PRELIMINARY NOTE

ISSN Online 1678-4456

Urease Test in Horses: associations between positive test results and bacteria other than *Helicobacter sp*.

Teste de Urease em Equinos: associações entre resultados de testes positivos e outras bactérias além de Helicobacter sp.

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ABSTRACT

This study was designed to examine positivity rates of the urease test (UT) in gastric samples obtained from adult horses and to determine whether positive test results are associated with the presence of bacteria other than *Helicobacter sp*. Gastric content samples and biopsy specimens from the glandular and non-glandular regions of the stomach were collected from 39 adult horses via gastroscopy. Samples were placed in tubes containing urease test medium and incubated in a water bath at 37 C for 24 h. Urease test-positive samples were submitted to bacterial culture and biochemical tests for potential isolation and identification of urease-producing bacteria. Positive UT results were obtained in eight horses. Laboratory analysis revealed the presence of *Providencia sp.*, *Escherichia coli, Pseudomonas aeruginosa*, *Citrobacter amalonaticus*, *Edwardsiella tarda*, *Klebsiella oxytoca*, *Proteus mirabilis,* and *Klebsiella pneumoniae*. In conclusion, changes in urease test medium color after more than 12 h of incubation suggest the presence of bacteria other than *Helicobacter sp*. However, relationships between positive UT results, types of bacteria detected, and their role in the etiopathogenesis of gastric ulcers in horses remain to be determined.

Keywords: Diagnosis. Gastric ulcer. Horses. Urease.

RESUMO

Este estudo foi delineado para avaliar as taxas de positividade do teste da urease (TU) em amostras gástricas obtidas de cavalos adultos e determinar se os resultados positivos dos testes estão associados à presença de outras bactérias além de *Helicobacter sp*. Amostras de conteúdo gástrico e amostras de biópsia da região glandular e não glandular do estômago foram coletadas de 39 cavalos adultos por meio de gastroscopia. As amostras foram colocadas em tubos contendo meio teste de urease e incubadas em banho-maria a 37ºC por 24 horas. Amostras positivas para teste de urease foram submetidas a cultura bacteriana e testes bioquímicos para potencial isolamento e identificação de bactérias produtoras de urease. Resultados positivos do TU foram obtidos em oito cavalos. A análise laboratorial revelou a presença de *Providencia sp*., *Escherichia coli*, *Pseudomonas aeruginosa*, *Citrobacter amalonaticus*, *Edwardsiella tarda*, *Klebsiella oxytoca*, *Proteus mirabilis* e *Klebsiella pneumoniae*. Conclui-se que alterações na cor do meio de teste de urease após mais de 12 horas de incubação sugerem a presença de outras bactérias além de *Helicobacter sp*. Porém, as relações entre resultados positivos de TU, tipos de bactérias detectadas e seu papel na etiopatogenia das úlceras gástricas em cavalos ainda não foram determinadas.

Palavras-chave: Diagnóstico. Úlcera gástrica. Cavalos. Urease.

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Received: February 15, 2024
Accepted: January 6, 2025

How to cite: Piffer MLT, Silva LCLC, Zoppa ALV, Benites NR, Melville PA, Delboni CC, Barros AMC, Belli CB. Urease Test in Horses: associations between positive test results and bacteria other than *Helicobacter sp*. Braz J Vet Res Anim Sci. 2025;62:e222055. https://doi.org/10.11606/issn.1678-4456.bjvras.2025.222055.

Helicobacter pylori is a common cause of chronic gastritis, gastric ulcers, and gastric adenocarcinomas in humans (De Groote et al., 2005). This organism has seldom been described in veterinary patients. Given the significance of gastric ulcers in horses, some aspects of this pathological condition must be elucidated.

The positive urease test (UT) is specific for *Helicobacter sp*. and is widely used in human medicine. Urease activity has been detected in horses. However, since other bacteria may also be involved in gastric lesions, further research is needed to investigate correlations between positive UT results and urease-producing bacteria other than *Helicobacter sp.* in the stomach.

This study aims to examine positivity rates of the urease test (UT) in gastric samples obtained from adult horses and to determine whether positive test results are associated with the presence of bacteria other than *Helicobacter sp*.

During gastroscopy, biopsy specimens from the glandular and non-glandular stomach and gastric content samples were collected from 39 healthy adult horses. Horses belonged to the São Paulo State Mounted Police or were referred to the Veterinary Hospital of the School of Veterinary Medicine and Animal Science of the University of São Paulo (FMVZ-USP) for gastroscopic examination. This project was approved by the FMVZ-USP Ethics Committee for the Use of Animals (CEUA nº 1666/2009) and funded by USP (2009.1.1077.10.4).

Three samples (one sample corresponding to gastric contents, one representative of the glandular mucosa, and one representative of the non-glandular mucosa) were collected per horse. Samples were placed in individual tubes containing urease test medium (Probac Brazil) and incubated in a water bath at 37 C for 24 h. The urease test medium is used to identify bacteria capable of hydrolyzing urea using the enzyme urease. This rapid process does not require bacterial multiplication and induces medium alkalinization. The turning point indicative of *Helicobacter sp.* occurs in the first 6 h of incubation and rarely after 12 h. After evaluating the three samples collected, positive samples were submitted to bacterial culture and biochemical tests to isolate and identify other urease-producing bacteria potentially implicated in positive UT results.

The material was initially inoculated in BHI broth (Brain and Heart Infusion broth) with incubation at 37 C for 24 h. In addition, the samples were also seeded on Sheep Blood Agar (5%) and MacConkey Agar, with incubation in aerobic conditions at 37 C, with readings in 24-96 h. The samples cultivated in BHI broth were seeded on Sheep Blood Agar (5%) and MacConkey Agar, with incubation performed like previously described for the initial cultivation. The isolated microorganisms were identified according to Lennette et al. (1985) and classified according to Krieg and Holt (1994) and Murray et al. (1990).

Gastroscopic examination revealed ulcers in the non-glandular stomach region in 21 horses. Ulcers were also detected in the glandular region in one case. Eight out of 39 horses tested positive for urease production (i.e., medium color change to pink after 12 h of incubation). Of these, only two had gastric ulcers.

Laboratory analyses of samples obtained from positive horses are shown in Table 1. Gastric lesions were mild to moderate, and the primary complaint was intermittent or recurrent abdominal discomfort in most cases. However, it was impossible to determine whether bacteria detected in positive samples were the primary or secondary cause of gastric lesions. Similar organisms were sometimes identified in biopsy specimens and gastric content samples after culture. This finding suggests gastric contents are a potential source of contamination of the intact or ulcerated mucosa.

Overall, there is limited information about the role of the gastric microbiota on ulcer development. Unlike other species, the relationship between gastric ulcers and Helicobacter infection in horses is subtle (Plaizier et al., 2018). Nonetheless, the detection of *Helicobacter* in the equine stomach using an *in silico* approach rather than PCR and bacterial culture has been reported (Vallejo-Cordoba et al., 2020).

Culture and biochemical tests revealed bacteria other than *Helicobacter sp*. in urease-positive samples. The urease test medium (Probac Brazil) is specific for *Helicobacter* detection, and changes in medium color are expected to

occur within the first six hours of incubation in positive cases. The fact that color changes in this study occurred after more than 12 h of incubation strongly indicates that other bacteria were present (Figure 1).

However, urease from different bacteria has different kinetic properties. These differences should be accounted for in test result interpretation. For example, urease from *C. pylori* has a higher affinity for the substrate than urease produced by species of *Proteus* or *Providencia* (Mobley, 2000). Even so, *UT detected Providencia* in this study.

The UT is easy to perform but does have some limitations. In a canine study (Diker et al., 2002), UT results were positive in 72% of stomachs, compared to 84% positivity of direct microscopic examination. Uneven distribution of bacteria in the stomach, bleeding, or use of medications that decrease stomach acid production (i.e., increase gastric pH) may also interfere with urease activity and lead to false-negative results (Patel et al., 2014).

Bacterial culture and biochemical tests revealed the presence of *Providencia sp*. in all samples obtained from horse 13. The sample collected from the non-glandular region of the stomach was also positive for *Escherichia coli*. However, *Escherichia coli* in that sample were urease-negative. Hence, the presence of that organism in the stomach was probably unrelated to positive UT results.

As to the role of *Escherichia coli* on gastric ulcer development in horses, in a study with five racehorses submitted to gastroscopy as part of poor performance evaluation (Voss et al., 2022), samples collected from glandular gastric lesions and adjacent normal mucosa were examined using 16S rRNA gene sequencing. Unlike, Husted et al. (2010), who detected significant associations between *Escherichia* and glandular gastric lesions, those samples were negative for *Escherichia*.

Providencia sp. is often found in the environment and is a common inhabitant of the gastrointestinal tract (Albert et al., 1995). Since only *P. rettgeri* and *P. stuartii* produce urease, these are the only species found in UT-positive samples (O'Hara et al., 2000).

Providencia sp. has been reported in recent research. In a study investigating crystallogenic properties of the gastric mucosa in 12 healthy individuals and 30 patients with ulcer complications, Martusevich et al. (2021) concluded that the procrystallogenic potential of the symbiosis between *Helicobacter pylori* and *Providencia* or *Morganella* may be an important factor in the pathogenesis of gastric ulcers

Table 1 – Results of culture and biochemical tests of gastric biopsy specimens and gastric content samples collected from horses with positive urease test results

Horse	Culture and Serum Biochemistry Results		
	Nonglandular	**Glandular**	**Content**
13	*Providencia sp. U+; Escherichia coli U-*	*Providencia sp.*	*Providencia sp.*
22	-	-	*Pseudomonas aeruginosa*
30	-	-	*Pseudomonas aeruginosa*
32	*Pseudomonas aeruginosa; Escherichia coli*	*Pseudomonas aeruginosa; Citrobacter amalonaticus; Edwardsiella tarda*	-
33*	*Pseudomonas aeruginosa; Klebsiella oxytoca*	-	-
34*	-	-	NEGATIVE FOR BACTERIAL GROWTH
35	*Proteus mirabilis; Klebsiella pneumoniae*	-	-
36	-	*Pseudomonas aeruginosa*	-

*Horses diagnosed with gastric ulcers on gastroscopic examination.

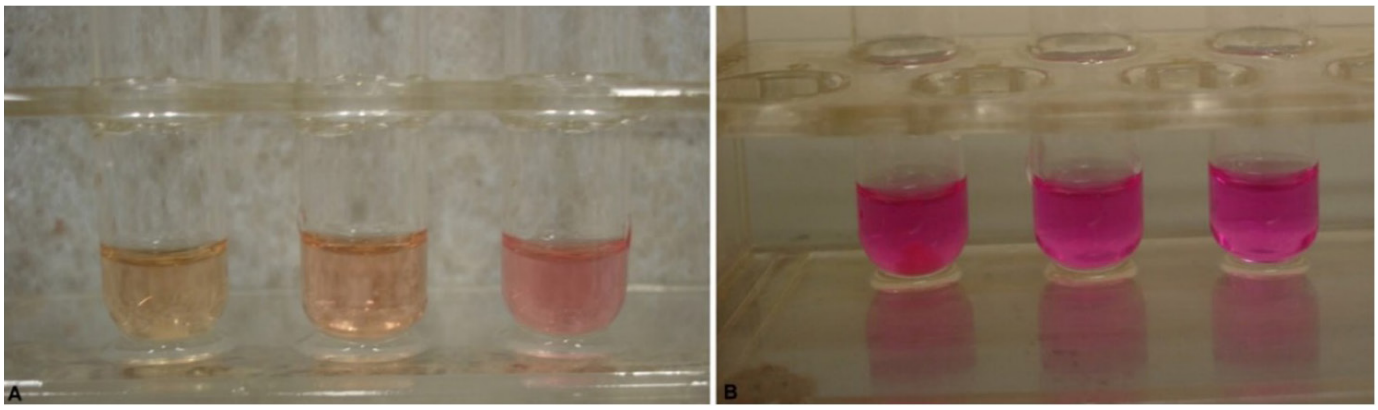

Figure 1 – Urease test results after 12 (A) and 20 h (B) of incubation. Note changes in urease test medium color from yellow to pink. The samples illustrated here are all from the same animal. The samples are considered positive if the color changes within the first 6 h.

through mucosal damage and progression to complications. This study detected Providencia in the glandular and non-glandular portions of the stomach and gastric contents. These findings will interest future studies investigating gastric ulcers in horses.

In our study, *Pseudomonas aeruginosa* was present in gastric content samples (horses 22 and 30) and biopsy specimens taken from the non-glandular (horses 32 and 33) and glandular (horses 32 and 36) portions of the stomach.

Pseudomonas aeruginosa is the most important species of the genus *Pseudomonas.* Most of these organisms can hydrolyze urea (Hirsh, 2004) and are not commonly found in the microbiota of healthy individuals. However, in a study with dogs with chronic gastric disease, Husnik et al. (2022) have shown that bacteria that may be part of the normal microbiota of the stomach, such as *Proteus mirabilis* and *Pseudomonas aeruginosa*, actually do produce urease and can lead to false-positive UT results. In horses, *P. aeruginosa* is more often involved in metritis and vaginitis (Hirsh, 2004).

Interestingly, some of the bacteria identified in our study are not commonly found in the equine stomach and are seldom implicated in gastroenteric disorders. For example, *Citrobacter amalonaticus* was detected in the glandular specimen of horse 32. *Citrobacter amalonaticus* is a Gram-negative, facultative anaerobic organism occasionally found in feces, blood, and the respiratory and urinary tracts, which rarely causes disease. Urease production has been reported in 11 to 89% of bacteria of the genus *Citrobacter* (Versalovic et al., 2011).

The UT can be used in horses. However, it is not specific for *Helicobacter sp.* and may indicate the presence of other urease-positive bacteria, especially when changes in medium color are delayed. Future studies are warranted to determine the contribution of these bacteria to gastric lesion development and/or persistence in this species. One of the limitations of this study is that there was much missing information in the animal's medical records, which greatly limited the ability to obtain information about the clinical manifestations presented by the horses and, consequently, made it impossible to make more robust interpretations of the results obtained.

Another relevant limitation is that the product used to determine the production of the urease enzyme by Helicobacter pylori was developed for diagnosis in humans, which limits the transposition of the results obtained to the analysis performed in animals.

The findings of this study revealed that several bacteria might colonize the equine stomach and may or may not be affected by hydrochloric acid production and the presence of other microorganisms commonly found in the glandular and non-glandular mucosa. In conclusion, changes in urease test medium color after more than 12 h of incubation of equine gastric biopsy specimens and gastric content samples submitted to the UT suggest the presence of bacteria other than *Helicobacter sp*.

Conflict of Interest

The authors declare no potential conflict of interest regarding this article's research, authorship, or publication.

Ethics Statement

This project was approved by the Ethics Committee for the Use of Animals at FMVZ-USP (CEUA No. 1666/2009) and funded by USP (2009.1.1077.10.4).

Acknowledgements

We thank the University of São Paulo for granting the Institutional Scientific Initiation Scholarship.

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Financial Support: The authors received financial support for the research from the University of São Paulo - USP (2009.1.1077.10.4).