Research Article

Helicobacter detection in the apparently normal donkeys' stomach: sampling, methods and implications for equine glandular gastric disease and serum antioxidant status

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Abstract

The involvement of *Helicobacter-like* in equine glandular gastric disease (EGGD) is not clear. Much evidence supports the presence of *Helicobacter* in the horse's stomach, but it is not so clear about the donkey. The present study was conducted to evaluate the presence of *Helicobacter* in the stomach of donkeys and evaluate the best method for its identification and its possible participation in EGGD and serum antioxidant status. Gastric juice sampling and biopsy from near margo plicatus (NMP) and pyloric antrum (PA) were done by gastroscopic method from 12 donkeys. Histopathology and rapid urease test (RUT) were used to identify *Helicobacter*. Total antioxidant capacity (TAC), total thiol (THIOL), nitric oxide (NO), and diphenyl-1-picrylhydrazyl (DPPH) were evaluated for the antioxidant status of serum. *Helicobacter* was detected only by the RUT method in one donkey (8.33%), but it is unlikely that this infection was effective in causing EGGD. RUT results for all three samples of gastric juice and NMP and PA were not different. Histopathology of NMP and PA did not show *Helicobacter* infection. *Helicobacter* is present in the stomach of a donkey but does not

change the antioxidant status of serum. RUT is more efficient than H&E histopathology for the assessment of *Helicobacter pylori* in the equine stomach, and RUT of gastric juice is preferable to tissue samples because it is easy, fast, and non-invasive. In conclusion, it is recommended to carry out more studies with more accurate methods to evaluate the effect of *Helicobacter* in gastric diseases of donkeys and other equine.

Abbreviations

equine glandular gastric disease (EGGD)

near margo plicatus (NMP)

pyloric antrum (PA)

rapid urease test (RUT)

hematoxylin and eosin (H&E)

total antioxidant capacity (TAC)

total thiol (THIOL)

nitric oxide (NO)

diphenyl-1-picrylhydrazyl (DPPH)

Introduction

Equine glandular gastric disease (EGGD) is one of the most important gastrointestinal diseases in horses [1]. Gastric microbiota changes in EGGD, but the cause is not completely clear [2,3]. However, there is no conclusive evidence that bacteria are always involved in the development of EGGD [4], and there is a possibility that microbiota changes are secondary and related to opportunistic bacteria. Opportunistic bacterial colonization has been seen in chronic EGGD [4,5]. Bacteria such as *Escherichia fergusoni, Enterococcus faecium, Streptococcus bovis* and Sarcina can be associated with EGGD lesions [2,6]. *Helicobacter pylori* is a suspected etiology in the pathogenesis of gastric ulcer in humans, dogs and cats [7,8]. However, the involvement of *Helicobacter* in EGGD is uncertain [9].

Helicobacter spp belongs to the *Campylobacter* genus and it is often believed that this bacterium is not effective in the etiopathology of EGGD [4]. However Helicobacter has been identified in different methods in the equine stomach [10–13] and feces [14]. Some studies did not find evidence of the involvement of *Helicobacter* species in the pathogenesis of EGGD [2,6,15]. Although Helicobacter was found in the stomach of horses, it had no significant relationship with EGGD [3]. In some reports, the presence of Helicobacter has been associated with EGGD [16–18].

The RUT method has been used in various studies to identify Helicobacter in horses [10,18]. Evaluation with RUT is based on the presence of bacterial urease enzyme and urea absorption from the culture medium. The sensitivity of RUT in horses was 40% compared to PCR [10] and 100% compared to histopathology [18]. Histopathological and immunohistochemical evaluation of glandular and non-glandular gastric samples of horses showed 81% Helicobacter infection [12].

Hyperlipidemia, kidney disease, and grain overload increase the risk of equine gastric ulcer disease (EGUS) in donkeys and can cause colic [19], but the importance of bacteria in the pathogenesis of EGUS in donkeys is unknown. However, some consider Helicobacter to be a possible cause of EGUSs in donkeys [20,21]. Also, people infected with Helicobacter have lower antioxidant levels than healthy people [22]. The present study gives a perspective on *Helicobacter's* presence in donkeys stomach using RUT of mucosal samples of gastric gland, RUT of gastric juice and histopathological evaluation (HPE) of biopsied tissue samples. It also explores the presence of EGGD and serum antioxidant levels in donkeys to evaluate the possibility of their relationship with the presence of *Helicobacter* in donkeys' stomach.

Results

Vital signs of animals, including body temperature, respiration rate and heart rate were normal in clinical examination. They had no clinical signs of diseases, including alimentary disorders and colic.

The results of gastroscopy showed that EGGD grade was zero in all donkeys and only one of the animals had a grade 1 EGGD.

Evaluation of RUT for gastric juice and glandular gastric tissue samples showed infection with urease positive *Helicobacter*-like infectious in only one of 12 animals (Fig 2). Both gastric juice RUT and glandular gastric (NMP and PA) mucosa RUT were positive in the same animal. In the gastroscopic evaluation of the animal with a positive RUT, there was no sign of even the mildest degree of EGGD.

Histopathological examination of NMP and PA gastric specimens did not confirm Helicobacter-like infection of any of the donkeys. No pathological changes were in the histopathological samples of NMP and PA.

There was no obvious difference between the antioxidant status of the animal that had positive RUT and the average of the rest of the animals that had negative RUT (Table 1).

The values obtained for the animal with a positive RUT is within the range of changes of the values obtained for other animals with a negative RUT, so it may be concluded that the presence of bacteria and a positive test did not cause obvious changes between the two groups of animals. (Table 1).

Discussion

The present study was an evaluation of the presence of *Helicobacter*-like bacteria in the donkey stomach, which was carried out by RUT and histopathology. The findings of the present study confirm the presence of *Helicobacter* in the donkey's stomach, but just like the horse [4] in the donkey, the presence of Helicobacter is not related to the development of EGGD.

Some studies emphasize the presence of *Helicobacter* in the equine stomach. In the genetic analysis conducted on the gastric mucosa of slaughtered Colombian horses, it was found that 23.3% of the samples were positive for *Helicobacter* species. The gene similar to *H. heilmannii* was identified. There was no significant relationship between the presence of Helicobacter and gastric ulcer [23]. Some species of Helicobacter, such as *H. equorum*, were able to multiply in the hindgut of horses in the experiments, but they did not cause any microscopic and clinical pathological complications [11]. PCR evaluation of gastric mucosa biopsies of horses (93% with gastric lesions) showed that only 14% were positive for *H. pylori* and all of the samples were negative for with *H. equorum. H. equorum* was found in the feces of only 8% of horses [24].

In some studies, no signs of *Helicobacter* likes have been found in equine gastric. Fluorescence in situ hybridization (FISH) and RUT were performed on healthy and unhealthy gastric mucosa of slaughtered Danish horses. There was no evidence of *Helicobacter* in the stomachs of healthy and unhealthy horses [6]. In a study that was conducted on the cytology brush sample of horse glandular mucosal, the microbiota was analyzed by DNA sequencing method and no evidence of *Helicobacter* presence was found [2]. No evidence of *Helicobacter* was reported in the study of the microbiota of stable horses' feces [25]. *Helicobacter* was not found in the gastric mucosa of healthy American horses[26]. *Helicobacter* was not found in gastric biopsies of Korean racing horses with gastric ulcer by

PCR and culture, and only in two cases *H. pylori* and *H. ganmani* were found by next generation sequencing techniques [15].

Studies have been done on the digestive microbiota of donkeys, but there were no clear signs of *Helicobacter* in the digestive system. Investigation of the digestive tract microbiota of slaughtered donkeys showed that the diversity in the stomach pylorus is less than in the cecum and large intestines [27]. Growing evidence shown that the diversity of the fecal microbiota of donkeys is age-dependent [28], gender-dependent [29], and the microbial community composition in wild asses is more complex than in domestic donkeys [30].

In the present study, RUT of all samples of gastric juice and glandular gastric mucosa of NMP and PA revealed Helicobacter infection in the infected stomach. According to the results obtained in the present study, it is possible to use gastric mucosal RUT and gastric juice RUT to evaluate Helicobacter-like infections. But since the use of gastric juice is less invasive and can be done with simpler equipment and less skill, it is the preferred method. As the results of the present study showed in equine, RUT is more sensitive than HPE for detecting *H. pylori* in humans [31]. A comparison of RUT and HPE in humans with gastritis showed that both tests have the same accuracy in detecting *H. pylori* infection. Since RUT is a cheap and faster technique, it can be a good alternative to HPE [32]. In humans, a new method for RUT has been used in which the mucosa is swept using a sweeping motion with an absorbent swab held with forceps. Compared to the conventional method of tissue sample collection, the sweeping RUT method had higher sensitivity and accuracy and faster detection time for *H. pylori* diagnosis [33]. The findings of the present study showed that there was no difference between the RUT of gastric juice, NMP and PA gastric tissue samples.

In the previous study the sensitivity of RUT in horses was 40% compared to detection by PCR as the gold standard [10]. Some *Helicobacter* isolates from horses were urease negative

[14] and had no pathological effect [11]. The use of RUT will not be suitable for detecting urease-negative *Helicobacter*. Therefore, RUT will not detect urease-negative *H. pylori.*

In the present study the use of H&E staining could not show Helicobacter infection in RUT positive samples. While Warthin-Starry special stain, Giemsa and Blue Toluidine staining methods have been able to identify Helicobacter as well as RUT in horses [18]. Therefore, it is possible that the use of special staining techniques can be used to detect Helicobacter in equine. However, in one study no Helicobacter contamination was found in the histopathology of the postmortem horses' stomachs although special stains including Gram, PAS and Warthin Starry were also used. [34].

Histopathological and immunohistochemical evaluation of samples collected from the mucosa and submucosa of slaughter horses was performed in Araguari, Brazil. In this evaluation, 81% of the horses had *Helicobacter* species in both the glandular and nonglandular regions, and the highest contamination was observed in the margo plicatus region [12]. The results of the present study showed that, there was no difference between the histopathological results of NMP and PA.

The results of the present study showed that serum antioxidant levels in Helicobacter-positive donkeys do not differ from Helicobacter-negative donkeys. This is while the serum antioxidant level in humans with *H. pylori* infection is different from healthy people and *H. pylori* positive patients have lower total thiol, native thiol and disulphide levels [22].

Previously, it was believed that antibiotics should be used to treat EGUS because *Helicobacter* was considered to be involved in the development of this syndrome [35]. In some studies, the microbiota of horses with EGGD is not different from the microbiota of healthy horses. For example *Lactobacillus salivarius* and *Sarcina ventriculi* have been found in the healthy and lesioned gastric mucosa of horses [6]. However, in one study Sarcina was

more in the mucosa involved with EGGD and may be involved in its pathogenesis [2]. Proteobacteria were more abundant in healthy mucosa than in EGGD [2].

The limitations of the present study were the small number of animals and the lack of more accurate *Helicobacter* diagnosis methods such as PCR or specific staining for histopathology samples. In addition to the valuable findings of the present study on miniature donkeys, perhaps these results can be extended to horses as well. This study was not repeated in horses due to financial limitations for buying horses.

It was concluded that donkeys, like horses, may positive for *Helicobacter-Like*, but it is unlikely that this infection will be effective in the development of EGGD. Glandular gastric infection with *Helicobacter-like* did not change the antioxidant status of the serum. The results obtained from the RUT methods for all three gastric juice, NMP and PA tissue samples were not different from each other. Histopathology with H&E staining of glandular gastric tissue of NMP and PA could not show *Helicobacter-Like* infection and the sampling sites did not did not make any difference to each other in this respect.

Materials and Methods

2.1. Animals

Miniature donkeys (n=12), including six females and six uncastrated males, were randomly obtained from West Azarbaijan province. The age of the animals was between three and seven years, their weight was between 150 and 200 kg and they had a body score of 3 to 4 out of 9 [36]. These animals received oral ivermectin (0.2 mg/kg, IVERGEN®, Laluk, Tehran, Iran) six weeks before the start of the study. The animals were housed in stables and, they were fed daily with alfalfa hay, and had constant access to water. Physical examination of the animals was performed and their clinical health was confirmed.

2.2. Experimental Design

In the precent study, donkeys were subjected to gastroscopy and gastric juice samples were taken for RUT and tissue samples of gastric glandular mucosa were taken for histological evaluations with hematoxylin and eosin (H&E) staining and RUT. Tissue samples were evaluated for the presence of *Helicobacter* spp. infection under a light microscope. The commercial RUT kit (Bahar Afshan, Tehran) was used to evaluate the presence of urease positive bacteria.

Blood samples were collected from donkeys and a complete blood cell count was performed immediately after sampling. Serum was obtained after clotting and centrifugation at 5000 rpm for 10 minutes. The serums were kept at -20°C until the measurements. To evaluate the oxidant-antioxidant status of serum, total antioxidant capacity (TAC), total thiol (THIOL), nitric oxide (NO), and diphenyl-1-picrylhydrazyl (DPPH) were evaluated [37]. The researchers and technicians involved in the trial, including those who performed the sampling and laboratory and histological analysis, were blinded to the previously obtained results.

2.3. Endoscopy

Food (12 hours) and water (four hours) withholding were applied before gastroscopy [38]. Sedation was performed using intravenous acepromazine at a dose of 0.05 mg/kg and xylazine at a dose of 0.5 mg/kg (NEUROTRANQ®, Alfasan, Woerden, Holland) [39]. Before the gastroscopic examination, gastric fluid was sucked using an endoscope catheter tube (7 fr, 3.5m, STORZ, Tuttlingen, Germany) for RUT (Fig 1). Gastroscopy (STORZ®, RP100, Tuttlingen, Germany) was performed by blowing air and washing food residues on the gastric mucosa. The entire gastric and the upper duodenum were examined, except for the part that was covered with a little water and food at the bottom of the stomach [40]. EGGD was evaluated with a grade from 0 to 4 by two expert investigators [41]. Glandular gastric mucosal biopsy of near margo plicatus (NMP) and pyloric antrum (PA) was performed using grasping forceps (3.5 m, STORZ, Tuttlingen, Germany) (fsigni). Prior to the biopsy, the sampling site was flushed with distilled water. Histopathological specimens were fixed in 10% formalin immediately after sampling.

2.4. Statistical analysis

Statistical analysis could not be performed due to the presence of only one animal with a positive rapid urease test, and only the descriptive statistics and range of parameters changes of other animals with a negative test were presented. The parameters values have been calculated with Excel software.

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Figure legends

Figure 1. Sampling of glandular gastric mucosa from near margo plicatus (NMP) and pyloric antrum (PA) for histopathology and RUT. Gastric juice (GJ) sampling for RUT.

Figure 2. Positive (A) and negative (B) RUT samples.

Table:

Table 1: Comparison of serum antioxidant status in donkey with positive RUT and donkeys with negative RUT

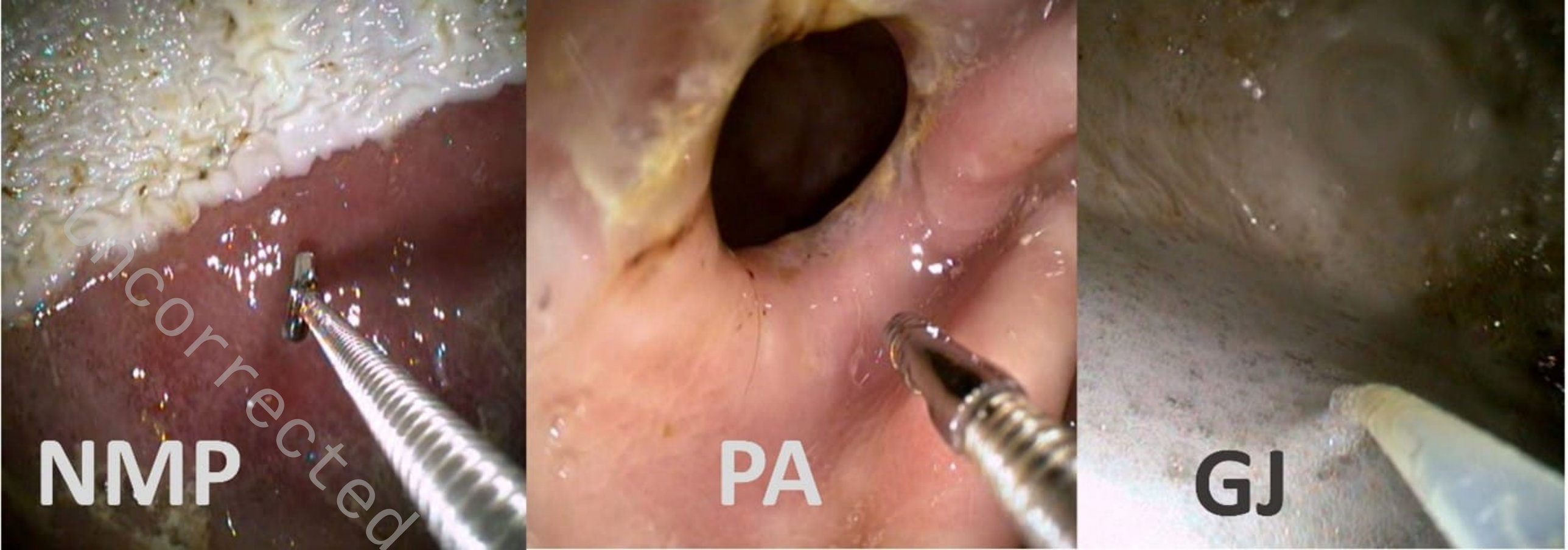
Online supplemental material

No supplemental material

Table 1.
Comparison of serum antioxidant status in donkey with positive RUT and donkeys with negative RUT

Antioxidant test	RUT Negative (mean)	RUT Positive	CI
TAC (nmol/mg)	26.45	22.35	(1.21-6.98)
DPPH (nmol/mg)	46.89	48.67	(-6.56-3.01)
NO (nmol/mg)	28.80	26.37	(8.05-4.05)
THIOL (nmol/mg)	17.50	16.74	(-1.98-3.51)

total antioxidant capacity (TAC), total thiol (THIOL), nitric oxide (NO), diphenyl-1-picrylhydrazyl (DPPH)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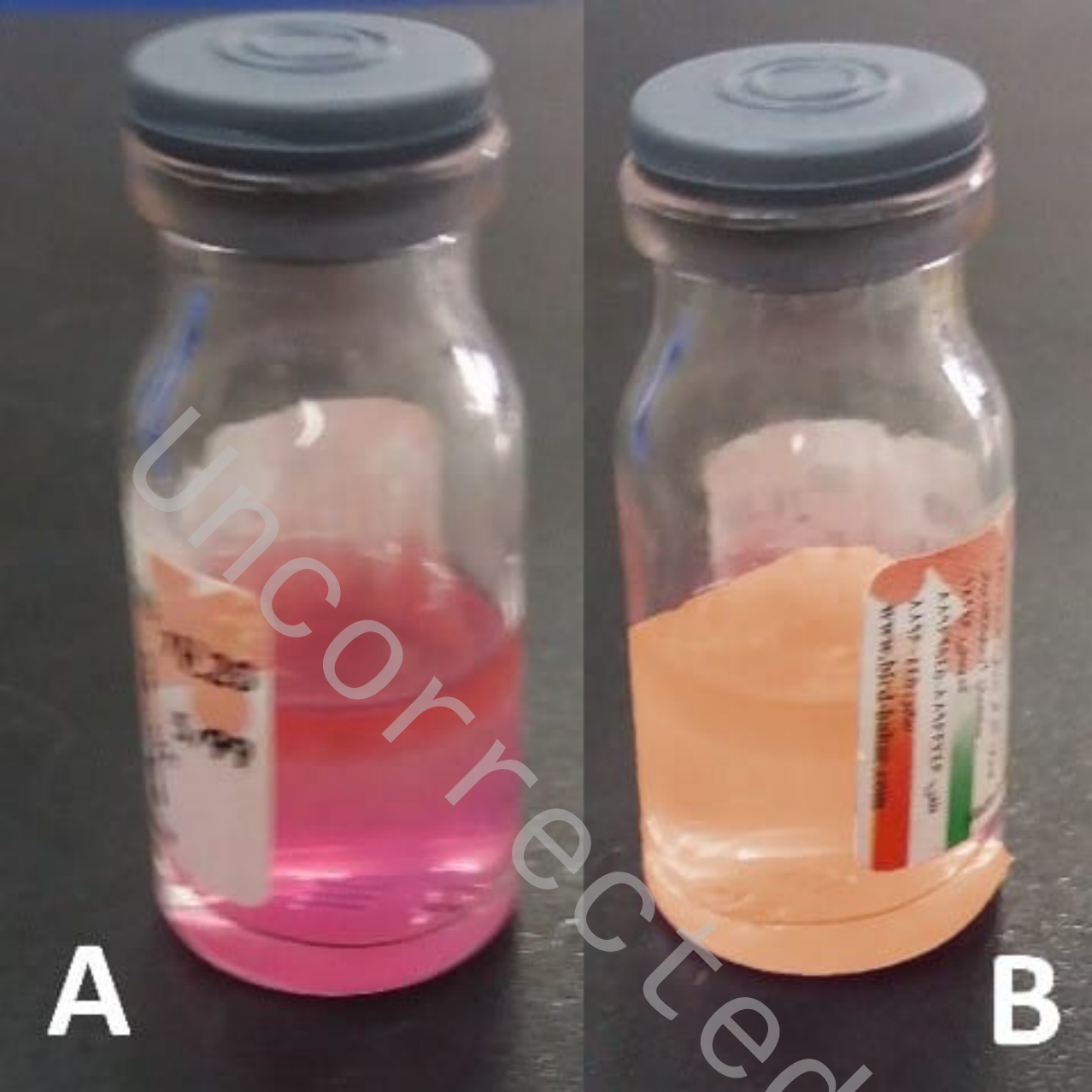
PA
GJ
uncorrected



عنوان مقاله: تشخیص هلیکوباکتر در معده الاغ‌های سالم انگاشته شده: نمونه‌برداری، روش‌ها و پیامدهای آن برای بیماری معده‌ی غده‌ای اسب‌سانان و وضعیت آنتی‌اکسیدانی سرم

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نویسنده مسئول: دکتر قادر جلیل زاده امین

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خلاصه فارسی: دخالت هلیکوباکتر در بیماری معده غده اسب (EGGD) مشخص نیست. شواهد زیادی وجود هلیکوباکتر در معده اسب را تایید می کند، اما در مورد الاغ این موضوع چندان واضح نیست. مطالعه حاضر به منظور بررسی وجود هلیکوباکتر در معده الاغ و بررسی بهترین روش برای شناسایی آن و مشارکت احتمالی آن در وضعیت EGGD و آنتی اکسیدانی سرم انجام شد. نمونه برداری از شیره معده و بیوپسی از نزدیک مارگو پلیکاتوس (NMP) و آنتروم پیلور (PA) از 12 الاغ به روش گاستروسکوپی انجام شد. هیستوپاتولوژی با رنگ آمیزی H&E و تست سریع اوره آز (RUT) برای شناسایی هلیکوباکتر استفاده شد. ظرفیت آنتی اکسیدانی تام (TAC)، تیول تام (THIOL)، نیتریک اکسید (NO) و دی فنیل-1-پیکریل هیدرازیل (DPPH) نیز برای ارزیابی وضعیت آنتی اکسیدانی سرم استفاده شد. هلیکوباکتر تنها با روش RUT در یک الاغ (8/33 درصد) شناسایی شد، اما بعید است که این عفونت در ایجاد EGGD موثر باشد. نتایج RUT برای هر سه نمونه شیره معده و NMP و PA تفاوتی با یکدیگر نداشتند. هیستوپاتولوژی NMP و PA با رنگ آمیزی H&E آلودگی به هلیکوباکتر را نشان ندادند. وجود هلیکوباکتر در معده الاغ وضعیت آنتی اکسیدانی سرم را به شکل واضحی تغییر نداد. RUT نسبت به هیستوپاتولوژی H&E برای ارزیابی هلیکوباکتر پیلوری در معده اسب کارآمدتر است و RUT شیره معده به دلیل آسان، سریع و غیر تهاجمی بودن به نمونه های بافتی ارجحیت دارد. در نهایت پیشنهاد می شود مطالعات بیشتری با روش های دقیق تر برای ارزیابی اثر هلیکوباکتر در بیماری های معده الاغ و سایر اسب‌سانان انجام شود.

واژگان کلیدی: سندرم زخم معده اسب، الاغ، میکروبیوم، آنتی اکسیدان