoANIu tool [31] and the digital DNA-DNA hybridization (dDDH) was inferred by means of the Genome-to-Genome Distance Calculator (GGDC) (formula 2) [32]. The OrthoANI and dDDH values obtained for all the analysed genomes revealed that the two new isolated strains belonged to the same species (OrthoANI: 99.6 %, dDDH: 95.9%), while being different from the other *Helicobacter* species described to date (OrthoANI ≤73.6 % and dDDH ≤36.5 %) (Fig. S1).

Phenotypic characterisation

The new *Helicobacter* strains were biochemically, physiologically, and morphologically characterised according to the recommendations of On *et al*. [33]. All tests were performed in two time-independent assays with freshly prepared media. Growth of both strains was examined at 25 °C, 37 °C and 42 °C under microaerobic conditions (using Anaerocult® C, Merck Millipore), and at 37 °C under aerobic and anaerobic conditions (using AnaeroGen, Oxoid) for 48 to 72 h on CBA. An oxidase test was carried out using Bactident Oxidase strips (Merck) and catalase activity was evaluated by adding a 3 % H_2O_2 solution and observing bubble production within 5 s. Indoxyl acetate hydrolysis was determined as previously described by Mills and Gherna [34]. The following biochemical analyses were performed using the API-Campy identification system (bioMérieux) as per the manufacturers' instructions: urease activity, reduction of nitrate, esterase activity, hydrolysis of hippurate, γ-glutamyl transferase activity, reduction of triphenyl tetrazolium chloride, alkaline phosphatase activity, production of H_2S, assimilation of glucose, and pyrrolidonyl-, L-arginine-, and L-aspartate arylamidase activity. In addition, nitrate reduction was also tested using plate method of Cook [35] and γ-glutamyl transferase activity was confirmed using DIATABS™ Diagnostic Tablets for Bacterial Identification (Rosco). Tolerance to 1 % glycine, 1.5 % NaCl and 2 % NaCl was determined as previously described [36]. The motility and susceptibility to cephalothin (30 µg, Oxoid) and nalidixic acid (30 µg, Oxoid) were also determined, according to the Minimal Standards recommended for the families *Campylobacteraceae* and *Helicobacteraceae* [33]. All test were performed using the type and reference strains *Helicobacter valdiviensis* (CECT 8410^T); *H. apodemus* RAT 1 (=CCUG 73261); *H. canicola* CAD 722 (=CCUG 73259); *Campylobacter jejuni* (DSM 4688^T); *C. coli* (DSM

4689^T); *C. lari* (DSM 11375^T); *C. subantarcticus* (LMG 24377^T); *C. insulaenigrae* (LMG 22716^T); *C. volucris* (LMG 24380^T) and *Escherichia coli* (ATCC 25922) as controls. Morphological characteristics of the strain A82^T were observed using a transmission electron microscope (LIBRA 120 PLUS, Zeiss) after negative staining with 2 % uranyl acetate for 30 s. This strain showed a spiral shape with a pair of unsheathed bipolar flagella (Fig. 4). Based on the phenotypic characterisation, the new strains can be affiliated to the *Helicobacter* genus, since they comply with the typical characteristics of the genus described both in its original description [37] and in its subsequent emendation [38], such as to be Gram negative curved rods, nonsporeforming, motile by flagella, microaerophilic, with oxidase activity, chemoorganotrophs and asaccharolytic. Table 1 shows the most important phenotypic characteristics differentiating the novel strains from the phylogenetically closest *Helicobacter* species. An extended comparison with all *Helicobacter* taxa can be found in Supplementary Table S1.

In summary, the phylogenetic, genomic, and phenotypic results obtained demonstrate that the two isolates recovered from wild bird faecal samples represent a novel taxon, distinct from other currently known *Helicobacter* species. The name *Helicobacter ibis* sp. nov. is proposed, with A82^T (=LMG 32718^T= CCCT 22.04^T) as the type strain.

Description of *Helicobacter ibis* sp. nov.

Helicobacter ibis (i'bis. L. gen. n. *ibis*, of an ibis, indicating the source of a new *Helicobacter* strain).

Cells are Gram-stain negative, non-endospore forming, curved and S-shaped rods, approximately 0.3 μm wide and 1.0–1.8 μm long. They are motile by means of a pair of bipolar unsheathed flagella and do not have periplasmic fibres. After 72 h of incubation on Columbia Blood Agar Base (supplemented with 5 % sheep blood) at 37 °C in a microaerobic atmosphere, colonies are small, 1-2 mm in diameter, non-haemolytic, with a grey pigmented, slightly convex, and round with smooth margins. Coccoid cells were observed in old cultures (>96 h). The bacterium grows under microaerobic conditions at 37 °C and at 42 °C, but not at 25 °C. Weak growth was observed at 37 °C under anaerobic conditions, but not aerobically. No growth was obtained on media containing 1 % glycine nor media supplemented with 1.5 % or 2 % NaCl. Isolates are oxidase, catalase, alkaline phosphatase, and γ-glutamyl transpeptidase positive, but urease and esterase negative, and do not hydrolyse indoxyl acetate. They reduce nitrate, while L- triphenyl tetrazolium chloride, arginine arylamidase and L-aspartate arylamidase production is variable. No pyrrolidonyl arylamidase, hippurate hydrolysis or H_2S production was detected. Cells are resistant to cephalothin but susceptible to nalidixic acid. The clinical significance of this species is unknown.

The type strain, A82^T (=LMG 32718^T= CCCT 22.04^T), was isolated from wild bird faecal droppings in the city of Valdivia, Chile. The DNA G+C content of the type strain is 31.9 mol%. The 16S rRNA gene sequence and draft genome assembly of the type strain have been deposited in GenBank under accession numbers ON950425 and JAQHXR000000000, respectively. The isolate WB-40 is an additional strain of this species, and its GenBank accession numbers for the 16S rRNA gene and draft genome sequences are ON950426 and JAQHXS000000000, respectively.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Reference

1. **Ochoa S, Collado L.** Enterohepatic *Helicobacter* species - clinical importance, host range, and zoonotic potential. *Crit Rev Microbiol* 2021; 47:728-761. https://doi.org/10.1080/1040841X.2021.1924117
2. **Flahou B, Haesebrouck F, Smet A**. Non-*Helicobacter pylori Helicobacter* infections in humans and animals. In: Backert S, Yamaoka Y (editors). *Helicobacter pylori* Research, Japan: Springer; 2016. Pp 233-3369.
3. **Dewhirst FE, Seymour C, Fraser GJ, Paster BJ, Fox JG.** Phylogeny of *Helicobacter* isolates from bird and swine feces and description of *Helicobacter pametensis* sp.

nov. *Int J Syst Bacteriol* 1994; 44:553-560. https://doi.org/10.1099/00207713-44-3-553

4. **Fox JG, Taylor NS, Howe S, Tidd M, Xu S, Paster BJ, Dewhirst FE.** *Helicobacter anseris* sp. nov. and *Helicobacter brantae* sp. nov., isolated from feces of resident Canada geese in the greater Boston area. *Appl Environ Microbiol* 2006; 72:4633-4637. https://doi.org/10.1128/AEM.02876-05
5. **Waldenström J, On SL, Ottvall R, Hasselquist D, Harrington CS, Olsen B.** Avian reservoirs and zoonotic potential of the emerging human pathogen *Helicobacter canadensis*. *Appl Environ Microbiol* 2003; 69:7523-7526. https://doi.org/10.1128/AEM.69.12.7523-7526.2003
6. **Collado L, Jara R, González S.** Description of *Helicobacter valdiviensis* sp. nov., an *Epsilonproteobacteria* isolated from wild bird faecal samples. *Int J Syst Evol Microbiol* 2014; 64:1913–1919. https://doi.org/10.1099/ijs.0.057141-0
7. **Aydin F, Saticioglu IB, Ay H, Kayman T, Karakaya E, Abay S.** Description of the two novel species of the genus *Helicobacter*: *Helicobacter anatolicus* sp. nov., and *Helicobacter kayseriensis* sp. nov., isolated from feces of urban wild birds. *Syst Appl Microbiol* 2022; 45:126326. https://doi.org/10.1016/j.syapm.2022.126326
8. **Fox JG, Chien CC, Dewhirst FE, Paster BJ, Shen Z, Melito PL *et al*.** *Helicobacter canadensis* sp. nov. isolated from humans with diarrhea as an example of an emerging pathogen. *J Clin Microbiol* 2000; 38:2546-2549. https://doi.org/10.1128/JCM.38.7.2546-2549.2000
9. **Fresia P, Jara R, Sierra R, Ferrés I, Greif G, Iraola G, Collado L.** Genomic and clinical evidence uncovers the enterohepatic species *Helicobacter valdiviensis* as a potential

human intestinal pathogen. *Helicobacter* 2017;22: e12425. https://doi.org/10.1111/hel.12425

10. **Ahmed NA, Gulhan T**. *Campylobacter* in Wild Birds: Is It an Animal and Public Health Concern? *Front Microbiol* 2022; 12:812591. doi: 10.3389/fmicb.2021.812591
11. **Aydin F, Karakaya E, Kayman T, Abay S, Saticioglu IB**. *Helicobacter turcicus* sp. nov., a catalase-negative new member of the *Helicobacter* genus, isolated from Anatolian Ground Squirrel (*Spermophilus xanthoprymnus*) in Turkey. *Int J Syst Evol Microbiol* 2022; 72:5338. https://doi.org/10.1099/ijsem.0.005338
12. **Hebert PD, Stoeckle MY, Zemlak TS, Francis CM**. Identification of birds through DNA barcodes. *PLoS Biol* 2004; 2:e312. https://doi.org/10.1371/journal.pbio.0020312
13. **Fox JG, Dewhirst FE, Shen Z, Feng Y, Taylor NS, Paster BJ *et al***. Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. *Gastroenterology* 1998; 114:755–763. https://doi.org/10.1016/S0016-5085(98)70589-X
14. **Vandamme P, Holmes B, Bercovier H, Coenye T.** Classification of Centers for Disease Control group eugonic fermenter (EF)-4a and EF-4b as *Neisseria animaloris* sp. nov. and *Neisseria zoodegmatis* sp. nov., respectively. *Int J Syst Evol Microbiol* 2006; 56: 1801-1805.
15. **Hill JE, Paccagnella A, Law K, Melito PL, Woodward DL, Price L *et al*.** Identification of *Campylobacter* spp. and discrimination from *Helicobacter* and *Arcobacter* spp. by direct sequencing of PCR-amplified *cpn60* sequences and comparison to cpnDB, a chaperonin reference sequence database. *J Med Microbiol* 2006; 55:393-399. https://doi.org/10.1099/jmm.0.46282-0

16. **Thompson JD, Higgins DG, Gibson TJ.** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; 22:4673–4680. https://doi.org/10.1093/nar/22.22.4673

17. **Saitou N, Nei M.** The Neighbor-Joining Method - a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987; 4:406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

18. **Kumar S, Stecher G, Li M, Knyaz C, Tamura K**. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol Biol Evol* 2018; 35:1547-1549. https://doi.org/10.1093/molbev/msy096

19. **Kimura M.** A Simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide-sequences. *J Mol Evol* 1980; 16:111-120.

20. **Andrews S**. FastQC: a quality control tool for high throughput sequence data 2010. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

21. **Bolger AM, Lohse M, Usadel B.** Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014; 30:2114-2120. https://doi.org/10.1093/bioinformatics/btu170

22. **Bankevich A, Nurk S, Antipov D, Gurevich A, Dvorkin M, Kulikov A *et al.*** SPAdes: a new assembly algorithm and its applications to single-cell sequencing. *J Comp Biol* 2012; 19: 455-477. https://doi.org/10.1089/cmb.2012.0021

23. **Mikheenko A, Prjibelski A, Saveliev V, Antipov D, Gurevich A.** Versatile genome assembly evaluation with QUAST-LG. *Bioinformatics* 2018; 34:i142-i150. https://doi.org/10.1093/bioinformatics/bty266

24. **Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW**. CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Research* 2015; 25:1043-1055. http://www.genome.org/cgi/doi/10.1101/gr.186072.114.

25. **Seemann, T.** Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014; 30:2068-2069. https://doi.org/10.1093/bioinformatics/btu153

26. **On SL, Lee A, O'Rourke JL, Dewhirst FE, Paster BJ, Fox JG, Vandamme P.** *Helicobacter*. In: Whitman WB, Rainey F, Kampfer P, Trujillo M, Chun J, DeVos P, Hedlund B, Dedysh S, editors. Bergey's manual of systematics of Archaea and Bacteria. New York (NY): Wiley; 2015. https://doi.org/10.1002/9781118960608.gbm01073

27. **de la Haba RR, López-Hermoso C, Sánchez-Porro C, Konstantinidis KT, Ventosa A**. Comparative genomics and phylogenomic analysis of the genus *Salinivibrio*. *Front Microbiol* 2019; 10:2104. https://doi.org/10.3389/fmicb.2019.02104

28. **Edgar RC**. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004; 32:1792-1797. https://doi.org/10.1093/nar/gkh340

29. **Price MN, Dehal PS, Arkin AP.** FastTree 2- approximately maximum-likelihood trees for large alignments. *PLoS One* 2010; 5:e9490. https://doi.org/10.1371/journal.pone.0009490

30. **Shimodaira H, Hasegawa M**. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol Biol Evol* 1999; 16:1114-1116. https://doi.org/10.1093/oxfordjournals.molbev.a026201

31. **Yoon SH, Ha SM, Lim J, Kwon S, Chun J.** A large-scale evaluation of algorithms to calculate average nucleotide identity. *Antonie Van Leeuwenhoek* 2017; 110:1281-1286. https://doi.org/10.1007/s10482-017-0844-4

32. **Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M.** Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013; 14:60. https://doi.org/10.1186/1471-2105-14-60

33. **On SLW, Miller WG, Houf K, Fox JG, Vandamme P.** Minimal standards for describing new species belonging to the families *Campylobacteraceae* and *Helicobacteraceae*: *Campylobacter*, *Arcobacter*, *Helicobacter* and *Wolinella* spp. *Int J Syst Evol Microbiol* 2017; 67:5296-5311. https://doi.org/10.1099/ijsem.0.002255

34. **Mills CK, Gherna RL**. Hydrolysis of indoxyl acetate by *Campylobacter* species. *J Clin Microbiol* 1987; 25:1560-1561. https://doi.org/10.1128/jcm.25.8.1560-1561.1987

35. **Cook GT.** A Plate Test for Nitrate Reduction. *J Clin Pathol* 1950; 3:359-362. http://dx.doi.org/10.1136/jcp.3.4.359

36. **On SL, Holmes B.** Effect of inoculum size on the phenotypic characterization of *Campylobacter* species. *J Clin Microbiol* 1991; 29:923-926. https://doi.org/10.1128/jcm.29.5.923-926.1991

37. **Goodwin CS, Armstrong JA, Chilvers T, Peters M, Collins MD, Sly L *et al*.** Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov., respectively. *Int J Syst Bacteriol* 1989; 39:397-405**.** https://doi.org/10.1099/00207713-39-4-397

38. **Vandamme P, Falsen E, Rossau R, Hoste B, Segers P, Tytgat R *et al*.** Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic

descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol* 1991; 41:88-103. https://doi.org/10.1099/00207713-41-1-88

39. **Eaton KA, Dewhirst FE, Radin MJ, Fox JG, Paster BJ, Krakowka S *et al***. *Helicobacter acinonyx* sp. nov., isolated from cheetahs with gastritis. *Int J Syst Bacteriol* 1993; 43:99-106. https://doi.org/10.1099/00207713-43-1-99

40. **Stanley J, Linton D, Burnens AP, Dewhirst FE, On SL, Porter A *et al*.** *Helicobacter pullorum* sp. nov. -genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. *Microbiology* 1994; 140: 3441-3449. https://doi.org/10.1099/13500872-140-12-3441

41. **Shen Z, Fox JG, Dewhirst FE, Paster BJ, Foltz CJ, Yan L, *et al*.** *Helicobacter rodentium* sp. nov., a urease-negative *Helicobacter* species isolated from laboratory mice. *Int J Syst Bacteriol* 1997; 47:627-634. https://doi.org/10.1099/00207713-47-3-627

42. **Simmons JH, Riley LK, Besch-Williford CL, Franklin CL**. *Helicobacter mesocricetorum* sp. nov., A novel *Helicobacter* isolated from the feces of Syrian hamsters. *J Clin Microbiol* 2000; 38:1811-1817. https://doi.org/10.1128/JCM.38.5.1811-1817.2000

43. **Robertson BR, O'Rourke JL, Vandamme P, On SL, Lee A**. *Helicobacter ganmani* sp. nov., a urease-negative anaerobe isolated from the intestines of laboratory mice. *Int J Syst Evol Microbiol* 2001; 51:1881-1889. https://doi.org/10.1099/00207713-51-5-1881

44. **Shen Z, Mannion A, Lin M, Esmail M, Bakthavatchalu V, Yang S *et al***. *Helicobacter monodelphidis* sp. nov. and *Helicobacter didelphidarum* sp. nov., isolated from grey short-tailed opossums (*Monodelphis domestica*) with endemic cloacal prolapses. *Int J Syst Evol Microbiol* 2020; 70:6032-6043. https://doi.org/10.1099/ijsem.0.004424

45. **Gruntar I, Kostanjšek R, Pirš T, Papić B.** *Helicobacter colisuis* sp. nov., isolated from caecal contents of domestic pigs (*Sus scrofa domesticus*). *Int J Syst Evol Microbiol* 2022; 72:5600. https://doi.org/10.1099/ijsem.0.005600

Figures

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of Helicobacter ibis sp. nov. within the genus Helicobacter. Bootstrap values (≥70 %) based on 500 replicates are shown at the nodes. Bar, 0.01 substitutions per nucleotide position.

Fig. 2. Neighbour-joining tree based on hsp60 gene sequences showing the phylogenetic position of Helicobacter ibis sp. nov. within the genus Helicobacter. Bootstrap values (≥70 %) based on 500 replicates are shown at the nodes. Bar, 0.05 substitutions per nucleotide position.

Fig. 3. Approximately maximum-likelihood phylogenomic tree based on 318 concatenated core protein sequences of members of the genus Helicobacter showing the position of the new isolated strains. Ultrafast bootstrap values ≥70 % are indicated above the branches. Bar, 0.1 substitutions per nucleotide position.

Fig. 4. Transmission electron micrograph of strain A82T; bar, 2 µm.

Table

Table 1. Phenotypic characteristics differentiating Helicobacter ibis sp. nov. from other related species of the genus Helicobacter.

Table 1. Phenotypic characteristics differentiating *Helicobacter ibis* sp. nov. from other related species of the genus *Helicobacter. Helicobacter* species: **1**, *H. ibis* sp. nov. (Data from this study); **2**, *H. pylori* (Data from Goodwin *et al*. [37]; Eaton *et al*. [39]); **3**, *H. pullorum* (Stanley *et al*. [40]); **4**, *H. rodentium* (Shen *et al*. [41]); **5**, *H. mesocricetorum* (Simmons *et al*. [42]); **6**, *H. ganmani* (Robertson *et al*. [43]); **7**, *H. canadensis* (Fox *et al*. [8]); **8**, *H. valdiviensis* (Collado *et al*. [6]); **9**, *H. monodelphidis* (Shen *et al.* [44]); **10**, *H. turcicus* (Aydin *et al*. [11])*;* **11**, *H. colisuis* (Gruntar *et al.* [45]). **+**, 100 % of strains positive; **-,** 100 % of strain negative; **(+)**, 70–94 % of strains positive; **(-)**, 7–33 % of strains positive; **±**, 42-66 % of strains positive; **W**, weak growth; **S**, sensitive; **R**, resistant; **V**, variable; **ND**, not determined; **M**, monopolar; **B**, bipolar.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Catalase	+	+	(+)	+	+	-§	+	+	+	-	+
Nitrate reduction	+	-	+	+	(+)	+	±	-	(-)	-	+
Urease	-	+*	-	-	-	-	-	±	-	-	-
Alkaline phosphatase	+	+	-	-	+	-	-	-	+	+	+
γ-Glutamyl transpeptidase	+	+	ND	-	-	ND	-	-	+	-	V
Indoxyl acetate hydrolysis	-	-	-	-	ND	-	+	+	-	+	-
Growth at 42 °C (microaerobic)	+	-	+	(+)	+	-	+	+	-	+	+
Growth at 37 °C (anaerobic)	W	-	-	+	-	+	-	-	-	+	+
Growth on 1 % glycine	-	-	-	+	-	-	+	+	-	-	-
Susceptibility to (30 µg per disc):											
Nalidixic acid	S	R	S	R	S†	S‡	R	V	R	R	R
Cephalothin	R	S	R	R	R	R‡	R	R	R	R	R
No. of flagella	2	4-8	1	2	2	2	1-2	1	7-14	2	2
Sheathed flagella	-	+	-	-	-	-	-	-	-	-	-
Flagellar arrangement	B	M	M	B	B	B	B	M	B	B	B

*Goodwin et al. [37] reported that some strains lose their urease activity after being repeatedly subcultured.

§Weak reaction detected (in two out of seven strains).

†One out of six strains was resistant.

‡The tests were assessed in plates with 32 mg L^{-1} of the respective antibiotic.