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Presence of *Helicobacter suis* on pork carcasses

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ABSTRACT

Helicobacter (*H.*) *suis* is a world-wide spread pathogen which not only colonizes the stomach of pigs, but is also the most prevalent gastric non-*H. pylori Helicobacter* (NHPH) species in humans. *H. suis* infections are associated with gastric lesions both in pigs and in humans. Recently, the presence of viable *H. suis* bacteria has been demonstrated in minced pork, suggesting that manipulation or consumption of contaminated pig meat is a possible route of transmission of this zoonotic agent. The main goal of this study was to determine the extent of pork carcass contamination with *H. suis* at slaughter. In two consecutive studies, the occurrence of *H. suis* DNA was assessed in scalding water, head and mouth swabs, mesenteric lymph nodes, palatine tonsils and on the chest, shoulder and ham region of pork carcasses from three slaughterhouses using qPCR with *ureA* gene based *H. suis*-specific primers. *H. suis* DNA was detected on carcasses in all slaughterhouses, in 8.3% of all 1083 samples. It was found in all sampled matrices, except for the palatine tonsils and scalding water samples. Contamination levels of dressed pork samples did not exceed 184 genomic equivalents per 100 cm^2 (shoulder, ham) or 300 cm^2 (chest). All positive PCR products were subjected to sequence analysis of the *ureA* gene to confirm the identification of *H. suis* bacteria. Using multilocus sequence typing (MLST) on a selection of the positive samples, 5 unique sequence types (STs) could be assigned. Multiple *H. suis* strains were present on samples derived from one specific pig herd. Since *H. suis* DNA was detected in 11% (n: 90) of the mesenteric lymph nodes derived at the slaughterhouse, it was determined whether these organisms can colonize the mesenteric lymph nodes after experimental infection. Despite high-level colonization of the porcine stomachs with the *H. suis* strain, no *H. suis* DNA was detected in the mesenteric lymph nodes at four weeks after experimental infection. This might indicate that its presence in these tissues of slaughtered pigs is due to contamination during the slaughter process, but further studies are necessary to confirm this. In conclusion, we demonstrate a relatively high prevalence of *H. suis* on pork carcasses.

1. Introduction

In 1990, Queiroz et al., studied the role of pigs as a possible reservoir for *Helicobacter* (*H.*) *pylori*-like organisms. Instead of *H. pylori*-like organisms, not previously described Gram negative, large spiral-shaped bacteria were found in the mucus layer of lumen and antral pits of the pig stomachs. Initially, the name "*Gastrospirillum suis*" was proposed. Subsequent characterization, however, showed that this bacterium belonged to the genus *Helicobacter* (De Groote et al., 1999) and after the first successful *in vitro* isolation, the name *H. suis* was accepted (Baele et al., 2008). *H. suis* bacteria have been detected in up to 60% of pig stomachs, with prevalences depending on the age of the animals, as well as on the geographic region (Hellemans et al., 2007; Kopta et al., 2010). Pigs, however, are not the only possible hosts for *H. suis* bacteria. These organisms have been detected in human gastric biopsies more frequently than any of the other gastric non-*H. pylori Helicobacter* (NHPH) species causing gastric diseases such as gastritis, gastric ulcers and gastric cancer (Debongnie et al., 1995, 1998; Haesebrouck et al., 2009; Joo et al., 2007; Morgner et al., 1995).

The exact routes of transmission from pigs to humans remain unclear. Recently, the presence of viable *H. suis* bacteria has been reported in minced pork, suggesting that manipulation or consumption of contaminated pork is a possible route of transmission of *H. suis* bacteria (De Cooman et al., 2013). To date, there are no other studies reporting the occurrence of *H. suis* bacteria in pork and no information on carcass contamination is available. The contribution of food of animal origin to human infection with *H. suis* therefore remains unclear.

The main aim of this study was, therefore, to investigate *H. suis* occurrence on pork carcasses. Samples were taken at seven stages of slaughter, in three abattoirs, and examined quantitatively for the presence of *H. suis* DNA. Multilocus sequence typing (MLST) was used to obtain insight in the heterogeneity of the *H. suis* population present on pork carcasses.

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2. Materials and methods

2.1. Sample collection and preparation

The slaughter process, which is generally standardized in all Belgian slaughterhouses, is as follows. After stunning, the animals are secured by the hind legs to an overhead conveyer rail. The gas or electric stunned animals are stuck immediately by cutting the main blood vessel in the chest. After bleeding, the animals are scalded and dehaired mechanically. The pigs are then secured to the overhead conveyer, singed by gas burners, eviscerated, and next cut along the midline by using an automatic splitting saw.

In a first study, 363 samples originating from three pig herds were collected in three different Belgian abattoirs (A, B, C). The samples were taken at five sampling points, but without following the same carcasses through the slaughter process. In each slaughterhouse, 30 samples from the head and 30 samples from the mouth were collected immediately after automatic splitting of the carcasses, using cotton swabs (Cultiplast®, LP Italiana Spa, Milano, Italy) which were moistened in buffered peptone water. Furthermore, 30 mesenteric lymph nodes were removed immediately after evisceration and placed into sterile plastic bags for transport to the laboratory. In the lab, the surface of the lymph nodes was seared and cut aseptically into small pieces. Next, the lymph nodes were homogenized and 40 mg was transferred into 1.5 ml Eppendorf tubes, which were then kept at −20 °C until DNA extraction. Thirty palatine tonsils were excised and treated the same way as the lymph nodes. Finally, 2 l of scalding water was collected from each slaughterhouse, after at least 2 h of slaughter activities.

A second study was carried out in slaughterhouse C that was visited on three occasions. On each occasion, all samples originated from only one pig herd, but again without following the same carcass through the slaughter process. Each time, 30 mesenteric lymph nodes were collected immediately after evisceration, 30 samples from the mouth were taken after mechanical splitting and 30 samples were collected from the ham, shoulder and chest region of carcasses on the slaughterline and once again of carcasses after 6 h of chilling by forced ventilation. Mesenteric lymph nodes were treated as described above. For collection of samples from the mouth and the carcasses, sponge-sticks (3M™ Sponge-Sticks, St. Paul, USA) were used, which were moistened in 10 ml buffered peptone water. A 100 cm^2 area of the ham and shoulder and a 300 cm^2 area of the chest was sampled, according to the EU Decision 2001/471/EC of 8 June 2001. All mouth and carcass samples were stored in plastic, sterile bags at −20 °C until DNA extraction.

2.2. DNA extraction

Head and mouth swabs, collected during the first study, were placed in Eppendorf tubes, containing 250 μl buffered peptone water. After vortexing for 1 min, the swabs were placed in tips, which were then placed in Eppendorf tubes, and then centrifuged at 9300 ×*g* for 5 min. The obtained pellet was kept and 100 μl Prepman Ultra (Invitrogen, Carlsbad, CA, USA) was added. Further conditions used for DNA extraction, were according to the manufacturer's protocol.

One liter of each scalding water sample, collected during the first study, was filtered using the PowerWater® DNA Isolation kit (Mobio Laboratories, Inc., Carlsbad, USA), applying small adaptations to the protocol, using a 0.22 μm filter membrane and adding an additional centrifugation step at 800 ×*g* for 5 min.

For DNA extraction from the tonsils and mesenteric lymph nodes, collected during the first and second study, the Isolate Genomic DNA Mini Kit (Bioline, London, UK) was used, following the manufacturer's instructions for isolation of DNA from animal tissue.

The mouth and carcass samples that had been collected during the second study and stored in plastic bags, were defrosted overnight at 4 °C. Then, 10 ml of buffered peptone water was added and the samples were homogenized in a stomacher for 90 s. Thereafter, most of the fluid was recovered from the bags and placed into 15 ml Falcon tubes for centrifugation (30 min at 3724 ×*g*). The obtained supernatant was removed, and 50 μl of PrepMan Ultra (Invitrogen) was added to the pellet. DNA extraction was performed according to the manufacturer's instructions.

2.3. Quantitative polymerase chain reaction

After DNA extraction, quantitative polymerase chain reaction (qPCR) was performed on all samples, using the same primers (UreSu 531 FW and UreSu 783 RV) and conditions as described by De Cooman et al. (2013). Briefly, qPCR involved an initial cycle at 95 °C for 15 min, followed by 40 cycles of denaturation (at 95 °C for 20 s), primer annealing (at 60 °C for 30 s) and chain extension (at 73 °C for 30 s). An external standard, comprising tenfold dilutions of a 1541 bp *ureA–ureB* gene segment, was used starting at 10^5 amplicons for each 10 μl of reaction mixture. The copy number concentration was calculated based on the expected length of the amplicon and its dsDNA amount. Sequences of the primers used, are given in Table 1. Both standard and samples were run in duplicate. Sequence analyses of the *ureA* gene were performed on 21 positive PCR-products to confirm the identification of *H. suis* bacteria.

2.4. Multilocus sequence typing

The five mouth swab samples with the highest quantity of *H. suis* equivalents on qPCR (originating from two pig herds of the second study) were selected for further typing using multilocus sequence typing (MLST). The variable regions of seven housekeeping genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureAB* and *yphC*) were amplified and sequenced according to the method described by Liang et al. (2013). Amplification was established with Accuzyme DNA polymerase, using the same conditions as previously described. Purification of PCR products and sequencing were performed by GATC Biotech (Cologne, Germany). Electropherograms were exported and converted to Kodon software (Applied Maths, Sint-Martens-Latem, Belgium).

2.5. Experimental H. suis infection in pigs

Twenty-four pigs were inoculated with *H. suis* strain HS1 (Baele et al., 2008) at the age of four weeks. All animals received 10^9 to 2×10^9 *H. suis* bacteria *per os* twice, with an interval of 48 h. At the age of eight weeks, the animals were euthanized and stomach samples were collected from the mucosa of the antrum, corpus and fundus, from each animal, as well as 3 mesenteric lymph nodes (one close to the stomach, one close to the jejunum and one close to the rectum). DNA was extracted from these samples, using the protocol described above for the tonsils and mesenteric lymph nodes. DNA extracts were frozen at −20 °C until further analysis. For the detection of *H. suis* DNA, qPCR was performed using the same primers and conditions as described before.

All laboratory animal experiments were approved by the Animal Care and Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

Table 1
Oligonucleotide primers used for amplification and quantitation (UreSu 531 FW and UreSu 783 RV) and establishing an external standard (UreSu 428 FW and UreSu 1968 RV) (De Cooman et al., 2013).

Primer	Sequence (5′ to 3′)	Amplicon size (bp)
UreSu 531 FW	CACCACCCCGGGGAAGTGATCTTG	253
UreSu 783 RV	CTACATCAATCAAATGCACGGTTTTTTCTTCG	
UreSu 428 FW	GGTGTTGCCCATATGATTCA	1541
UreSu 1968 RV	CGAATCCTAGAGTCAGCAA	