Buck, et al.; **Ultrastructural and molecular characterization of non-*Helicobacter pylori* species in the gastric mucosa of naturally infected pigs.** Braz J Vet Pathol, 2018, 11(2), 42 – 49.

42

Original Full Paper

Ultrastructural and molecular characterization of non-*Helicobacter pylori* species in the gastric mucosa of naturally infected pigs

Letícia Y. Buck[1], Victor Marutani[1], Elis Lorenzetti[2], Amauri A. Alfieri[2], Ana Paula L. Bracarense[1*]

[1]Laboratory of Animal Pathology, Universidade Estadual de Londrina, Campus Universitário, Caixa Postal 10011, 86057-970, Londrina, Paraná, Brazil.
[2]Laboratory of Molecular Biology, Universidade Estadual de Londrina. Campus Universitário, Caixa Postal 10011, 86057-970, Londrina, Paraná, Brazil.
*Corresponding author: anapaula@uel.br

Abstract

Infection by *Helicobacter* spp. has been associated with gastritis and ulcers in pigs and humans. Association between *Helicobacter* species and lesions can contribute to determine specific pathogenicity. The aim of this study was to describe ultrastructural aspects of *Helicobacter* spp. and identify *Helicobacter* species by PCR assay. Gastric samples from 13 naturally infected sows were analyzed. From these, 12 were positive for *Helicobacter* spp. 16S rRNA gene and seven were identified as *H. suis*. The species was not identified in five samples and all samples were negative for urease gene. The sequencing of rRNA gene of five samples showed similarity with *H. suis* and *H. heilmannii* type I. Seven samples positive for *Helicobacter* genus generated no sequenceable fragments. On ultrastructural study, three samples showed helical bacteria measuring 4 to 6 μm long, 0.5 to 0.8 μm width, 4 to 8 spirals and 2 to 6 bipolar flagella. Two samples showed bacteria measuring 9 to 10 μm in length, 0.5 μm width, 22 to 24 spirals and no flagella, characterizing *Helicobacter* non-*H. pylori*, but these samples were negative for *H. suis*. In conclusion, the results indicate that adult pigs are commonly infected by helical bacteria presenting different ultrastructural characteristics, suggesting that mixed infection is frequent.

Key words: sows, scanning electron microscopy, PCR, ultrastructure.

Introduction

Helicobacter pylori (*H. pylori*) was the first species to be identified colonizing the stomachs of humans and is associated with the development of gastritis, ulcers, and gastric cancer (21). The discovery of the association between infection with *H. pylori* and gastric diseases increased the interest of the involvement of this bacterium in the development of gastropathy (25). Different species of *Helicobacter* have been identified within the gastric mucosa in various animal species, with or without associated gastric diseases (10).

H. pylori is the most prevalent species worldwide and can colonize the stomach of approximately 50% of a population (11). There is evidence that *Helicobacter* non-*H. pylori* can cause disease in humans, and that pigs are reservoirs of these bacteria with zoonotic potential (12). The contact with pigs increases the risk of becoming infected by *Helicobacter* non-*H. pylori* (6). The diagnosis of *Helicobacter* infection can be achieved through invasive (culture, histology, urease test) or non-invasive (serology, stool antigen test, urease breath test) methods (3).

Helicobacter suis (*H. suis*) is the most prevalent *Helicobacter* non-*H. pylori* species in humans. Gastric infection by *H. suis* in humans is observed in 36.6% of the samples (28). In addition, this agent might be transmitted to humans by manipulation or consumption of contaminated pork (6). In pigs, *Helicobacter* non-*H. pylori* infection has been associated with gastritis and ulcerative lesions of the pars oesophagea (22), and changes in epithelial proliferation and E-cadherin expression (2). Gastric ulceration in naturally *H. suis*-infected pigs was

associated with changes in the expression of inflammatory markers and acid secretion (8).

H. suis is a very fastidious microorganism and is extremely difficult to isolate, being considered uncultivable until 2008 (1). In subsequent years, 10 isolates originated from the stomachs of pigs have been cultured in vitro (15). Considering the difficulty in the culture of this microorganism, it is interesting to identify other methods for the detection and characterization of *Helicobacter* species. Therefore, the ultrastructural morphology associated with molecular biology techniques have been employed as useful tools in identifying infecting species. In this study we aimed to identify specific species of *Helicobacter* in the gastric mucosa of sows and to carry out the ultrastructural characterization of these species to try to better understand the bacterium species that might be involved in gastric diseases.

Material and methods

Samples

Samples of the stomach of 13 clinically normal, pluriparous, post-weaning sows (Large White crossed with Landrace) from a research facility were collected. The mean weight of the pigs was 170 ± 4 kg, with age varying between 15 to 24 months. All animals were fed twice daily and water was provided *ad libitum*.

Food and water were fasten for 12 h before gastroscopy and gastric biopsy. All animals were anaesthetized with ketamine (4 mg/kg, intramuscular) and continuous infusion of midazolam (0.5 mg/kg/h). Biopsy was performed with a flexible colonic endoscope (2.3 mm diameter biopsy channel) and two samples from three pre-determined gastric regions (cardia, fundus, and pyloric antrum) were collected side-by-side. Tissue fragments were sampled for ultrastructural analysis (maintained in glutaraldehyde plus paraformaldehyde solution) or polymerase chain reaction (maintained at -20°C). Mucosal appearance, content and the presence of bleeding at the biopsy site were evaluated during the endoscopic procedure. The study was approved by the institutional Ethics Committee for Animal Experimentation (number 5907).

Ultrastructural analysis

Gastric biopsies were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 20 h. The samples were then washed with sodium cacodylate buffer (0.1 M, pH 7.2) and treated with 1% osmium tetroxide in sodium cacodylate buffer for 1 h, subjected to gradual dehydration in ethanol (70, 80, 90 and 100%), and dried to the critical point (CPD 030 Critical Point BALTEC Dryer, Leica Microsystems, Liechtenstein).

After drying the samples were glued on stubs using carbon plate and coated with gold (Sputter Coater BALTEC SDC 050, Leica Microsystems, Liechtenstein). The specimens were analyzed using a FEI Quanta 200 scanning electron microscope.

Polymerase chain reaction (PCR)

Samples were treated with lysis buffer consisting of sodium dodecyl sulfate (SDS) and proteinase K at final concentration of 1% and 0.2 mg / mL, respectively. After homogenization, samples were incubated at 56ºC water bath for 3 h. The resulting material was subjected to DNA extraction and purification by the technique of phenol/chloroform/isoamyl alcohol followed by silica/guanidine isothiocyanate. A sample of autoclaved ultrapure water was used as negative control in all procedures. The material obtained remained stored at -20□C until PCR amplification.

PCR reactions were performed in a 25 µL final volume using 5 µL of DNA extracted, 0.5 µL (20 pmol) of each primer, 0.4 mM of each dNTP, 1 X PCR buffer (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 1.25 units of Platinum® Taq DNA polymerase (InvitrogenTM Life Technologies, São Paulo, Brazil), and 3 mM of MgCl2. Specific primers pairs were used in PCR assays designed to amplify amplicons of specific genes. These included the 16S gene for *Helicobacter* spp. (23), *H. suis* (7), *H. pylori* (5), *H. heilmannii* (19), *H. bizzozzeronni* (19), and *H. felis* (9) and the ureA and ureB genes of the *Helicobacter* genus (20). Amplification was performed in a thermocycler under time and temperature conditions determined for each primer pair. In all amplification reactions were used as known positive control a strain of *Helicobacter* spp., *H. pylori*, *H. suis*, *H. heilmannii*, *H. bizzozzeronni*, and *H. felis*. The DNA samples *H. baculiformis* (M50), *H. salomonis* (R1053), and *H. cynogastricus* (JKM4) (kindly provided by Dr. Smet, Ghent University, Belgium) were used as positive controls for the ureA and ureB genes in PCR assay and as negative control a sample of sterile distilled water was used.

The amplified products were analyzed by electrophoresis on a 2% agarose gel in TBE buffer, pH 8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA), stained with ethidium bromide (0.5 µg/mL) and visualized under UV light. Positive *Helicobacter* genus samples were tested for all five *Helicobacter* species-specific reactions.

Sequencing and phylogenetic analysis

The PCR amplicons were purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), quantified in a Qubit Fluorometer using Quant-iT dsDNA BR Assay Kit (Invitrogen Life Technologies, Eugene, Oregon, USA), and sequenced in an ABI3500 Genetic

Analyzer sequencer with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) using the forward and reverse primers. Sequence quality analyses and contig assembly were performed using Phred and CAP3 software (http://asparagin.cenargen.embrapa.br/phph/) respectively, and the sequences were accepted if base quality was ≥ 20. Sequence similarity searches were performed with sequences deposited in GenBank using the basic local alignment search tool (BLAST) software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree based on nucleotide sequences was obtained using the neighbor-joining method with the Kimura two-parameter model using MEGA software package (version 5.05). Bootstrapping was statistically supported with 1,000 replicates. Sequence identity matrix was performed using the BioEdit software version 7.1.3.0.

Results

All pigs exhibited moderate to severe hyperemia of gastric glandular mucosa, while moderate parakeratosis was observed in pars oesophagea of all animals. Four animals had focal ulcers at the aglandular region.

Ultrastructural analysis by scanning electron microscopy identified helical microorganisms in five samples (Table 1); helicobacteria were arranged as large colonies adhered to the surface of the gastric mucosa or as individualized bacteria immersed in the mucus next to the epithelium (Fig. 1).

Table 1. Results of different assays performed to identify *Helicobacter* species in samples from the stomachs of naturally infected sows. Polymerase chain reaction (PCR) to identify *Helicobacter* genus (16S rRNA) and *H. suis*. GenBank accession number resulting from the sequences obtained in this study. Presence (+) or absence (-) of *Helicobacter* in samples analyzed by scanning electron microscopy (SEM).

Samples	PCR for 16S rRNA	GenBank acession number	PCR for *H. suis*	SEM
1	+	KR066790	+	No *Helicobacter*
2	+	KR066791	+	No *Helicobacter*
3	+	Not sequenceable	+	No *Helicobacter*
4	+	Not sequenceable	-	Presence of *Helicobacter*
5	+	Not sequenceable	-	No *Helicobacter*
6	-	-	-	No *Helicobacter*
7	+	Not sequenceable	-	Presence of *Helicobacter**
8	+	KF282619	-	Presence of *Helicobacter*
9	+	KR066792	+	Presence of *Helicobacter*
10	+	KR066793	+	No *Helicobacter*
11	+	Not sequenceable	+	Presence of *Helicobacter**
12	+	Not sequenceable	+	No *Helicobacter*
13	+	Not sequenceable	-	No *Helicobacter*

+ or - means a positive or negative PCR reaction for the presence of *Helicobacter* spp. (16S rRNA) or *H. suis*; SEM – scanning electron microscopy; **Helicobacter* measuring 9 to 10 µm in length.

The morphology of the bacteria observed in three gastric samples was characterized by 4-6 µm in length, 0.6 to 0.8 µm in diameter, 4-8 spiral with an interval of 0.75 µm, no periplasmic fibrils and two from six bipolar flagella (Fig. 2). In two samples (7 and 11), two types of helical bacteria were observed: (i) 4-6 µm in length helical bacteria as described above; (ii) helical bacteria measuring 9 to 10 µm in length and 0.5 µm in diameter. The number of spirals observed in this bacterium was 22 (sample 7) and 24 (sample 11) with an interval between spirals of 0.4 µm. No flagella was observed at the end of these bacteria (Fig. 3A).

Bacteria with different characteristics, as coccoid bacteria, large bacilli, small rods, rod chain with transverse septa, and palisade disposition were also observed (Fig. 3B). These bacteria were on the surface of seven gastric samples, including the five specimens that were positive for *Helicobacter* spp. infection (Table 1).

Primers were used to identify the segment of the 16S and the ureA and ureB genes, and species-specific primers. From 13 tested samples, 12 were positive for the 16S gene of *Helicobacter* spp. and seven of these for *H. suis*; however, quality sequence was only obtained from five samples (Table 1). The ureA and ureB genes were not amplified; the 16S PCR assays designed to amplify *H. pylori*, *H. heilmannii*, *H. felis* and *H. bizzozzeronni* yielded negative results.

The 12 positive samples were subjected to 16S rRNA gene sequencing, but only five fragments generated were viable for analysis (Table 1). The strains analyzed in this study presented 100% of nucleotide identity each other and 99.1% to 100% of nucleotide identity with *H. suis* and *H. heilmannii* type 1 strains (Fig. 4).
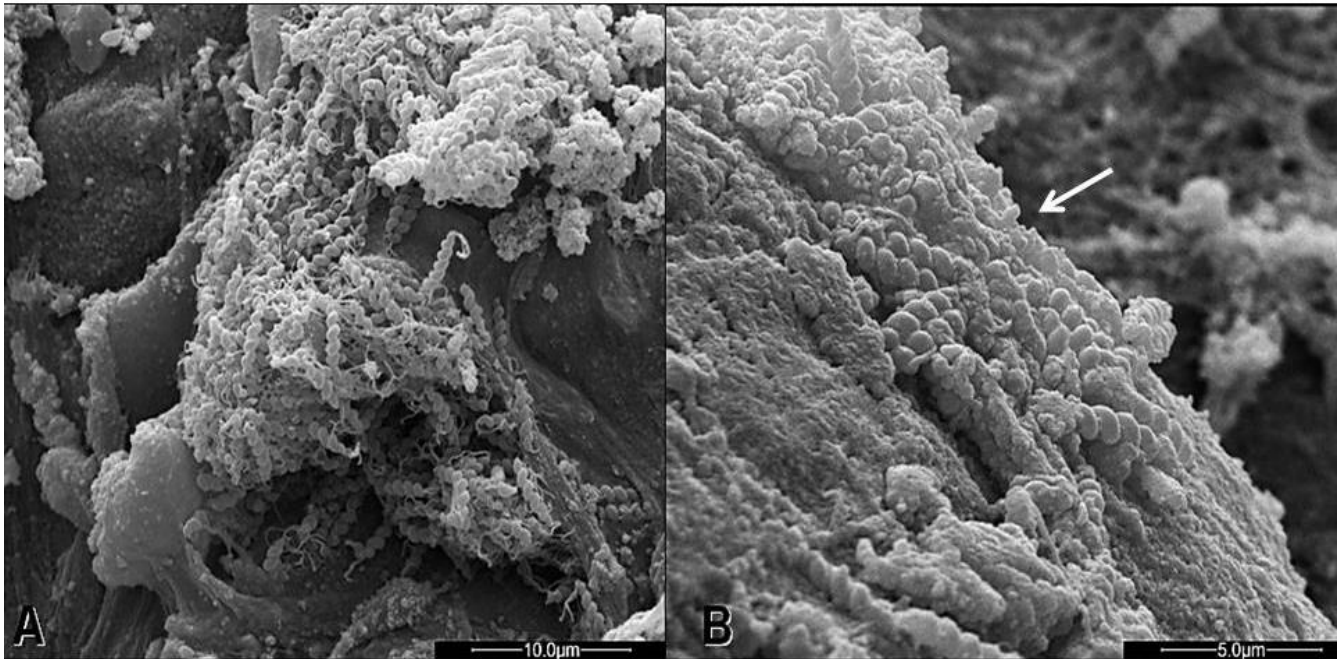
Figure 1. Scanning electron microscopy of pig gastric glandular mucosa naturally infected with *Helicobacter* spp. Presence of *Helicobacter* non-*H. pylori* adhered to gastric surface. **A.** Colony of helical bacteria presenting morphological characteristics of *Helicobacter* non-*H. pylori*. Bar=0 μm. **B.** *Helicobacter* non-*H. pylori* (arrow) on mucus glandular surface.   Bar=5μm.
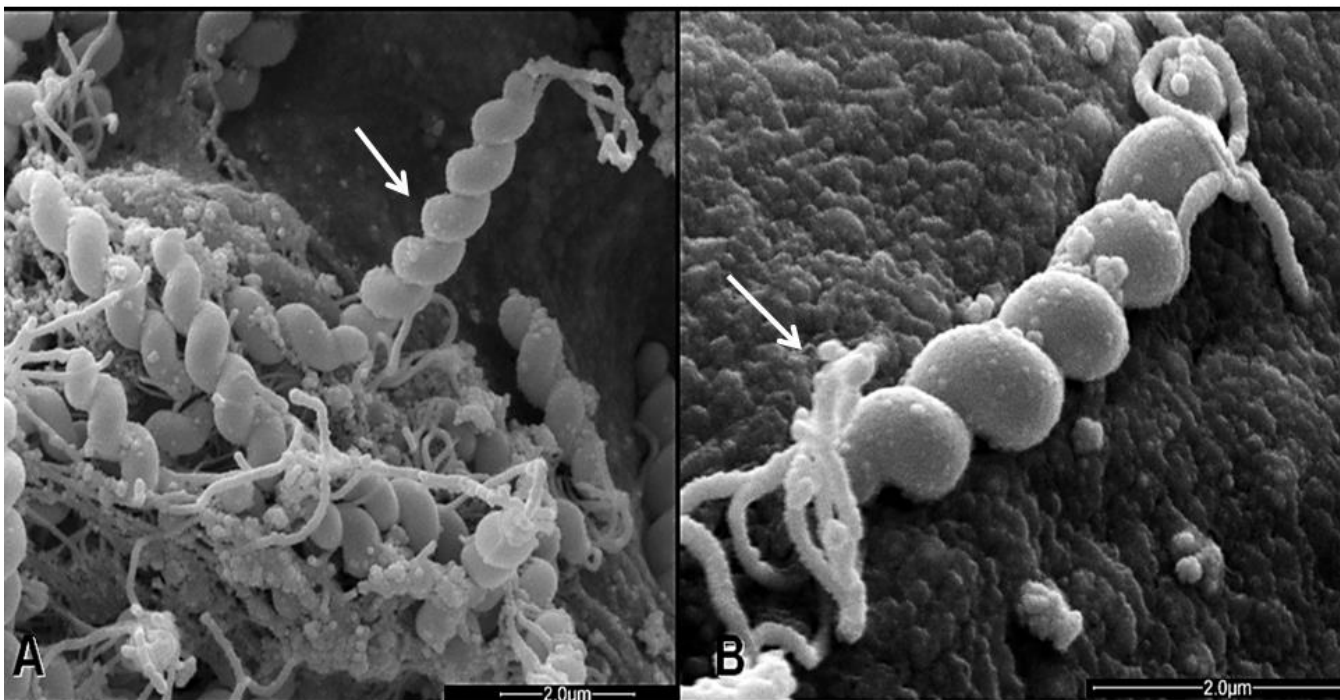
Figure 2. Scanning electron microscopy of pig gastric glandular mucosa. **A.** Bacteria with typical morphology of *Helicobacter* non-*H. pylori* (arrow); 4 to 6 μm in lenght, 4 to 6 spiral and multiple bipolar flagella. Bar=2 μm. **B.** Helical bacteria presenting 3 to 4 bipolar flagella (arrow), 5 μm in lenght and 6 spiral with no evidence of periplasmatic fibers. Bar=2 μm.
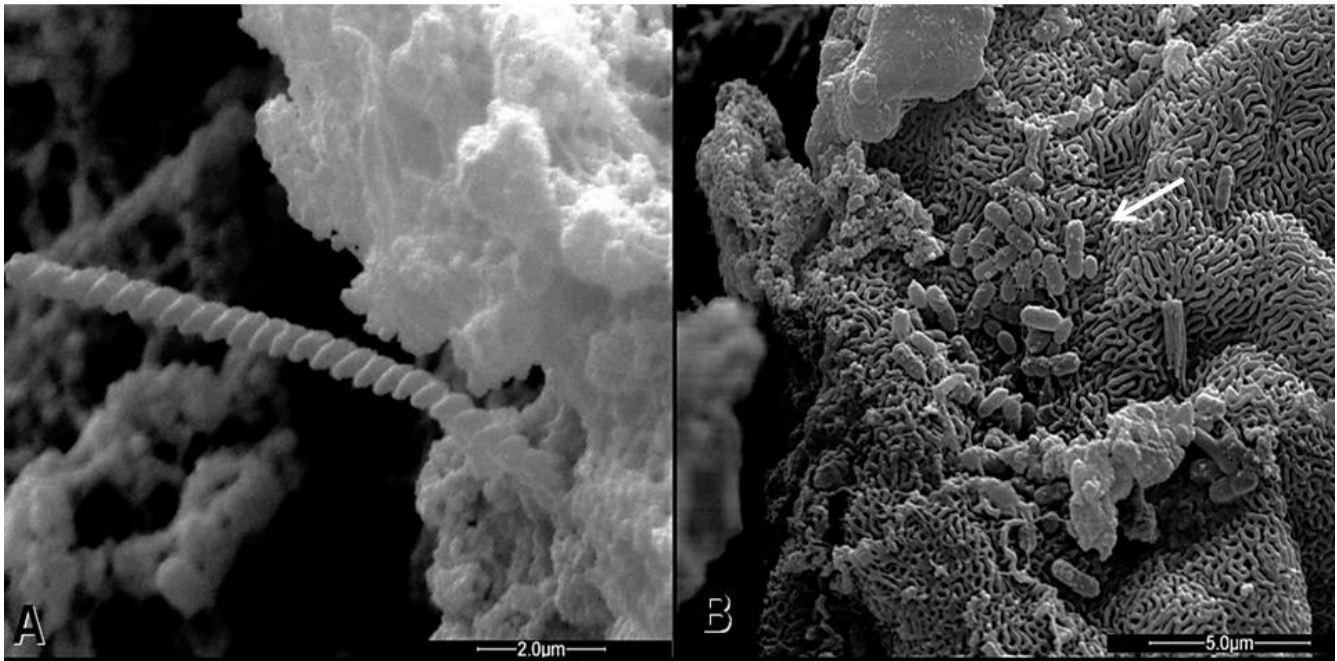
Figure 3. Scanning electron microscopy of pig gastric glandular mucosa. **A.** Spiral bacteria (sample 7) presenting 7 to 8 µm in length, 22 spiral and no flagella. Bar 2=µm. **B.** Bacteria exhibiting cocoid morphology or small rods (arrow) with 0.8 µm in length adhered to glandular gastric surface. Bar 5=µm.

Discussion

First efforts to characterize the different species of spiral organisms were based primarily on morphological features (17). The size and diameter of the organism, the number of coils and flagella were used to characterize different species in humans and animals (27); however, this methodology is not an accurate method for species identification (12). In a previous study with slaughtered pigs, we detected *Helicobacter* infection in 70% of the samples by polymerase chain reaction (2) accompanied by histological lesions in glandular gastric mucosa, and changes in epithelial proliferation and E-cadherin expression. However, the morphology of the bacteria in natural infections, and the real identity of the bacteria remain unclear. This study was designed to address these aspects characterizing *Helicobacter* specific species in older pigs (15 to 24 months) using molecular and morphological assays.

Scanning electron microscopy is not a highly sensitive diagnostic method, because we evaluated a small area of the gastric surface that can show no infection. It is known that *Helicobacter* genus have a tendency to colonize the stomach of animals heterogeneously (25). However, morphological assessment of bacteria using electron microscopy is important for species characterization (27). During this study, SEM was useful to confirm the morphological characteristics of bacteria colonizing the stomachs of naturally infected pigs. In addition, we observed the capacity of the bacteria to penetrate the gastric mucous along the surface, suggesting a close interaction with the mucosa. Moreover, the animals showing gastric ulcers at endoscopic evaluation were positive for *Helicobacter* reinforcing the association between infection and histological changes.

The first ultrastructural characterization of spiral microorganisms from pig gastric mucosa identified *Gastropirillum suis* (17), predominantly by transmission electron microscopy, with morphological features similar to those observed in three samples in this study (4 µm in length, 3 to 8 spiral turns and two to six bipolar flagella). In addition, most of the characteristics observed in the present study were compatible to those described for *H. suis* (12). However, some of these features were also attributed to *H. heilmannii* and *H. bizzozeronni* in other domestic pet animals (27). Alternatively, we have observed in two samples a different phenotype of spiral bacteria (greater length, smaller diameter, 22 to 24 spiral turns and no flagella). These characteristics correspond with the morphology described for *H. pullorum*, "*Helicobacter westmeadii*" and *H. cholecystus* species (26). Other species (*H. bilis, H. heilmannii* type 2 and *H. pullorum*) have been reported infecting the stomach of pigs (18). These data reinforce the necessity to investigate other species of *Helicobacter* colonizing pigs' gastric mucosa.
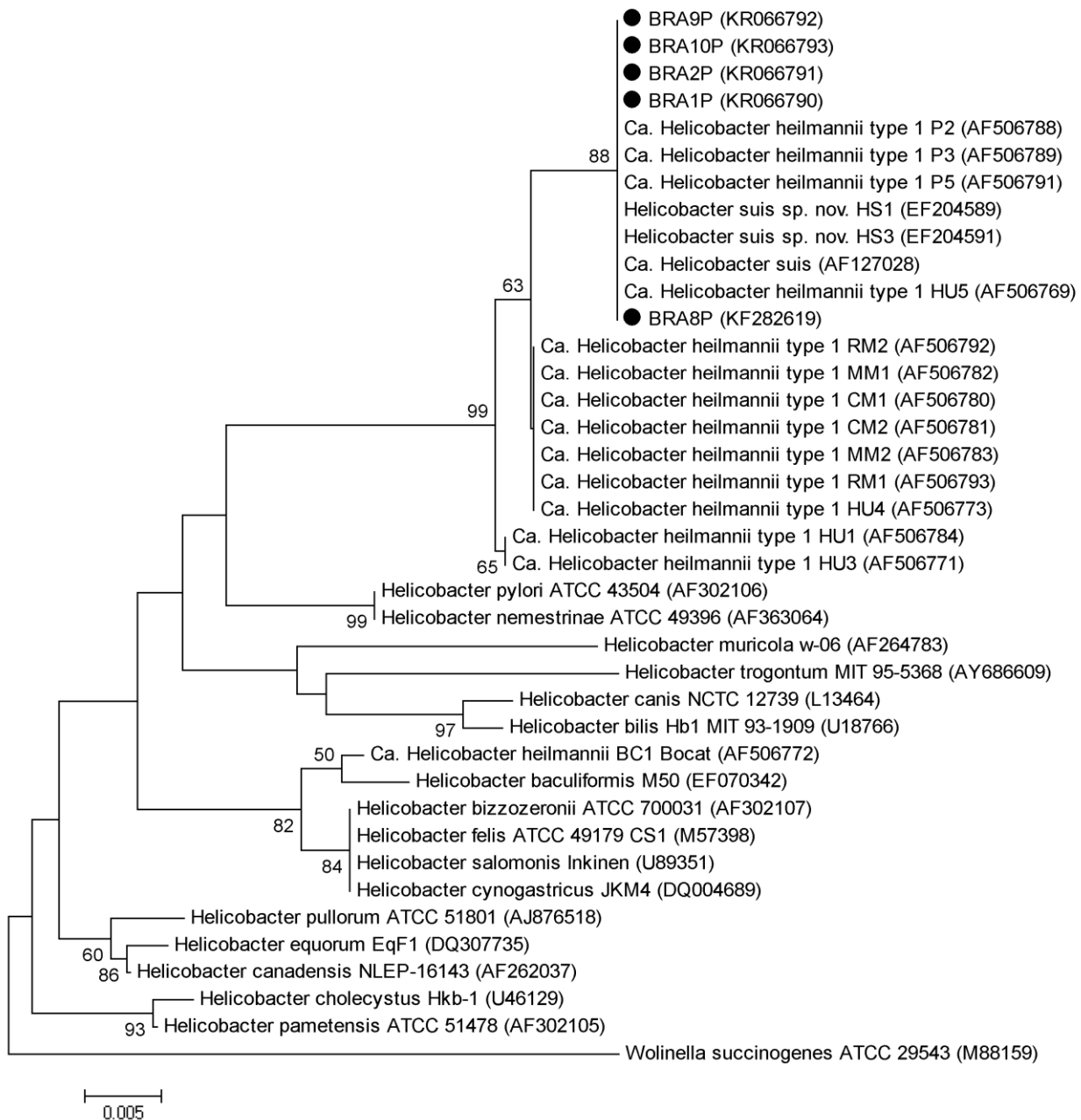
Figure 4. Neighbor-joining phylogenetic tree reconstruction using the Kimura two-parameter model based on partial (358 nt)16S rRNA gene of *Helicobacter* spp. Bootstraps values (1,000 replicates) higher than 50% are shown. GenBank accession numbers are given in parentheses. The sequences from this study are marked with filled circles.

Other types of bacteria have also been observed laying the gastric mucosa; coccoid forms, large bacilli or small rods were observed isolated or in conjunction with spiral bacteria in this study. The morphology of the microorganisms does not enable accurate identification, but their presence indicates that other genuses of bacteria are able to colonize the stomach of pigs. Another hypothesis is that *Helicobacter* can alter their morphological features according to the environment in which they are located, losing helical shape or assuming coccoid forms (29). However, morphological alterations of some species of *Helicobacter* were only reported under culture conditions; no studies relative to morphological alterations in vivo were located when major databases were accessed.

Twelve of the 13 pigs evaluated were positive for 16S *Helicobacter* genus by PCR. This high infection rate was expected as Hellemans and colleagues (13) reported that older animals, such as sows, may have a prevalence up to 90%. Interestingly, in this study, *H. suis* was identified

in 58% of the positive samples for the genus differing from literature data, in which this species is reported as the most frequent in pigs (3). PCR with specific primers for *H. suis* is the most specific and sensitive method for the detection of this agent in stomachs of pigs, and genus positive samples were also positive for *H. suis* (22).

During this study, five samples were PCR positive for the 16S rRNA gene but negative for all the specific species investigated. Furthermore, in three of these samples, helical morphology compatible with *Helicobacter* non-*H. pylori* bacteria were observed by SEM. In previous studies, the identification and analysis of ureA and ureB gene has been used to determine the infective species (20, 16). There was no positive sample for both ureA and ureB genes in this study. Our hypothesis is that the infecting species do not have this gene or the gene differs from other *Helicobacter* species known. Matsui and colleagues (16) reported that despite the presence of urease genes, this urease activity in stomach may be undetectable in *H. suis* infection. So far, there is no clear answer on the urease-negative strains of *H. suis* and how these bacteria can colonize the stomachs of humans and other animals, such as pigs. Probably these samples might have been a different species or strain that was not identified by the techniques and primers used during this study.

As other species have been described in the stomach of pigs, such as *H. pullorum* and *H. bilis* (4, 24), it is necessary to evaluate the possible presence of these species alone or in association with *H. suis*, as well as their association in producing alterations in gastric mucosa. Other assays, such as immunohistochemistry and fluorescent in situ hybridization are reported as specific diagnostic methods for *Helicobacter* detection (14). In this study, morphological and molecular differences were observed in bacteria infecting sows, indicating that mixed infections of *Helicobacter* occur in natural conditions.

In conclusion, our observations suggest that *H. suis* is not the only species of the genus *Helicobacter* that colonizes the stomach of pigs. Molecular biology techniques and morphological evaluation of *Helicobacter* non-*H. pylori* are useful for the characterization of these bacteria.

Acknowledgements

References

1. Baele M, Decostere A, Vandamme P, Ceelen L, Hellemans A, Mast J, Chiers K, Ducatelle R, Haesebrouck F. Isolation and characterization of *Helicobacter suis* sp. nov. from pig stomachs. Int J System Evol Microbiol. 2008;58:1350-8.

2. Bracarense AP, Yamasaki L, Silva EO, Oliveira RL, Alfieri AA. *Helicobacter* spp. infection induces changes in epithelial proliferation and E-cadherin expression in the gastric mucosa of pigs. J Comp Pathol. 2013;149:402-9.

3. Calik Z, Karamese M, Acar O, Aksak Karamese S, Dicle Y, Albayrak F, Can S, Guvendi B, Turgut A, Cicek M, Yazgi H. Investigation of *Helicobacter pylori* antigen in stool samples of patients with upper gastrointestinal complaints. Braz J Microbiol. 2016;47:167-71.

4. Cantet F, Magras C, Marais A, Federighi M, Megraud F. *Helicobacter* species colonizing pig stomach: molecular characterization and determination of prevalence. Appl Environ Microbiol. 1999;65:4672-6.

5. Clayton CL, Kleanthous H, Coates PJ, Morgan DD, Tabaqchali S. Sensitive detection of *Helicobacter pylori* by using polymerase chain reaction. J Clin Microbiol. 1992;30:192-200.

6. De Cooman L, Houf K, Smet A, Flahou B, Ducatelle R, De Bruyne E, Pasmans F, Haesebrouck F. Presence of *Helicobacter suis* on pork carcasses. Int J Food Microbiol. 2014;187:73-6.

7. De Groote D, Van Doorn LJ, Van den Bulck K, Vandamme P, Vieth M, Stolte M, Debongnie JC, Burette A, Haesebrouck F, Ducatelle R. Detection of non-pylori *Helicobacter* species in "*Helicobacter heilmannii*"-infected humans. Helicobacter. 2005;10:398-406.

8. De Witte C, Devriendt B, Flahou B, Bosschem I, Ducatelle R, Smet A, Haesebrouck F. *Helicobacter suis* induces changes in gastric inflammation and acid secretion markers in pigs of different ages. Vet Res. 2017;48:34.

9. Ferrero RL, Labigne A. Cloning, expression and sequencing of *Helicobacter felis* urease genes. Mol Microbiol. 1993;9:323-33.

10. Flahou B, Haesebrouck F, Smet A, Yonezawa H, Osaki T, Kamiya S. Gastric and enterohepatic non-*Helicobacter pylori* Helicobacters. Helicobacter. 2013;18:66-72.

11. Goh KL, Chan WK, Shiota S, Yamaoka Y. Epidemiology of *Helicobacter pylori* infection and public health implications. Helicobacter. 2011;16:1-9.

12. Haesebrouck F, Pasmans F, Flahou B, Chiers K, Baele M, Meyns T, Decostere A, Ducatelle R. Gastric helicobacters in domestic animals and nonhuman primates and their significance for human health. Clin Microbiol Rev. 2009;22:202-23.

13. Hellemans A, Chiers K, De Bock M, et al. Decostere A, Haesebrouck F, Ducatelle R, Maes D. Prevalence of 'Candidatus *Helicobacter suis*' in pigs of different ages. Vet Rec. 2007;161:189-92.

14. Kocsmár É, Szirtes I, Kramer Z, Szijártó A, Bene L, Buzás GM, Kenessey I, Bronsert P, Csanadi A, Lutz L, Werner M, Wellner UF, Kiss A, Schaff Z, Lotz G. Sensivity of *Helicobacter pylori* detection by Giemsa staining is poor in comparison with immunohistochemistry and fluorescent in situ hybridization and strongly depends on inflammatory activity. Helicobacter. 2017;22:12387.

15. Liang J, Ducatelle R, Pasmans F, Smet A, Haesebrouck F, Flahou B. Multilocus sequence typing of the porcine and human gastric pathogen *Helicobacter suis*. J Clin Microbiol. 2013;51:920-6.

16. Matsui H, Takahashi T, Murayama SY, Uchiyama I, Yamaguchi K, Shigenobu S, Matsumoto T, Kawakubo M, Horiuchi K, Ota H, Osaki T, Kamiya S, Smet A, Flahou B, Ducatelle R, Haesebrouck F, Takahashi S, Nakamura S, Nakamura M. Development of new PCR primers by comparative genomics for the detection of *Helicobacter suis* in gastric biopsy specimens. Helicobacter. 2014;19:260-71.

17. Mendes EN, Queiroz DM, Rocha GA, Moura SB, Leite VH, Fonseca ME. Ultrastructure of a spiral micro-organism from pig gastric mucosa ("*Gastrospirillum suis*"). J Med Microbiol. 1990;33:61-6.

18. Mladenova-Hristova I, Grekova O, Patel A. Zoonotic potential of *Helicobacter* spp. J Microbiol Immunol Infect. 2017;S1684-1182:30055-5.

19. Neiger R, Dieterich C, Burnens A, Waldvogel A, Corthésy-Theulaz I, Halter F, Lauterburg B, Schmassmann A. Detection and prevalence of *Helicobacter* infection in pet cats. J Clin Microbiol. 1998;36:634-7.

20. O'Rourke JL, Solnick JV, Neilan BA, Seidel K, Hayter R, Hansen LM, Lee A. Description of 'Candidatus *Helicobacter heilmannii*' based on DNA sequence analysis of 16S rRNA and urease genes. Int J Syst Evol Microbiol. 2004;54:2203-11.

21. Plummer M, Franceschi S, Vignat J, Forman D, de Martel C. Global burden of gastric cancer attributable to *Helicobacter pylori*. Int J Cancer. 2015;136(2):487-90.

22. Proietti CP, Bietta A, Brachelente C, Lepri E, Davidson I, Franciosini MP. Detection of *Helicobacter* spp. in gastric, fecal and saliva samples from swine affected by gastric ulceration. J Vet Sci. 2010;11(3):221-5.

23. Riley LK, Franklin CL, Hook RR, Jr., Besch-Williford C. Identification of murine helicobacters by PCR and restriction enzyme analyses. J Clin Microbiol. 1996;34:942-6.

24. Roosendaal R, Vos JH, Roumen T, van Vugt R, Cattoli G, Bart A, Klaasen HL, Kuipers EJ, Vandenbroucke-Grauls CM, Kusters JG. Slaughter pigs are commonly infected by closely related but distinct gastric ulcerative lesion-inducing gastrospirilla. J Clin Microbiol. 2000; 38:2661-4.

25. Rossi M, Hanninen ML. *Helicobacter* spp. other than *H. pylori*. Helicobacter. 2012;17:56-61.

26. Solnick JV, Schauer DB. Emergence of diverse *Helicobacter s*pecies in the pathogenesis of gastric and enterohepatic diseases. Clin Microbiol Rev. 2001;14:59-97.

27. Stoffel MH, Friess AE, Burnens A, Schmassmann A, Neiger R. Distinction of gastric *Helicobacter* spp. in humans and domestic pets by scanning electron microscopy. Helicobacter. 2000;5:232-9.

28. Van den Bulck K, Decostere A, Baele M, Driessen A, Debongnie JC, Burette A, Stolte M, Ducatelle R, Haesebrouck F. Identification of non-*Helicobacter pylori* spiral organisms in gastric samples from humans, dogs, and cats. J Clin Microbiol. 2005;43:2256-60.

29. Utriainen M, Jalava K, Sukura A, Hanninen ML. Morphological diversity of cultured canine gastric *Helicobacter* spp. Comp Immunol Microbiol Infect Dis. 1997;20:285-97.