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DETECTION AND MOLECULAR STUDY OF *GIARDIA INTESTINALIS* IN HORSES AT BAGHDAD CITY, IRAQ

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Abstract

This study was carried out to detection *Giardia* spp. in the feces of horses from equestrian club by traditional methods regarding the effect of age, sex, and months on the infection rate and Identification of *Giardia* spp. Assemblages (A-H) by PCR, sequencing and phylogenetic analysis. A total of 180 (108 males and 72 females) faecal sample were collected from horses during a period extend from 1st /October 2019 to end of March 2020. The results of infection was 26.11% (47/180). Both sex were subjected to the infection, 25% (27/108) in males and 27.7% (20/72) in females, without significant differences ($p>0.001$). DNA was extracted from 100 fecal samples the result was 67% have *Giardia* positive. *Giardia intestinalis* assemblage b were found. The Iraqi isolates mainly appeared in three clades: the first one includes: MT465320, MT465319, MT465321, and MT465317. One isolate appeared divergent from the rest which is deposited in the gene bank as (MT465316) while the rest appeared closely related to the isolate from Egypt. The off-grouped sequences were seen in the sequences originated from Norway, Thailand, and Kenya. According to the molecular study and phylogenetic tree, *Giardia intestinalis* assemblage b are considered the main species that distribution in horses of Baghdad city which recorded for the first time in Iraq.

Key words : *Giardia*, horses, Baghdad, bg gene.

Introduction

Giardia is common anaerobic flagellated protozoan parasite that causes *Giardiasis*. It is a unicellular microscopic protozoan parasite that can infect the intestine and cause enteric disease in wide range of vertebrates, including humans, felids, canids, primates, rodents, ungulates and ruminants (Thompson and Monis, 2004; Cacciò and Ryan, 2008).

Giardia is a complex parasite have about 40 species described from different animals, but several of them are synonyms. *Giardia* (*lamblia, intestinalis and duodenalis*) species including 8 different genetic groups called assemblages (A-H) each one need a different host (Lasek-Nesselquist *et al.,* 2010; Yaoyu and Xiao, 2011). The assemblages (A & B) occur in humans and animals, the assemblages (C- H) are less host range, the assemblages (C & D) happened in canis, the assemblage (F) occur in cats, the assemblage (E) occur in cattle, the assemblage G occur in rats, and the assemblage H occur in aquatic mammals (Ey *et al.,* 1997; Thompson, 2004; Lasek-Nesselquist *et al.,* 2010). Besides, the assemblages (A & B) obtained from human isolates which classified into four sub-assemblages (*i.e.*, AI, AII, BIII, & BIV) with multi-locus genotyping (MLG) (Cacciò *et al.,* 2008; Lebbad *et al.,* 2010). Recently, the assemblages (A, B, & E) of *Giardia intestinalis* have been reported in horses (Traub *et al.,* 2005; Veronesi *et al.,* 2010; Santín *et al.,* 2013). The horses can be possible reservoirs for human infections or for other animals (Iijima *et al.,* 2018; Naguib *et al.,* 2018).

The parasite have two forms the trophozoite and cyst with direct life cycle (Adam, 2001). *Giardia* infection can be transmitted by faecal-oral pathways, direct with infected host, person-person transmission, indirectly transmission with food-borne or water-borne and ingesting *Giardia* cysts that contaminate water and food (Risebro *et al.,* 2007; Naguib *et al.,* 2018; Demircan *et al.,* 2019).

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The symptoms of *Giardiasis* are somewhat variable, and range from acute to chronic diarrhoea and may be absence of symptoms in some cases (Cacciò and Ryan, 2008). *Giardia* can cause weight loss, abdominal pain, dehydration and malabsorption in children or young animals, it have been described to cause mild symptoms or absents of symptoms in healthy animals. Because of the economic losses caused by *Giardia* and its effect on the physical and mental development of affected children, therefore *Giardia* is a disease that has been neglected by the World Health Organization since 2004 (Savioli *et al.,* 2006).

Giardia in horses was first reported in South Africa (Allain *et al.,* 2017)**,** then in other countries, including USA (XIAO and HERD, 1994), Canada (Olson *et al.,* 1997) and China (Qi *et al.,* 2015) While *Giardia* causes diarrhea in horses (Manahan, 1970; Kirkpatrick and Skand, 1985), usually the infected horses do not indicate any clinical signs and subclinical effects. The Assemblages (A, B, & E) of *Giardia* have been identified in horses in Italia (Santín *et al.,* 2013).

The direct smear and faecal floatation with high density solution are the most common techniques for the identification of *Giardia* cysts. Also the immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) for antigen detection are additional tests that confirm the presence of the parasites. Out of all, the most sensitive technique that helps to identify the species, assemblages (A-H) and genotypes of *Giardia* is the molecular analysis (Tangtrongsup and Scorza, 2010).

In Iraq there is no information about assemblage and serotyping of disease *Giardiasis* in horses. Butty, 2011 discover *Giardia* in horses by wet mount, flotation, Lugol's iodine and Giemsa stain in Mosul, therefor this study was considered for investigate about the disease in the horses at Baghdad city.

Materials and Methods

Animals

The study were conducted on the horses that raise in the equestrian club this club contain approximately 4000 horses and located in AL-Ameria / Baghdad. The horses was include 108 males and 72 females of different ages (<1- 3 year include 62, >3- <6 year include 87 and 6-20 are 31).

Samples collection

One hundred and eighty faecal samples (about 30-50 g) were collected from each horse, 30 samples were collected for each month during the pried extend from 1st /October 2019, to the end of March 2020, each fecal sample was collected using sterile disposable latex glove, and placed into individual plastic box with ice, which were sealed, labeled, and transported immediately to the Parasitology laboratory, at Veterinary Medicine College-University of Baghdad. Small amount from each fecal sample (about 50 mg) of feces placed in Eppendorf tube then labeled and stored at -20°c used later for DNA extraction.

Primers

The primers for PCR amplification were used according to (Demircan *et al.,* 2019) based on (beta-giardin gene) bp 511. The forward (5'-GAACGAACGAGATCGAGGTCCG-3') and reverse (5'-CTCGACGAGCTTCGTGTT-3')

Microscope examination

As soon as fecal samples reach laboratory were processed immediately using traditional methods, these include: Direct smear As in (Adeyemo *et al.,* 2018), Floatation Concentration Method As in (Anne M. Zajac, 2007). The infection rate was recorded for each months regarding the sex and ages groups then the result undergo statistical analysis.

Molecular study

DNA extraction

DNA was extracted from one hundred faecal samples randomly collected and previously stored at -20°c, the samples placed at room temperature until using addbio DNA extraction kit /Korea.

Result of prevalence

A total of 180 fecal samples from horses were examined for detection *Giardia* infection during period from 1st /October 2019 to end of March 2020 the total rate of infection was 26.11% (47/180) (Table 1).

Table 1: Infection rate with *Giardia* in horses.

Host	No. of examined	No. of infected	Percentage
Horses	180	47	26%

The study revealed that both sex were subject to infection with *Giardia* in males was 25% (27/108) in males and in females was 27.7% (20 /72) in females, without significant difference (Table 2).

Infection rate with *Giardia* according to age's group

The study showed that the infection rate with *Giardia* was high 28.7% (25/87) at age group >3-<6 years and 27.4% (17/62) at age group <1-3 years, while at age 6-20 group was recorded less infection rate 16.1% (5/31)

with significant differences (P≤0.01) (Table 3).

Table 2: Infection rate with *Giardia* in horses according to sex.

Sex	No. of examined	No. of infected	Percentage
Males	108	27	25.00%
Females	72	20	27.78%
Total	180	47	26.1%
P-value	.....	.....	0.307 NS

NS: Non-Significant.

Table 3: The infection rate according to age of horses.

Age group	No. of examined	No. of infected	Percentage
< 1-3 years	62	17	27.4%
> 3-<6 years	87	25	28.7%
6-20 years	31	5	16.1%
Total	180	47	26.1%
P-value	.....	.....	0.0015 **

** (P≤0.01) -H.S.

Infection rate according to months

The prevalence of *Giardia* were recorded all over the months of the study in horses. The highest infection rate was recorded in February 36.6% (11/30) and November 30% (9/30), while the lowest rate was recorded in January 16.6% (5/30) with significant deference (P≤0.01) (Table 4).

Table 4: Infection rate with *Giardia* during the period of the study.

Months	No. of examined horses	No. of infected horses	Percentage
October	30 sample per month	8	26.6%
November		9	30%
December		7	23.3%
January / 2020		5	16.6%
February		11	36.6%
March		7	23.3%
Total	180	47	26.1%
P-value	.....	.....	0.0087 **

** (P≤0.01) -H.S.

Microscopic examination

Microscope examination of fecal samples by direct smear show *Giardia* cyst stain with lougalÊs iodine appear rounded with 2-4 nuclei and axostyle (Fig. 1 A-B). Also trophozoite which appear pear like shape with tow nuclei and flagella (Fig. 2).

Result of molecular study

Detection of *Giardia* infection by PCR technique

The molecular detection and genotyping of *G. duodenalis* in the faecal samples were achieved through the Polymerase Chain Reaction (PCR) and the sequencing respectively.

The use of conventional PCR technique to identify *Giardia* spp. and assemblage by using beta-giardin gene and primer have 511 base pare. The result showed that from 100 DNA sample 67 (67%) was identified of *G.duodenalis* (Fig. 4).

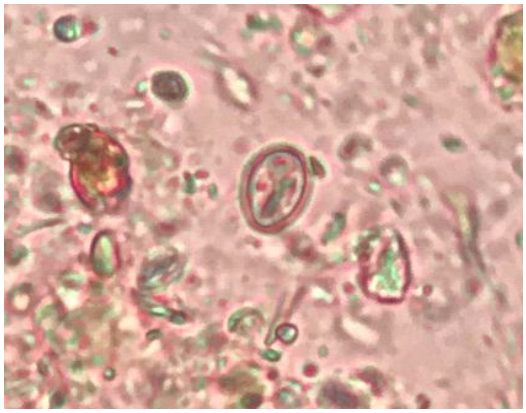

Fig. 1: Giardia cyst in the feces by direct smear with Lugol's iodine stain detected at 40x.

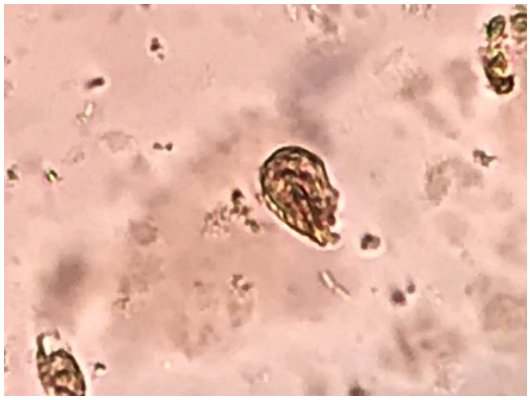

Fig. 2: Giardia trophozoite by direct smear at 100x.

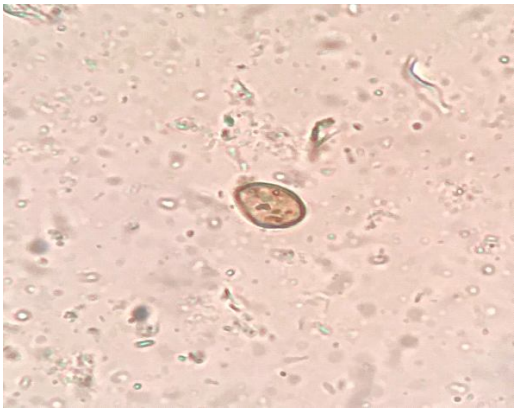

Fig. 3: Giardia cyst by NaCl flotation method appear rounded with two nuclei at 40x.

Phylogenetic analysis

Phylogenetic tree of *Giardia* sp. for beta-giardin (bg) gene.generated by maximum likelihood method from a nucleotide sequence alignment in MEGA7, with a bootstrap of 1000 replicates to provide support for individual nodes. The resulted phylogram depicts splitting the sequences. Noticeably, the sequences were revealed closely related to the *Giardia* intestinalis assemblage B (accession number LC508615.1)

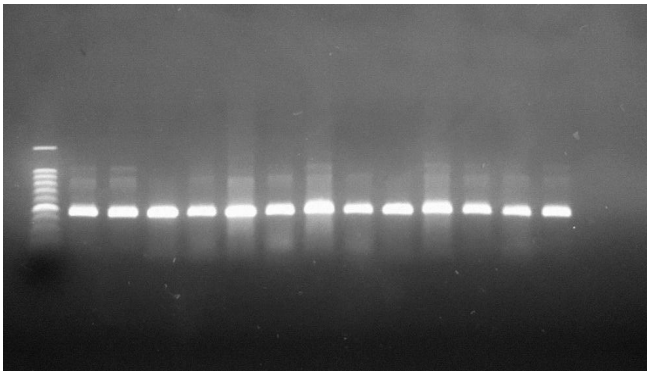

Fig. 4: Gel electrophoresis image using (1%) agarose shows

Phylogenetic tree analysis of the currently identified sequences referred as red triangle with their corresponding accession numbers. As can be seen, the Iraqi strains mainly appeared in three clades: the first one includes: MT465320, MT465319, MT465321, and MT465317. One isolate appeared divergent from the rest which is deposited in the gene bank as (MT465316) while the rest appeared closely related to the isolate from Egypt. The off-grouped sequences were seen in the sequences originated from Norway, Thailand, and Kenya (Fig. 5).

Discussion

Giardiasis infection occurred in many animals species and human worldwide and the parasite varied according to host species, season of sample collection, geographical location and type of diarrhoea (Lee *et al.,* 2016; Deng *et al.,* 2017; Nguyen *et al.,* 2018).

The investigation about *Giardia* parasite in horses was conducted on 180 faecal samples during period of 6 months at Baghdad city. The total infection rate with *Giardia* in present study was (26.1%), this result may related to animal husbandry, breed, type of nutrition and

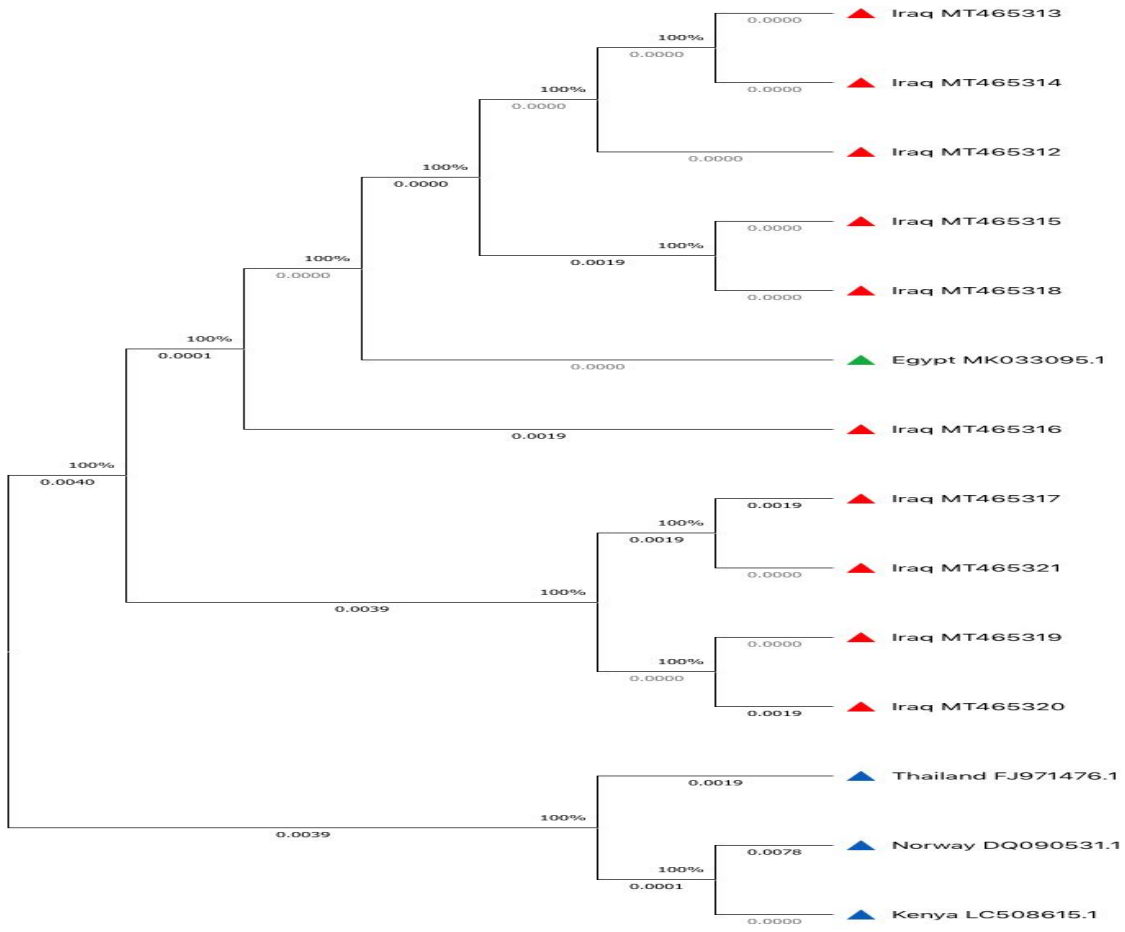

Fig. 5: Phylogenetic tree analysis of Giardia (beta-giardin) gene.

lack of attention to animal hygiene. This result was agree with result of (Butty, 2011), which recorded total infection rate 19.63% in Nineveh, Iraq. Also with Santín *et al.,* (2013) result which recorded prevalence rate 34 (17.4%) out of 195 in Colombia and Demircan *et al.,* (2019) which recorded the prevalence of *Giardia* was 25 (16.6%) out of 150 sample in Kayseri, Central Anatolia Region in Turkey.

The present study was higher than result of (Li *et al.,* 2020), which recorded prevalence rate 2.8% (9/325) in Shandong Province, Xinjiang Autonomous Region, and Inner Mongolia Autonomous Region in northern China during 2015–2019. Also the result of this study was higher than (Qi *et al.,* 2020), result which recorded 48 (7.7%) out of 621fecal samples collected from 15 cities in China and 34 (8.5%) out of 326 was the prevalence of *Giardia* recorded in Jordan by Mukbel *et al.,* (2017). Qi *et al.,* (2015) revelled the prevalence of *Giardia* parasite in horses was 1.5% (4/262). Also the result of this study was higher than (Traversa *et al.,* 2012), which recorded prevalence rate 8.6% in Italia. The infection rate of present study was lower as compared to 83% 10/12 in USA recorded by (Traub *et al.,* 2005).

The difference in *Giardia*sis prevalence in varied areas may be associated with feeding situations, different local climatic conditions, detection methods, sampling time, sample size and animal husbandry practices as well as different susceptibility of different breeds of horse. Also the nature of horse husbandry with restricted movement, limited grazing and low density, might have played a role in rising the infection rate compared to other parts of the world.

The present study revealed that both sex were subjected to the infection with *Giardia*, the total rate of infection was 25% (27/108) in males and 27.7% (20/72) in females, without significant differences. Similar result was recorded in the prevalence of *Giardia* parasite in horses between both sex by (Demircan *et al.,* 2019). The result of present study also was agree with (Qi *et al.,* 2020) study which found no significant difference between males and females horses. Also with (Santín *et al.,* 2013) was found no significant difference between males and females infection and (De Souza *et al.,* 2009).

Regarding the age group the present study revealed that animals older than 6 years are less infected with *Giardia* parasite, the total infection rate in >3-<6 years was 25 (28.7%) out of 87, <1-3 years old are 17 (27.4%) out of 62, and 6-20 years old are 5 (16.1%) out of 31. The present study agree with (Demircan *et al.,* 2019) which show the highest infection rate was observed in horse aged between 3-6 year (23.9%), followed by adults aged 7-10 years (14.2%), with the lowest infection rate observed in horse aged 11-15 years (11.7%). This study also agree with (Butty, 2011) which show the highest infection rate was at 5-6 years old.

The result of the study was disagree with (Kostopoulou *et al.,* 2015) which found infection in foal's age less than 30 day was more susceptible for infection with *Giardia*. (Veronesi *et al.,* 2010) show that the infection with *Giardia* common in foal more than older horses. That differences may be due to variant in immune status of animals Santín *et al.,* (2013) found that no significant differences were recorded according to age group. also Qi *et al.,* (2020) found that no significant differences were recorded according to age group. Mukbel *et al.,* (2017) found no significant differences was recorded according to age. Similar result were observed by (Qi *et al.,* 2015) which found no significant differences based on age. Atwill *et al.,* (2010) show that no relation between age and prevalence of *Giardia*. Low levels of infection in old horses may be to their strong immunity, making them less susceptible to infection.

The present study revelled a significant differences ($Pd \leq 0.01$) between rate of infection according to months, the highest infection rate was recorded in February 11/30 (36.6%), November 9/30 (30%), October 8/30 (26.6%), March 7/30 (23.3%), and December 7/30 (23.3%) while the lowest rate was recorded in January 5/30 (16.6%). This study disagree with (Butty, 2011) which show no significant differences between rate of infection during the months of the study. The differences in the percentage of infection according the months may be related to different factors such as number of samples, environmental condition, age, sex, immunity status, stress factor.

Although *Giardia*sis is an important infection of both humans and animal, there have been limited molecular studies on the characterization and prevalence of the parasite in horses in worldwide (Veronesi *et al.,* 2010; Traversa *et al.,* 2012; Santín *et al.,* 2013; Kostopoulou *et al.,* 2015; Qi *et al.,* 2015; Deng *et al.,* 2017). This is the first study to investigate the *Giardia* infections in horses using molecular methods in Iraq.

In this study *Giardia intestinalis* of 10 positive sample for bg gene the assemblage B was the dominant assemblage.

A sequence analysis of 48 *G. duodenalis* (SSU rRNA)- positive samples identified three assemblages of *G. duodenalis* 75.0% (36/48) were infected with assemblage B, 20.8% (10/48) with assemblage A, and 4.2% (2/48) with assemblage E. Assemblage B was the

dominant assemblage and was detected at almost all *G. duodenalis* positive.

To date, three main *G. duodenalis* assemblages have been detected in horses throughout the world, including zoonotic assemblages A and B and livestock-specific assemblage E. Assemblage B is the predominant genotype. In China, assemblage A (n = 2) and two assemblage B (n = 2) samples were identified in grazing horses in Xinjiang Uygur Autonomous Region (Qi *et al.,* 2015) and 22 *G. duodenalis* tpi positive samples were identified as assemblage A (n = 5), assemblage B (n = 14), or assemblage G (n = 3) in Sichuan Province (Deng *et al.,* 2017).

In a previous study in Colombia, 34 *G. duodenalis* isolates from horses were identified as assemblage A (n =2) or assemblage B (n =32). Six *G. duodenalis* isolates from horses were identified as assemblage B in New York State and four from horses in Western Australia as assemblage A (Traub *et al.,* 2005). In contrast, in a study in Italy, all 20 *G. duodenalis*-positive isolates from horses were identified as assemblage E (Veronesi *et al.,* 2010), whereas another study in Italy identified 37 *G. duodenalis*-positive isolates from horses as assemblage A (n =16), B (n = 11), or E (n = 10) (Traversa *et al.,* 2012). In this study, assemblage B (n =36) was more prevalent than assemblage A (n =10) or E (n =2), supporting the data cited above. Assemblages A and B are responsible for the majority of known human *G. duodenalis* disease. They also infect a wide range of animals, including livestock, nonhuman primates, companion animals, and wild animals, and are even found in raw urban wastewater (Yaoyu and Xiao, 2011). Assemblage E is primarily specific to hoofed livestock, although it is also regularly detected in other animals in China, including rabbits, horses, flies, pigs, cats, dogs, nonhuman primates, and Parasitol chinchillas (Li *et al.,* 2017). Surprisingly, in Egypt, assemblage Ewas the secondmost frequent genotype in 20 *G. duodenalis*-positive human faecal samples, accounting for three cases (15%, 3/20) (Foronda *et al.,* 2008). In another study in Brazil, 15 of 44 samples from children (34%, 15/44) were identified as assemblage E (Fantinatti *et al.,* 2016). These studies indicate that assemblage E may also have zoonotic and anthropozoonotic profiles in the transmission of *G. duodenalis*. Therefore, the presence of assemblages A, B, and E in the racehorses in our study suggests that *G. duodenalis* is transmitted between species and that race-horses constitute a potential zoonotic risk to humans.

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