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The Relative Frequency of *Histomonas meleagridis* Infection in Commercial and Backyard Turkey Flocks in Golestan, Mazandaran, Gilan, and Tehran Provinces of Iran

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Abstract

Background: Histomoniasis is caused by the protozoan Histomonas meleagridis with an intermediate host of Heterakis gallinarum, which results in ulceration of the ceca walls, enlargement of the ceca by large casts, mesenteric inflammation, and liver necrosis. This disease is very important in the turkey breeding industry that is growing in Iran**.**

Objectives: The present study aims to evaluate the relative frequency of *Histomonas meleagridis* infection in different turkey flocks to draw a cross-sectional picture of *Histomonas meleagridis* infection in Golestan, Mazandaran, Gilan, and Tehran provinces of Iran.

Methods: This study is a cross-sectional survey of Histomonas meleagridis infection during spring. Dropping samples were taken from backyard and commercial turkey flocks. After taking the fecal samples, Giemsa staining under a light microscope was investigated. To confirm the diagnosis of infection, a PCR test was performed.

Results: Out of 240 samples (from 19 flocks), 20 were detected by direct microscopic observation of *Histomonas meleagridis,* and 15 samples were confirmed by PCR.

Conclusions: The results of this study showed that the relative frequency of *Histomonas meleagridis* infection was lower than in similar studies in other parts of the world. This may be due to the less widespread use of turkey production in Iran. The growth of the turkey production industry in Iran over the last decade, as well as forecasts of further growth over the next few years, evaluate histomoniasis as a necessity.

Keywords:

Giemsa staining, Histomonas meleagridis, Histomoniasis, PCR, Turkey

Introduction

Histomonas meleagridis is a protozoan pathogen of birds, mainly turkeys, and the causative agent of blackhead disease. Blackhead disease or histomoniasis is a disease of the liver and ceca leading to necrosis and inflammation of liver tissue and typhlitis. Mortality in turkeys varies from nearly 100% in susceptible young poults to less than 10% or subclinical infection in mature turkeys with good gut health and immunity (Hess *et al.,* 2015). Infection with *H. meleagridis* is transmitted in turkey flocks through direct and/or indirect routes. *Heterakis gallinarum*, a cecal nematode, is present mostly in chicken and less in turkey`s ceca. *Heterakis gallinarum* eggs reserve and protect *H. meleagridis* from harsh environmental conditions that can increase the stability of these protozoa. Earthworms also play an important role in the epidemiology of blackhead disease by concentrating *H. gallinarum* eggs in their body and subsequently *H. meleagridis* (Beckmann *et al.,* 2021). Currently, drugs used for the prevention and treatment of histomoniasis are banned for use in food-producing animals in North America and European Union (Liebhart *et al.,* 2017). Additionally, even though numerous studies have been conducted on histomoniasis vaccination (Mitra *et al*., 2018; Lagler *et al*., 2021; Mitra *et al*., 2021), unless such important viral diseases like Newcastle Disease (Morovati *et al*., 2022) no commercial vaccine is available for turkeys yet.

In Iran, according to the official reports of the Ministry of Agriculture Jihad in 1393 and 1398, turkey production has been growing in most regions of the country in recent years by increasing more

than 1 million commercial turkey production from 1393 to 1398 (Annual Report of Ministry of Jihad Agriculture, 1393, 1398). After starting its turkey industry approximately 20 years ago, Iran ranks third in turkey meat production in Asia (Ehsan *et al*., 2020). Backyard poultry plays a vital role in the economy of rural and suburban people and these poultry raising systems have low hygienic protocols and mostly raise chicken and turkey near each other or even together leading to increased potential risk of histomoniasis in the backyard and commercial turkey flocks. Studies indicate that histomonosis can be considered as re-emerging infectious disease in chicken flocks of intensive production systems. Despite these facts, there is a paucity of information on the prevalence of infection with *H. meleagridis* in commercial or backyard turkey flocks from Iran. Therefore, this study was conducted to investigate the relative frequency of *H. meleagridis* infection in the backyard and commercial turkey flocks in Golestan, Mazandaran, Gilan which are the m provinces of Iran by both parasitological and molecular methods.

Materials and Methods

Sample collection

The backyard and commercial turkeys raised in 4 provinces of Iran (Tehran, Golestan, Mazandaran, and Gilan) were included in this survey. Based on the sample size formula ($N= Z^2* p * q/ e^2$) (estimated prevalence is 15 percent), at least 196 samples were required but, in this study, 240 samples were taken during spring (Table 1). The cecal-dropping samples were collected with disposable spoons and in plastic

zip-lock bags. Samples were transferred to the laboratory near the ice pack at 4º C temperature immediately after taking. As the authors know the optimum temperature for DNA extraction is -20°c.

Table 1: Flock's population number

Commercial Flocks	**Flock Population (Bird)**	**Backyard Flocks**	**Flock Population**
Golestan No. 1	3000	Golestan No. 1	30
Mazandaran No. 1	7000	Golestan No. 2	5
Gilan No. 1	2500	Golestan No. 3	20
Gilan No. 2	9000	Golestan No. 4	5
Tehran No. 1	3000	Mazandaran No. 1	150
Tehran No. 2	2500	Gilan No. 1	10
Tehran No. 3	6000	Gilan No. 2	10
Tehran No. 4	5000	Gilan No. 3	20
Tehran No. 5	3000	Gilan No. 4	20
-	-	Tehran	10

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Parasitological examination of cecal droppings

Slide smears were taken from fresh cecal dropping samples, fixed with methanol for 30 seconds, and stained with Giemsa for 25 minutes. Then, slides were observed under a light microscope with low and high-power fields to detect *H. meleagridis.*

Direct detection of Histomonas meleagridis by using PCR

Samples were also used for PCR detection of *H. meleagridis*. First, a dropping was homogenized in PBS solution and filtered by sterile cotton bandage gauze to avoid excessive fecal materials. Then, samples were boiled for 15 minutes to release the DNA from parasites. The DNA amplification was done as previously described by Huber *et al*. (2005). A small subunit ribosomal RNA gene was used to generate the forward and reverse primers, HIS5F (5'-CCTTTAGATGCTCTGGGCTG-3') and HIS5R (5'-CAGGGACGTATTCAACGTG-3'), respectively, for the detection of *H. meleagridis* (Huber *et al.,* 2005). Did the nucleotide sequences and references of the primers use to detect the parasites in samples? Positive and negative controls should be included in all PCR runs, also a negative control must be included in all DNA extractions. Kindly re-write about those, that I mentioned above.

Statistical analysis

The results were analyzed using the SPSS version. 24. The relative frequency of infection was described descriptively with a 95% confidence interval. Chi-square and Fisher's exact tests were used to analyze the qualitative data (differences in infection between native and commercial turkeys and differences in infection between provinces). *P* value ≤0.05 was considered significant. Also, the agreement coefficient of two direct parasite observation tests was calculated through Giemsa staining and molecular PCR test.

Results

Frequency of *Histomonas meleagridis* infection

The frequency of infection with *Histomonas meleagridis* by province and diagnosis method are shown in Table 2. Of the 19 flocks surveyed, nine were commercial and 10 were backyard flocks. A total of five flocks (one commercial and four backyards) were positive for *H. meleagridis* infection. From 240 samples, 181 samples from commercial flocks and 59 samples from backyard flocks were collected. One sample (0.55%, with 95% confidence interval: -10.68-79.79%) and 14 samples (23.73%, with 95% confidence interval) collected from commercial and backyard flocks, respectively, were positive for *H. meleagridis* infection (Table 3). Using Fisher's exact test, a statistically significant difference was observed between positive cases (based on Giemsa staining and PCR test) and production type (P <0.001).

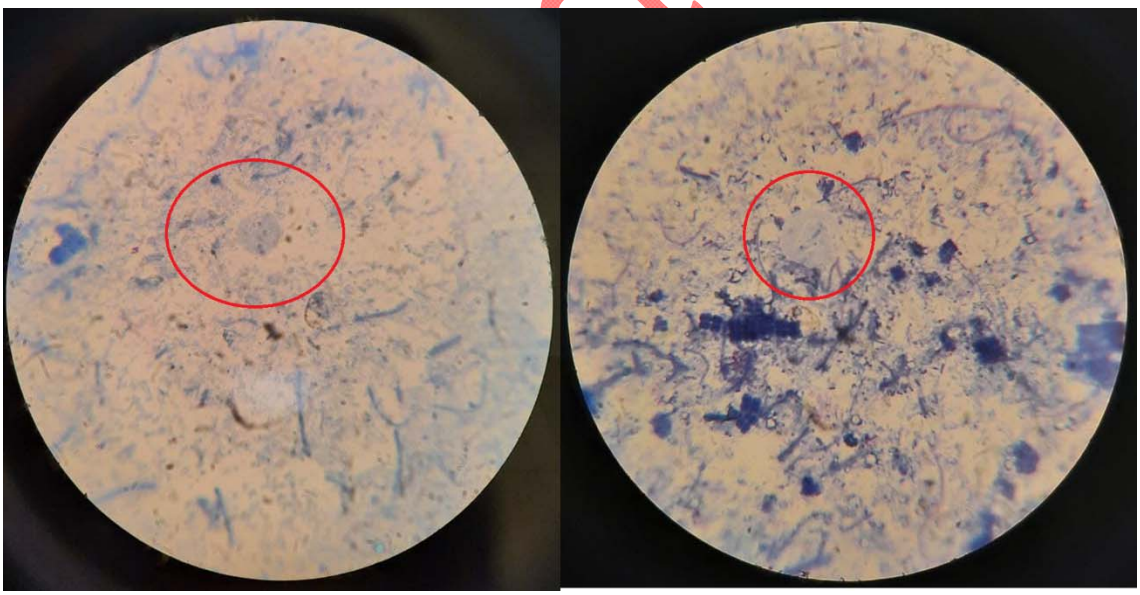

Figure 1. *Histomonas meleagridis* in Giemsa staining under the light microscope (100x).

Round bodies with foamy cytoplasm shown in the red circles are *Histomonas meleagridis* parasites.

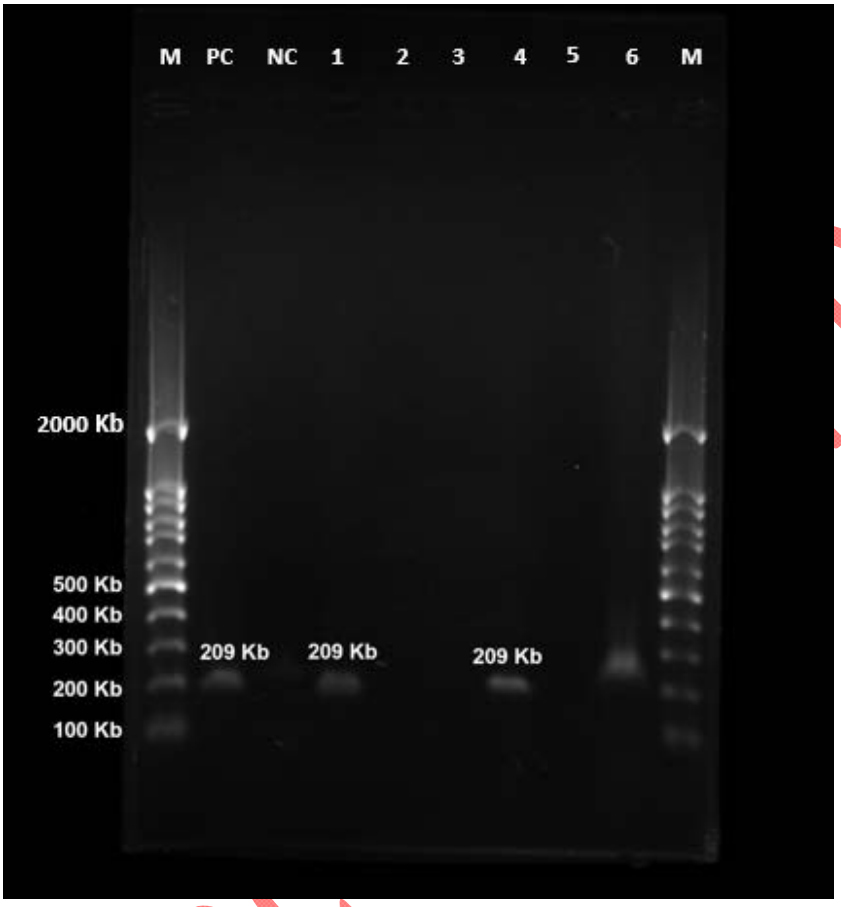

Figure 2. PCR Result- The result of the PCR test and the observation of the 209 kilobase band. Row M indicates 100-2000 kbp ladder, row PC indicates positive control, row NC indicates negative control, and rows 1 to 6 are samples. Samples 1 and 4 are positive, and the rest are negative.

Table 2. Frequency distribution of Giemsa staining and PCR results by province

Provinces	Giemsa Staining (Positive)	PCR (Positive)
Golestan	61 (5)	61 (3)
Mazandaran	52 (11)	52 (11)
Gilan	74 (4)	74 (1)
Tehran	53 (0)	53 (0)
Sum	240 (20)	240 (15)

Table 3. The number of PCR-positive flocks by province and breeding type

Provinces	Commercial Flocks No. (Positive)	Backyard Flocks No. (Positive)	Total Flocks No. (Positive)
Golestan	1 (1)	4 (2)	5 (3)
Mazandaran	1 (0)	1 (1)	2 (1)
Gilan	2 (0)	4 (1)	6 (1)

Tehran	5 (0)	1 (0)	6 (0)
Total	9 (1)	10 (4)	19 (5)

Relationship among *Histomonas meleagridis* infection, age, and flock size

In this study, samples taken from birds were divided into two age groups: adult (more than 30 weeks) and immature (less than 30 weeks). Table 4 shows the relationship between infection rates in terms of maturity. There was no statistically significant difference between positive cases (based on Giemsa staining) and maturity using the chi-square test (P = 0.127). In terms of flock size, commercial flocks were divided into two groups low numbers (below 2000 birds per commercial unit) and high numbers (above 2000 birds per commercial unit) (Table 5). Using Fisher's exact test, a statistically significant difference was observed between the positive cases (based on Giemsa staining) and the number of birds kept in industrial units (P = 0.039), but using the same test, no statistically significant difference between the positive cases (based on PCR test) and the number of birds kept in industrial units was observed (P = 0.199)

Table 4. The number of -positive flocks by Maturity

Sexual Maturity (Age)	**Giemsa Staining (Positive)**	**PCR (Positive)**
Mature	33 (5)	33 (2)

Immature	207 (15)	207 (13
Total	240 (20)	240 (15)

Table 5. The number of -positive flocks by Flock Size

Flock Size	Giemsa Staining (Positive)	PCR (Positive)
Small Commercial Units	36 (2)	36 (1)
Large Commercial Units	145 (0)	145 (0)
Total	181 (2)	181 (1)

Compatibility of direct microscopic examination of stained feces smears with Giemsa and PCR in terms of diagnosis of *Histomonas meleagridis* infection

Table 6 exhibits the degree of correlation between these two tests in measuring the infection with *H. meleagridis*. The agreement coefficient between Giemsa and PCR tests was 0.85, which indicates a relatively good agreement between the two tests. It should be noted, however, that in this study, only samples that were directly observed with *H. meleagridis* infection or were suspicious of infection were PCR-tested.

Table 6. The degree of concordance between direct microscopic observation tests and PCR test

	PCR		
	Negative	**Positive**	**Total**

	Negative	220	0	220
Giemsa	**Positive**	5	15	20
	Total	225	15	240

Discussion

Histomoniasis can be classified as a recurrent disease. As the global trend to grow poultry without the usage of antibiotics increases to control some condition like Salmonella infection (Gholipour-Shoshod et al., 2023), the disease is re-emerging in poultry and turkey flocks. Therefore, the study of the status of infection with the parasite *Histomonas meleagridis* can be a good prediction of the importance of this disease in the country (Jones *et al*., 2020, Hess *et al*. 2015).

The results of this study showed that out of 240 samples taken from 19 commercial and backyard flocks, 20 and 15 samples, respectively, were positive for *H. meleagridis* by direct observation and PCR. In some samples, the rate of infection was very low, which explains why some samples that are positive in microscope observation are negative via PCR.

The frequency of *H. meleagridis* infection has been the subject of various investigations in many parts of the world. In 2010, Hawke and co-workers studied 156 clinically histomoniasis-suspected and found that 65 (41.7%) were infected with *H. meleagridis* (Jahantigh *et al*. 2015). In another study in

China, out of 304 suspected histomoniasis, 288 samples were confirmed to be infected with *H. meleagridis* through histopathology, however, only 276 samples were then confirmed by PCR (Xu *et al,* 2018). In Vietnam, Ngoyan *et al*. (2015) reported 12.9% positive samples by direct observation among 194 samples taken from 36 healthy flocks. In the present study, out of 240 samples taken from 19 flocks, 10 infected flocks were confirmed by PCR and the contamination percentage was 26.32. In the case of backyard turkeys tested in this study, out of 59 samples, 14 samples were positive by PCR, which indicates contamination of 23.7% in these birds (Ngoyan *et al*. 2015).

Various outbreaks of the disease have been reported in European and American countries in recent years since the ban on the use of drugs and antibiotics in poultry farming (Liebhart *et al.,* 2017). In another study, Bilic *et al*. (2020) showed a link between the occurrence of histomoniasis and some bacteria like *E. coli* (Bilic and Hess, 2020). Therefore, the less observation of *H. meleagridis* infection in Iranian commercial turkey flocks may be attributed to the widespread use of antibiotics in Iran. However, the rate of infection in this study was 26.32%, which was close to infection in most parts of the world.

The occurrence of *H. meleagridis* infection in turkey flocks of Iran's neighboring countries has been investigated (Al-Alousi *et al*., 2008, Abdullah *et al*., 2014, Al-Moussawi, *et al*., 2016). In Iraq, Al-Alousi *et al*. (2008) confirmed the infection with *Histomonas meleagridis* in local chickens in villages in the Fallujah region of Iraq (Al-Alousi *et al*., 2008). Later, Abdullah *et al*. (2014) reported histomoniasis in the Iraqi Kurdistan region in 42 turkeys with suspected clinical signs of the disease by parasitological and histopathological studies (Abdullah *et al*., 2014). In another 2016 study, Al-Moussawi *et al*. reported

contamination of turkey nematodes in the Al-Nasiriyah area with *Heterakis gallinarum*, the intermediate host of *H. meleagridis* (Al-Moussawi *et al*., 2016). In the Van region of Turkey, histomoniasis was diagnosed in turkeys (Gunerhan *et al*. 2018). The investigations around Iran clearly show the presence of histomoniasis in the neighboring countries which may lead to the transfer of infection to the border provinces of Iran.

Studies have also been performed on the occurrence of *H. meleagridis* infection in Iran. In 2017, the rate of infection with *H. meleagridis* in chickens in Lorestan province and reported 31% infection rate (Badparva and Kheirandish, 2017). In two case studies, cases of *Histomonas* infection in turkeys in Mashhad and infection in Quebec Choker were reported (Razmi *et al*., 2006, Abbasnia *et al*., 2018). In 2018, Farjanikish *et al*. also examined the morphology of histones in Japanese quail (Farjanikish and Beyraghi, 2018). According to the available information, no comprehensive study has been conducted on the rate of infection with *H. meleagridis* in turkeys in Iran.

In this study, infection was observed in the northern provinces of the country, namely the Golestan, Gilan, and Mazandaran provinces. No *H. meleagridis* infection was observed in turkeys kept in Tehran province which may be due to the much lower breeding of backyard birds in Tehran province compared to the northern provinces. Simultaneous breeding of chickens, turkeys, and other backyard birds is seen in most parts of the northern provinces. Considering the extent of hosting *Heterakis gallinarum* (intermediate host of *H. meleagridis*) (Cupo and Beckstead, 2019), the possibility of more infection with this worm in birds of northern provinces than birds in Tehran province is another possible reason for not observing *H.*

meleagridis in Tehran. Recent epidemiological studies showed that a turkey house within 3 miles of a chicken house was 4.6% more likely to experience an outbreak of histomoniasis than a house outside of this diameter (Jones et al., 2020 J. Appl. Poult. Res. 29:496–501)

In this study, the sampling of flocks was performed cross-sectionally. According to previous studies, the possibility of infection with *H. meleagridis* is higher in warmer seasons. Because this parasite is not very resistant to low temperatures (Hauck *et al.,* 2010). Therefore, to measure the prevalence of contamination more accurately, it is better to conduct sampling in all seasons in more comprehensive studies to show a more accurate estimate of the contamination rate. This was not possible in this study due to the limitations of the Covid-19 pandemic.

The correlation between histomoniasis and turkey age is an issue that has been shown in previous studies as it has been reported to be more common at 9 weeks of age (Hauck *et al.,* 2018). However, in this study, no significant relationship was observed between the rate of infection with *H. meleagridis* and the age of turkeys. It is noteworthy to mention that in our study the presence/ absence of *H. meleagridis* in turkey feces and the presence of histomoniasis was not investigated. Therefore, the lack of connection between infection and not the presence of disease seems to be justified.

In farms, management procedures play a key role in causing diseases. Backyard production and commercial production differ greatly in terms of biosecurity level, wild bird handling, keeping different species of birds, and farmers' knowledge. Therefore, it is imperative to study the level of infection in these two types of production. Histomoniasis has been studied extensively. Studies by Hauck *et al*. in

2010 and 2018 showed infection in commercial birds, and also in backyard birds (Hauck, 2010, Hauck *et al*., 2018, Callait-Cardinal *et al*. 2018). There are also numerous reports of infection in backyard birds (Al-Alousi *et al*., 2008, Karaman *et al*., 2009, Abdullah *et al*., 2014, Gunerhan *et al*., 2018). Study results suggest that the type of production influences the rate of infection with H meleagridis in turkeys. In the present study, one infected sample was observed out of 181 samples taken from commercial birds. Of the 59 samples taken from native birds, 19 were positive by direct microscopic observation and 14 were confirmed by PCR. This issue may indicate the more significant importance of this disease in backyard and semi-commercial breeding.

Another important factor in the spread of poultry diseases in commercial units is the number of birds kept and their density. Histomoniasis is no exception. A 2010 study by Callait *et al*. found that there was no association between flock size and histomoniasis (Callait-Cardinal *et al*. 2010). While the results of the present study show that there is a significant relationship between the rate of infection with *H. meleagridis* in commercial flocks and the dimensions of the farm. Flocks with fewer than 2,000 birds are more likely to be infected. This is probably due to the seriousness of quarantine and biosecurity issues in larger collections.

In conclusion, the study on "The relative frequency of *Histomonas meleagridis* infection in commercial and backyard turkey flocks in Golestan, Mazandaran, Gilan, and Tehran provinces of Iran" provides valuable insights into the prevalence and risk factors associated with this infection in turkey flocks. The study found the infection in both commercial and backyard turkey flocks, with backyard flocks being more susceptible. The findings of this study highlight the presence of *Histomonas meleagridis* infection in

different turkey flocks in Iran. It also calls for further research to identify more effective preventive and control measures, which can help reduce the impact of the infection on turkey production in Iran and other parts of the world. Because H. meleagridis enveloped in cecal content may allow for oral infection, litter quality and better litter management could be critical to control lateral transmission.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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بررسی فراوانی نسبی آلودگی به *هیستوموناس مله اگریدیس* در گله‌های بوقلمون صنعتی و بومی در استان‌های گلستان، مازندران، گیلان و تهران در ایران

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زمینه مطالعه: هیستومونیازیس توسط تک یاخته *هیستوموناس مله‌اگریدیس* با میزبان واسط *مترا*کیس *گالیناروم* ایجاد می شود که منجر به زخم شدن دیواره های سکوم، بزرگ شدن آن توسط کست های بزرگ، التهاب مزانتریک و نکروز کبد می شود. این بیماری در صنعت رو به رشد پرورش بوقلمون در ایران بسیار حائز اهمیت است.

هدف: هدف از مطالعه حاضر بررسی فراوانی نسبی عفونت *هیستوموناس مله اگریدیس* در گله های مختلف بوقلمون به منظور ترسیم تصویر مقطعی از این عفونت *هیستوموناس* در استان های گلستان، مازندران، گیلان و تهران در ایران می باشد.

روش کار: این مطالعه یک بررسی مقطعی از عفونت *هیستوموناس ملهاگریدیس* است. نمونه برداری از گله های بوقلمون بومی و صنعتی انجام شد. پس از گرفتن نمونه مدفوع، مشاهده انگل در رنگ آمیزی گیمسا در زیر میکروسکوپ نوری مورد بررسی قرار گرفت. برای تایید تشخیص عفونت، آزمایش PCR انجام شد.

نتایج: از 240 نمونه، 20 نمونه با مشاهده میکروسکوپی مستقیم *هیستوموناس ملهاگریدیس* و 15 نمونه با روش PCR تایید شد.

نتیجه‌گیری نهایی: نتایج این مطالعه نشان داد که فراوانی عفونت *هیستوموناس ملهاگریدیس* نسبت به مطالعات مشابه در سایر نقاط جهان کمتر است. این مسئله ممکن است به دلیل حجم پرورش کمتر بوقلمون در ایران باشد. با توجه به اینکه صنعت تولید بوقلمون در ایران در دهه اخیر رشد چشمگیری داشته است و با پیش بینی روند رو به رشد این صنعت در سال های آینده، اهمیت مطالعه هیستومونیازیس بیش از پیش احساس می شود.

کلمات کلیدی: بوقلمون، پی سی آر، رنگ‌آمیزی گیمسا، *هیستوموناس ملهاگریدیس*، هیستومونیازیس