Helicobacter species and gastric ulceration in horses: a clinical study

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ABSTRACT: The goal of this study was to gather more clinical information about the relationship between *Helicobacter* species and gastric ulceration in horses. Twenty seven privately owned patients were selected for the clinical study. All horses were gastroscopically examined and biopsies were taken from the glandular mucosa. Stomach biopsies were examined using a PCR assay specific for *Helicobacter pylori* and/or *Helicobacter equorum.* In addition, faecal samples from thirteen horses were examined using a PCR assay specific for *H. equorum*. Twenty five horses (25/27; 93%) had lesions in their stomach. Gastric biopsies from twenty two horses were examined using the *H. pylori* specific PCR and the *ure* gene was detected in three of them (3/22; 14%). *H. equorum* DNA was not found in the stomach of any of the ten horses examined. *H. equorum* was diagnosed in only one faecal sample from the thirteen horses examined (1/13; 8%) and that case was not associated with gastric pathology. This study shows the possibility of sporadic detection of the *ure* gene in the equine stomach affected by mucosal ulceration.

Keywords: EGUS; pyloric stenosis; PCR; *Helicobacter pylori*; *Helicobacter equorum*

Equine gastric ulcer syndrome (EGUS) is a common condition in horses that could reach a prevalence as high as 94% (Murray et al., 1996). There are many risk factors that seem to be associated with EGUS (Buchanan and Andrews, 2003; Sanchez, 2004; Bezdekova et al., 2005, 2008). Infection with *Helicobacter pylori* is of primary importance in the etiology of gastric ulceration in man (Marshall and Warren, 1984). The role of *Helicobacter* sp. in the horse is unknown. There is a report of *Helicobacter* infection in a foal (Green et al., 1990), but a further publication by the same authors reports the failure to detect *H. pylori* in foals with gastric ulceration (Green et al., 1991). The possibility of *Helicobacter* infection in the equine stomach was discussed by Scott et al. (2001). Belli et al. (2003) reported the finding of the urease enzyme in a gastric sample from one horse suffering from gastric ulceration and this may have been due to the presence of *Helicobacter* urease-positive species. Recently more information has been published regarding the possible microbial etiology of EGUS. Hepburn (2004) found *Helicobacter* genus-specific (*Helicobacter*-like) DNA in biopsies of healthy and slightly affected equine stomachs. Contreras et al. (2007) detected *Helicobacter* genus-specific DNA in the gastric mucosa of asymptomatic Thoroughbred horses, even when *H. pylori* specific PCR detection was not successful.

In addition a new *Helicobacter* species, *Helicobacter equorum,* was isolated from faecal samples of two clinically healthy horses (Moyaert et al., 2007a). These bacteria colonize the distal gastrointestinal tract (Moyaert et al., 2007b). Moyaert et al. (2007c) subsequently studied the zoonotic potential of *H. equorum* which they concluded was low. The aim of the present study was to gather more information about the connection between known *Helicobacter* species and gastric ulceration in horses.

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MATERIAL AND METHODS

Horses

Twenty-seven privately owned horses were selected for this study. The horses were randomly chosen from all the horses admitted to the Equine Clinic, University of Veterinary and Pharmaceutical Sciences in Brno for further examination which included gastroscopy and had a history of weight loss and/or partial anorexia and/or recurrent colic episodes. They included eight different breeds (Thoroughbred – 15, Czech warm-blood – 4, Holstein warm-blood – 2, Paint horse – 1, Standardbred – 1, Achal-Teke – 1, Hanoverian warm-blood – 1, Quarter cross – 2), aged from 1 to 10 years. Ten stallions, eleven mares and six geldings were presented. Horses were numbered according to the time of admission to the clinic (Table 1).

Table 1. Summary of horses examined and results of gastroscopic and PCR examination

Horse No.	Breed	Age (years)	Sex	*H. pylori*	*H. equorum*		Nonglandular mucosa	Glandular mucosa
				Biopsy PCR	Biopsy PCR	Feces PCR	(EGUS degree 0–4)	(EGUS degree 0–4)
1	Th	1	stallion	P	NE	NE	4	4
2	CW	10	mare	P	NE	NE	3	4
3	HW	3	stallion	P	NE	NE	3	4
4	Th	5	stallion	N	NE	P	1	0
5	Th	10	gelding	N	NE	N	1	0
6	Th	6	gelding	N	NE	N	0	0
7	Th	5	stallion	N	NE	N	2	0
8	Th	7	gelding	N	NE	N	1	0
9	Th	6	gelding	N	NE	N	2	0
10	Th	5	mare	N	NE	N	0	0
11	Th	2	mare	NE	N	N	3	0
12	QC	2	mare	N	N	N	3	4
13	CW	10	stallion	NE	N	N	4	0
14	CW	6	mare	NE	N	N	2	1
15	Th	6	stallion	NE	N	N	3	0
16	Paint	1	stallion	NE	N	N	2	0
17	St	4	stallion	N	NE	NE	3	2
18	Th	9	mare	N	NE	NE	2	2
19	Achal	1	mare	N	NE	NE	4	0
20	Th	5	stallion	N	NE	NE	3	3
21	Han	7	gelding	N	NE	NE	4	3
22	Th	3	mare	N	NE	NE	4	3
23	Th	8	mare	N	NE	NE	2	0
24	HW	6	gelding	N	N	NE	3	4
25	CW	6	mare	N	N	NE	3	4
26	Th	1	stallion	N	N	NE	3	4
27	QC	1	mare	N	N	NE	3	4

EGUS = equine gastric ulcer syndrome, Th = Thoroughbred, CW = Czech warm-blood, HW = Holstein warm-blood, QC = Quarter cross, Paint = (Paint horse), St = Standardbred, Achal = Achal-Teke, Han = Hanoverian warm-blood, P = positive, N = negative, NE = not examined

Gastroscopic examination

All the horses were gastroscopically examined. Gastroscopy was a part of the routine diagnosis assessment. Food was withheld from each horse from 14 to 20 hours before endoscopic examination. The horses were sedated with xylazine (0.5 mg/kg *i.v.)* and gastroscopy performed using a 13-mm-diameter, 3.3-m flexible videoendoscope (Dr. Fritz^{TM}). The procedure included viewing of the whole nonglandular mucosa and *margo plicatus*, the proximal part of the glandular mucosa, pyloric antrum, pylorus and in most cases duodenum. Lesions on nonglandular mucosa were scored on a scale of 0–4 according to Andrews et al. (1999): Grade 0 – intact epithelium with no appearance of hyperkeratosis, grade 1 – intact mucosa with areas of hyperkeratosis, grade 2 – small single or multifocal lesions, grade 3 – large single or multifocal lesions or extensive superficial lesions, grade 4 – extensive lesions with areas of deep ulceration. Glandular lesions were also scored on a scale of 0–4 according to a modified version of the previously mentioned scoring system: Grade 0 – intact mucosa, grade 1 – intact mucosa with areas of hyperemia, grade 2 – small superficial lesions up to three in number, grade 3 – small superficial lesions over four in number, grade 4 – extensive deep single or multiple lesions.

Biopsy

Three or more biopsy samples were taken from the pyloric region of all horses with the use of transendoscopic forceps (27/27). They were stored in saline solution and kept frozen until further examination.

DNA was isolated from twenty two pyloric biopsies (22/27) and examined using a commercial PCR assay specific for *H. pylori* genes *cagA*, *ure*, *vacA* (Prague OK s.r.o., Czech Republic) according to the manufacturer's instructions.

The gastric biopsy samples from ten horses (10/27) were examined using a PCR assay specific for *H. equorum*. DNA was extracted after 16 hours incubation with proteinase K using a DNAeasy Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. One microliter of each eluate was later used for the PCR reaction as described above.

In conclusion, biopsy samples from five horses (5/27) were examined for the presence of both *H. pylori* and *H. equorum*. Seventeen horses (17/27) were examined exclusively for *H. pylori* and five (5/27) exclusively for *H. equorum*. Particular PCR tests were selected on the basis of availability of the test at the time of examination and the owner's readiness to finance the test expenses.

Faecal samples

Faeces from thirteen horses were examined (13/27). The faecal samples were taken from the rectum and immediately processed. DNA was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen, Germany) and 4 µl of each eluate were examined using the PCR assay specific for *H. equorum* (Moyaert et al., 2007b). The PCR mixture of 8.5µl contained 0.5 IU of HotStarTaq Plus DNA Polymerase (Qiagen), 2.5 mmol/l $MgCl_2$, 1 × PCR Buffer (Qiagen) supplemented with bovine serum albumin (New England BioLabs) to give a final concentration of 0.16 µg/µl, 200 µmol/l each dNTP and 0.2 pmol/ml of each primer (Generi Biotech, Czech Republic) and sterile distilled water. The conditions for amplification were modified: one cycle at 95°C for 5 min; 40 cycles of 30 s denaturation at 94°C, 40 s annealing at 68°C, and 60 s elongation at 72°C; and a final extension cycle of 10 min at 72°C. All PCR products were subjected to electrophoresis in 1% agarose gel and visualised on a UV transiluminator after ethidium bromide staining. The 1 074-bp PCR product was subjected to direct sequencing for confirmation.

The selection of horses for faecal sampling was made on the basis of availability of the test at the time of examination and the owner's readiness to finance the test expenses.

RESULTS

Gastroscopic examination

Gastroscopy revealed lesions in the stomach mucosa of twenty-five out of twenty-seven (93%) horses. Twenty five out of twenty-five horses (100%) had lesions on the nonglandular gastric mucosa. Fourteen from twenty-five horses (56%) had lesions on the glandular stomach. Of these horses all had lesions in both mucosal regions of the stomach. Eight from twenty-five (32%) horses had serious pyloric ulceration (grade 4) and pyloric stenosis (Table 1). Duodenal lesions were not found.

Biopsy samples

A positive result was obtained with the *H. pylori ure* gene primers in gastric biopsies from three of the 22 horses examined (3/22; 14%). However, no amplicon was produced with the *cagA* and *vacA* primers. The three positive horses had pyloric stenosis (EGUS grade 4). Nineteen horses (19/22; 86%) were negative for *H. pylori* (*ure* gene) stomach biopsies. This group of horses included individuals with serious pyloric ulcerations (EGUS grade 4) as well as horses with healthy pyloric mucosa (EGUS grade 0) (Table 1).

H. equorum DNA was not found in biopsies from any of the ten horses examined (0/10). This group also included individuals with seriously damaged pyloric mucosa (EGUS grade 4) (Table 1).

Faecal samples

H. equorum DNA was detected in a faecal sample from one out of the thirteen (8%) horses examined. This horse had healthy glandular gastric mucosa and hyperkeratosis of the nonglandular mucosa. The faecal samples of the twelve horses examined for *H. equorum* returned a negative PCR assay result (Table 1).

DISCUSSION

There are many risk factors that seem to be associated with EGUS including intermittent feeding, diet, exercise, transport stress, stall confinement and administration of various drugs (Buchanan and Andrews, 2003; Bezdekova et al., 2005, 2008). Infection with *H. pylori* is of primary importance in the etiology of gastric ulceration in man and it also represents an important factor in gastric cancer (Marshall and Warren, 1984; Collier and Stoneham, 1997). Nineteen named *Helicobacter* species colonize the lower intestinal tract of animals, many of which also colonize humans (Fox, 2002). Recently, *H. equorum* was added to that group, but its zoonotic potential has not been demonstrated (Moyaert et al., 2007a, c).

In human medicine, intravital diagnosis of *Helicobacter* infection is made by endoscopy when multiple biopsies are taken. These show acute ulceration, inflammation and the presence of a comma-shaped organism revealed by the use of Giemsa staining. In addition, a urease activity test is performed. In horses, the main diagnostic procedure for EGUS is gastroscopic examination with or without the taking of a biopsy and its further investigation. The detection of *Helicobacter* species using PCR, immunofluorescence, confocal microscopy, the detection of serum antibodies to *Helicobacter* sp. and the finding of typical histologic changes in a horse was mentioned but not further discussed by Murray (2005). *Helicobacter* is uniquely able to colonize the stomach via the action of cytoplasmic urease. Positive urease testing was performed by Belli et al. (2003) in a horse with gastric ulceration. A rapid urease test was positive in seven horses from fifteen examined in the work of Hepburn (2004). The occasional finding of spiral-shaped bacteria in the histology of the equine stomach is described by Contreras et al. (2007), but the isolation of bacteria was not successful. Our study was based on PCR assays and its comparison with gastroscopic findings. The other diagnostic methods described above were not used.

Scott et al. (2001) found a positive PCR reaction for *Helicobacter* species in three horses. Only nonglandular mucosa were examined. Contreras et al. (2007) detected *Helicobacter*-like DNA in the gastric glandular and nonglandular mucosa in Thoroughbred horses. *Helicobacter* colonizes glandular types of mucosa in man and other species. It lives on the mucosal surface between the protecting mucus and mucosa, and *Helicobacter* bacteria should not be able to colonize nonglandular (squamous) mucosal types (Fox, 2002). Discussion regarding the microbial etiology in equine gastric ulceration is focused on the glandular mucosal part of the equine stomach (Hepburn, 2004; Murray, 2005). Our study was based on glandular mucosa biopsy and its further examination, similarly to Hepburn's study (2004).

Contreras et al. (2007) took biopsies *post mortem* and examined them using a *Helicobacter* genus-specific assay targeting the 16S rRNA gene. *Helicobacter*-like DNA was detected on both mucosal types in eleven horses out of twenty and a high incidence of *H. pylori* DNA was found (99%). However, *H. pylori* specific PCR assays (*cagA* and *glmM* genes) were not positive in this study. This finding most probably indicates that the detected DNA was from an unknown *Helicobacter* species different from *H. pylori* (Contreras et al., 2007; Haesebrouck et al., 2009). Our study also included *cagA* gene detection and we also failed to confirm

its presence in equine gastric biopsies, but the *ure* gene was detected.

The same diagnostic method as in the Contreras's study (16S rRNA gene detection) was used by Hepburn (2004). *Helicobacter*-like DNA was detected in all twelve horses examined (Hepburn, 2004). In our study, a commercially available *H. pylori* PCR kit was used with specific assays targeting the *cagA*, *ure* and *vacA* genes. Positive PCR assays for *H. pylori* were performed on three horses, but only for the *ure* gene. To our knowledge, the *ure* gene has not been detected in horses before. This result suggests the presence of a urease positive microorganism in equine stomach. There are several possible explanations for the failure to amplify the other two genes, *cagA* and *vacA*, from our samples. Based on the high sequence similarity between so far described genes of *H. pylori* and *Helicobacter*-like DNA from horses (Contreras et al., 2007), it seems unlikely that these two genes would be completely absent and/or very different in the two genomes. However, we cannot exclude the possibility that their sequences differ to such an extent that the multiplex PCR protocol optimized for human samples failed to amplify all three genes in equine samples. We did not use the *Helicobacter* genus-specific assay (16S rRNA) described above, therefore the presence of *Helicobacter*-like DNA in our study is not confirmed. Unfortunately, sequence analysis of DNA found in three positive horses was not performed at the time of sample processing.

The *ure* gene from *H. pylori* DNA was found in three horses in our study and these horses had seriously damaged glandular mucosa in the pyloric region and nonglandular mucosa also. However, other similarly affected horses were negative in the PCR assay for *H. pylori*. Negative *H. pylori* PCR assay results were also obtained in horses with healthy gastric mucosa in our study. In the study of Contreras et al. (2007), the horses examined were clinically healthy, but gastric ulceration or gastritis was found *post mortem* in some of them. However, the detection of *Helicobacter*-like DNA was not associated with stomach disease and even healthy stomachs were found to be positive using *Helicobacter* genus-specific PCR assay processing (Contreras et al., 2007). In the study of Hepburn (2004), the presence of *Helicobacter* genus-specific material was also described. Horses in that study had mainly normal gastric mucosa (both types) or presented only slight mucosal damage and did not show any clinical signs of gastrointestinal disease (Hepburn, 2004). Our study included only horses which suffered from clinically evident digestive problems and a high EGUS prevalence was expected. Our results show the possibility the presence of the *ure* gene in seriously ulcerated glandular gastric mucosa but this finding is not consistent.

H. equorum, a new *Helicobacter* species, was isolated from the faecal samples of two clinically healthy horses (Moyaert et al., 2007a). Experimental colonization of the equine gastrointestinal tract revealed that *H. equorum* is able to colonize the caecum, colon and rectum (Moyaert et al., 2007b). This seems to exclude any role of this bacterium in EGUS etiopathogenesis. The negative results for *H. equorum* DNA detection from gastric biopsies in horses from our study confirm this notion.

Faecal samples examined in our study returned only one positive PCR reaction for *H. equorum*, which represents 8% of the examined horses. A similarly low *H. equorum* prevalence (0.8–7.9%) in faeces of adult horses was described by Moyaert et al. (2007c), but to evaluate the prevalence of *H. equorum* positivity in equine feces under our conditions, more extensive study is needed. A high prevalence of this microorganism was demonstrated in 1–6 month-old foals (67.8%; Moyaert et al., 2009). Sequencing of the PCR product revealed a high similarity to the sequences reported by Moyaert et al. (2007c) combining the features of two strains of *H. equorum* (GenBank accession numbers DQ307735 and DQ307736). No association between the presence of *H. equorum* and gastrointestinal disease has yet been demonstrated (Moyaert et al., 2007b). Gastroscopy on the horse which was positive for *H. equorum* revealed healthy gastric glandular mucosa and an only slightly affected nonglandular mucosal part (hyperkeratosis was present). Further examination of the digestive tract did not reveal any pathology in that patient.

Our results show the possibility of sporadic detection of the *ure* gene in an equine stomach with severe ulceration of the glandular mucosa. *H. equorum* was found in feces, but was not detected in the equine stomach. *H. equorum* was not associated with stomach pathology in the one detected case in our study. Further research is needed to evaluate the role of *Helicobacter* species in horses.

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