Original Article

The Relative Frequency of *Histomonas meleagridis* Infection in Turkey Flocks in Some Provinces of Iran

Ali Salavati, Seyed Mostafa Peighambari, Azam Yazdani, Jamshid Razmyar*

Department of Avian Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, 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 Iran's growing turkey breeding industry.

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

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

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

Conclusion: The results of this study showed that the relative frequency of *H. meleagridis* infection was lower than in similar studies in other parts of the world. This finding may be due to Iran's less widespread use of turkey production. Considering the growth of the turkey production industry in Iran over the last decade and its further production over the next few years, it is necessary to evaluate histomoniasis.

Keywords: Giemsa staining, *Histomonas meleagridis*, Histomoniasis, PCR, Turkey

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*** Corresponding Author:**
Jamshid Razmyar, Associate Professor.
Address: *Department of Avian Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.*
Phone: *+98 (21) 61117192*
E-mail: *jrazmyar@ut.ac.ir*

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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 <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 or indirect routes. *Heterakis gallinarum*, a cecal nematode, is primarily present in chickens and less in turkeys' ceca. *H. gallinarum* eggs reserve and protect *H. meleagridis* from harsh environmental conditions, which can increase the stability of these protozoa. Earthworms also play an essential 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 to prevent and treat histomoniasis are banned for food-producing animals in North America and the 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, turkey production has been growing in most regions of the country in recent years by increasing more than 1 million commercial turkey production from 2013 to 2018 (Ministry of Agriculture Jihad, 2013, 2018). 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 is a re-emerging infectious disease in chicken flocks of intensive production systems. Despite these facts, there is a lack of information on the prevalence of infection with *H. meleagridis* in commercial or backyard turkey flocks from Iran. Therefore, this study investigated the relative frequency of *H. meleagridis* infection in backyards and commercial turkey flocks in Iran's Golestan, Mazandaran, and Guilan provinces. They are the major provinces of Iran for turkey production. We used both parasitological and molecular methods to detect the infection.

Materials and Methods

Sample collection

The backyard and commercial turkeys raised in 4 provinces of Iran (Tehran, Golestan, Mazandaran, and Guilan) were included in this survey. Based on the sample size formula ($N=Z^2\times p\times q/e^2$) (estimated prevalence is 15%), at least 196 samples were required. However, 240 samples were taken during spring in this study (Table 1). The cecal-dropping samples were collected with disposable spoons and in plastic zip-lock bags. Samples were transferred to the laboratory on ice packs at 4°C temperature immediately after collection. The optimum temperature for DNA extraction was -20°C.

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, the slides were observed under a light microscope with low and high-power fields to detect *H. meleagridis*.

Direct detection of *H. meleagridis* using PCR

Samples were also used for PCR detection of *H. meleagridis*. First, a drop was homogenized in PBS (phosphate-buffered saline) 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 detecting *H. meleagridis* (Huber et al., 2005).

Statistical analysis

The results were analyzed using the SPSS software, version 24. The relative frequency of infection was described descriptively with a 95% CI. The chi-square and Fisher exact tests were used to analyze the qualitative data (differences in infection between native and commercial turkeys and differences in infection between provinces). $P\leq0.05$ was considered significant. Also, the agreement coefficient of two direct parasite observation tests was calculated through Giemsa staining and molecular PCR test.

Table 1. Flock's population number

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

Results

Frequency of *H. meleagridis* infection

The frequency of infection with *H. meleagridis* by province and diagnosis method are shown in Table 2. Of the 19 flocks surveyed, 9 were commercial, and 10 were backyard flocks. A total of 5 flocks (1 commercial and 4 backyards) were positive for *H. meleagridis* infection (Figure 1). From 240 samples, 181 samples from commercial flocks and 59 samples from backyard flocks were collected. One sample (0.55% with 95% CI, -10.68%, 79.79%) and 14 samples (23.73% with 95% CI) collected from commercial and backyard flocks, respectively, were positive for *H. meleagridis* infection (Table 3). Using the Fisher exact test, a statistically significant difference was observed between positive cases (based on Giemsa staining and PCR test) and production type ($P<0.001$).

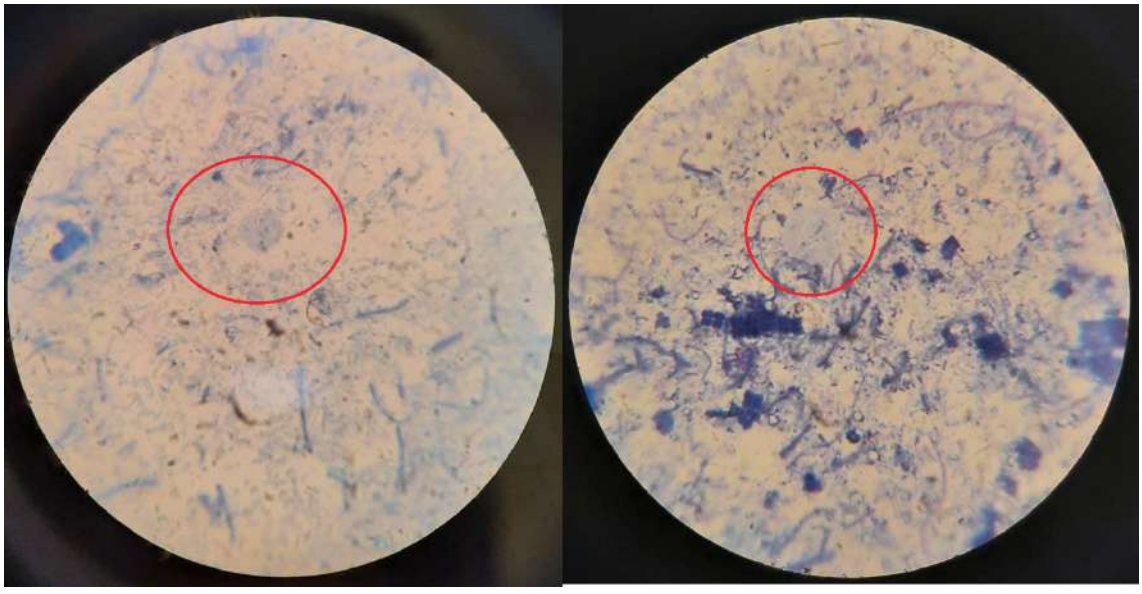

Figure 1. *H. meleagridis* in Giemsa staining under the light microscope (x100)

Note: Round bodies with foamy cytoplasm shown in the red circles are *H. meleagridis* parasites.

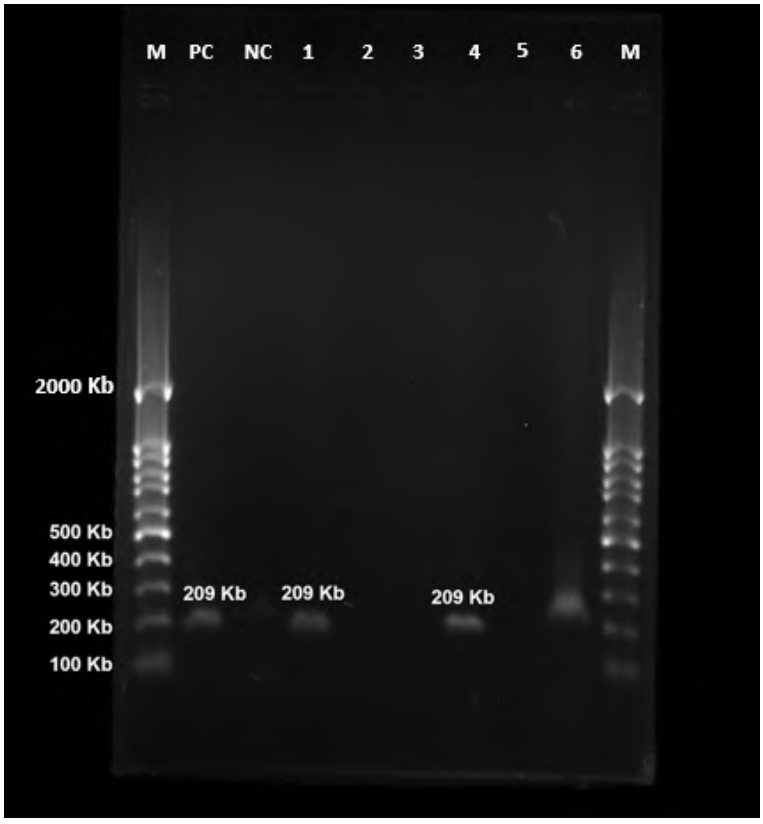

Figure 2. The result of the PCR test and observation of 209 kb band

According to Figure 2, row 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.

Relationship among *H. meleagridis* infection, age, and flock size

In this study, samples taken from birds were divided into two age groups: Adult (>30 weeks) and immature (<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 the Fisher 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 2. Frequency distribution of Giemsa staining and PCR results by province

Province	No. (%)	
	Positive	
	Giemsa Staining	PCR
Golestan	61(5)	61(3)
Mazandaran	52(11)	52(11)
Guilan	74(4)	74(1)
Tehran	53(0)	53(0)
Total	240(20)	240(15)

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

Province	Flocks No. (Positive)		
	Commercial	Backyard	Total
Golestan	1(1)	4(2)	5(3)
Mazandaran	1(0)	1(1)	2(1)
Guilan	2(0)	4(1)	6(1)
Tehran	5(0)	1(0)	6(0)
Total	9(1)	10(4)	19(5)

Table 4. The positive flocks by maturity

Sexual Maturity (Age)	No. (%) Positive	
	Giemsa Staining	PCR
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	No. (%) Positive	
	Giemsa Staining	PCR
Small commercial units	36(2)	36(1)
Large commercial units	145(0)	145(0)
Total	181(2)	181(1)

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

Variables		No. PCR		
		Negative	Positive	Total
Giemsa	Negative	220	0	220
	Positive	5	15	20
	Total	225	15	240

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

Table 6 presents 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 tested by PCR.

Discussion

Histomoniasis can be classified as a recurrent disease. As the global trend to grow poultry without antibiotics increases to control conditions 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 *H. meleagridis* can be a good prediction of the importance of this disease in the country (Jones et al., 2020; Hess et al., 2015).

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 infection rate 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 confirmed by PCR (Xu et al., 2018). Ngoyan et al. (2015) reported 12.9% positive samples by direct observation among 194 samples taken from 36 healthy flocks in Vietnam. 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 (Nguyen 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*. 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 infection rate in this study was 26.32%, which was close to the infection rate in most parts of the world.

The *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 *H. meleagridis* in local chickens in villages in the Fallujah region of Iraq. 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. In another 2016 study, Al-Moussawi et al. reported contamination of turkey nematodes in the Al-Nasiriyah area with *H. 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 infection rate of *H. meleagridis* in chickens in Lorestan Province was reported to be 31% (Badparva & Kheirandish, 2017). Two case studies reported Histomonas infection in turkeys in Mashhad City, Iran, and in Quebec Choker (Razmi et al., 2006; Abbasnia et al., 2018). In 2018, Farjanikish et al. (2018) also examined the morphology of histones in Japanese quail. According to the available information, no comprehensive study has been conducted on the infection rate of *H. meleagridis* in turkeys in Iran.

In this study, infection was observed in the northern provinces of the country, namely the Golestan, Guilan, and Mazandaran provinces. No *H. meleagridis* infection was observed in turkeys kept in Tehran Province, possibly due to the much lower breeding of backyard birds in Tehran Province compared to the northern provinces. The simultaneous breeding of chickens, turkeys, and other backyard birds is seen in most northern provinces. Considering the extent of hosting *H. gallinarum* (intermediate host of *H. meleagridis*) (Cupo & 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)

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 procedure 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 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 infection rate with *H. meleagridis* and the age of turkeys. Notably, our study did not investigate the presence or absence of *H. meleagridis* in turkey feces and histomoniasis. Therefore, the lack of connection between infection and the presence of disease seems to be justified.

In farms, management procedures play a key role in causing diseases. Backyard and commercial production differ significantly in biosecurity level, wild bird handling, keeping different species of birds, and farmers' knowledge. Therefore, studying the infection rate in these two production types is imperative. Histomoniasis has been studied extensively.

Research conducted by Hauck and colleagues has demonstrated the presence of Histomonas meleagridis in both commercial and backyard turkey flocks (Hauck, 2010; Hauck 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 production type influences turkeys' infection rate with *H. meleagridis*. In the present study, one infected sample was observed out of 181 samples from commercial birds. Of the 59 samples from native birds, 19 were positive by direct microscopic observation, and PCR confirmed 14. 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 no association between flock size and histomoniasis (Callait-Cardinal et al., 2018). While the results of the present study show a significant relationship between the infection rate of *H. meleagridis* in commercial flocks and the dimensions of the farm. Flocks with fewer than 2000 birds are more likely to be infected. This is probably due to the seriousness of quarantine and biosecurity issues in larger collections.

Conclusion

In conclusion, the study on the relative frequency of *H. meleagridis* infection in commercial and backyard turkey flocks in Golestan, Mazandaran, Guilan, 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 commercial and backyard turkey flocks, with backyard flocks being more susceptible. The findings of this study highlight the presence of *H. 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 management could be critical to control lateral transmission.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization and supervision: Jamshid Razmyar; Methodology: Seyed Mostafa Peighambari and Jamshid Razmyar; Laboratory tests: Azam Yazdani and Ali Salavati; Data analysis and visualization: Hesameddin Akbarein; Sampling and writing the original draft: Ali Salavati; Review and editing: Seyed Mostafa Peighambari.

Conflict of interest

The authors declared no conflict of interest.

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مطالعه پژوهشی

فراوانی نسبی آلودگی به هیستوموناس مله اگریدیس در گله‌های بوقلمون در چند استان ایران

علی صلواتی، سید مصطفی پیغمبری، اعظم یزدانی، *جمشید رزم یار

گروه بیماری‌های طیور، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران.

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چکیده

زمینه مطالعه: هیستومونیازیس توسط تک یاخته هیستوموناس مله‌اگریدیس با میزبان واسط هتراکیس گالیناروم ایجاد می‌شود که به زخم شدن دیواره‌های سکوم، بزرگ شدن آن توسط کست‌های بزرگ، التهاب مزانتریک و نکروز کبد منجر می‌شود. این بیماری در صنعت روبه‌رشد پرورش بوقلمون در ایران بسیار حائز اهمیت است.

هدف: مطالعه حاضر باهدف بررسی فراوانی نسبی عفونت هیستوموناس مله اگریدیس در گله‌های مختلف بوقلمون به منظور ترسیم تصویر مقطعی از این عفونت هیستوموناس در استان‌های گلستان، مازندران، گیلان و تهران در ایران انجام شد.

روش کار: این مطالعه یک بررسی مقطعی از عفونت هیستوموناس مله اگریدیس است. نمونه‌برداری از گله‌های بوقلمون بومی و صنعتی صورت گرفت. پس از گرفتن نمونه مدفوع، مشاهده انگل در رنگ‌آمیزی گیمسا در زیر میکروسکوپ نوری مورد بررسی قرار گرفت. برای تأیید تشخیص عفونت، آزمایش PCR انجام شد.

نتایج: از ۲۴۰ نمونه، ۲۰ نمونه با مشاهده میکروسکوپی مستقیم هیستوموناس مله اگریدیس و ۱۵ نمونه با روش PCR تأیید شد.

نتیجه‌گیری نهایی: نتایج این مطالعه نشان داد فراوانی عفونت هیستوموناس مله اگریدیس نسبت به مطالعات مشابه در سایر نقاط جهان کمتر است. این مسئله ممکن است به‌دلیل حجم پرورش کمتر بوقلمون در ایران باشد. باتوجه‌به اینکه صنعت تولید بوقلمون در ایران در دهه اخیر رشد چشمگیری داشته است و با پیش‌بینی روند روبه‌رشد این صنعت در سال‌های آینده، اهمیت مطالعه هیستومونیازیس بیش‌ازپیش احساس می‌شود.

کلیدواژه‌ها: رنگ‌آمیزی گیمسا، هیستوموناس مله اگریدیس، هیستومونیازیس، PCR، بوقلمون

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* **نویسنده مسئول:**
دکتر جمشید رزم یار
نشانی: تهران، دانشگاه تهران، دانشکده دامپزشکی، گروه بیماری‌های طیور.
تلفن: ۶۱۱۱۷۱۹۲ (۲۱) ۹۸+
رایانامه: *jrazmyar@ut.ac.ir*

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