

Prophylactic and Therapeutic Methods Against Histomoniasis in Poultry

Nguyen Van Phuong & Bui Khanh Linh

Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi
131000, Vietnam

Abstract

Histomoniasis, commonly known as blackhead disease, is a parasitic disease in poultry caused by the protozoan *Histomonas meleagridis*. The availability of various compounds for chemotherapy in the 1970s resulted in the successful control of blackhead disease. Since the ban of antihistomonal drugs in the European Union, the disease has re-emerged, resulting in up to 100% mortality in turkey flocks. This has renewed the interest of scientists with numerous publications focusing on prophylactic strategies. This review summarizes the literature on the preventive and curative options for the control and treatment of histomoniasis. Two main approaches to the prophylaxis of the disease were found, which included chemotherapies and plant substrate products. Histostat-50 and paromomycin were the only available drugs that showed antihistomonal activity despite some concern about their threat to human health and antibiotic resistance. None of the plant substrate products provided potential protection to birds against blackhead disease. The use of attenuated histomonads could be an alternative for the prevention of the disease, but the production of this vaccine prototype is still challenging due to advanced technique requirements.

Keywords

Histomoniasis, *Histomonas meleagridis*, parasitic in poultry, prophylactic, therapeutic

Historical treatment options

Histomoniasis, also known as blackhead disease, histomonosis, or infectious enterohepatitis, is a parasitic disease caused by the protozoan *Histomonas meleagridis*, and was first described by Cushman (1893). The disease can cause inflammatory lesions in the caeca and liver of gallinaceous birds, and may lead to flock mortality of 80-100% in turkey flocks (Swayne *et al.*, 2013). Although apparent clinical signs are not observed in infected chickens, the disease can also cause significant mortality in this host (Reece *et al.*, 1986).

Since the 1970s, histomoniasis has been successfully controlled

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Correspondence to
phuongnv@vnua.edu.vn

ORCID
Nguyen Van Phuong
<https://orcid.org/0000-0003-2529-6891>

<https://vjas.vnua.edu.vn/>

by using antflagellate feed additives such as dimetridazole and nifursol (Hafez *et al.*, 2005). In subsequent years, very little was published on the prevention and treatment of the disease (**Figure 1**). Between the 1990s and 2003, the European Union successively banned all preventive and curative drugs against *H. meleagridis* for use in food-producing animals because of public concerns about toxicity and carcinogenicity of the antihistomonal drugs (Byrne, 2001; Hafez *et al.*, 2005). As a result of the removal of these treatments, there have been numerous outbreaks of histomoniasis in the layer, free-range chicken and turkey flocks, which have caused considerable economic losses. A case that demonstrated such losses was reported in Germany where an outbreak occurred in a free-range layer flock of hens resulting in 6% increased mortality and 11% decreased egg production (Esquenet *et al.*, 2003). Between 2004 and 2008, about 65 flocks in Germany were infected with *H. meleagridis*. In 10 farms of commercial turkeys, a total of 144,000 birds were detected as infected with the mortality rate rising by 40% within one week (Hafez *et al.*, 2005; Hauck *et al.*, 2010).

Outbreaks occur not only in such developed countries but have also been noticed in some developing countries including Thailand, Vietnam, and Brazil (Somkid & Niwat, 2006; Nguyen *et al.*, 2015; Araújo *et al.*, 2015). A

mortality of 70-80% was recorded in an outbreak in Thailand in 2006. In 2015, an outbreak of histomoniasis in free-range chickens with a mortality of 43.7% was reported in Brazil (Araújo *et al.*, 2015). The standards for drug usage differ between regions, and in many cases, drugs are used without sufficient scientific evidence of their efficacy.

Another factor complicating the treatment of histomoniasis was the finding that a strain of *H. meleagridis* was partially resistant to nitarsone, the only approved prophylactic antihistomonal in the U.S. (Abraham *et al.*, 2014). Additionally, arsenical residue was recently found in the livers of roxarsone-treated chickens resulting in the withdrawal of this substance from the U.S. market (Kawalek *et al.*, 2011).

This situation has led *H. meleagridis* to become of renewed scientific interest, with a number of recent studies (**Figure 1**) focusing on different prophylactic strategies including the use of chemotherapy drugs such as arsenical, synthesized nitroheterocyclic compounds, and anthelmintics (van der Heijden, 2009). Plant substances have also become an alternative treatment of this parasitic disease. Among all essential oils and extracts, Natustat and Protophyt are two commercially available plant products that have been tested as possible comments. We acknowledge the insightful

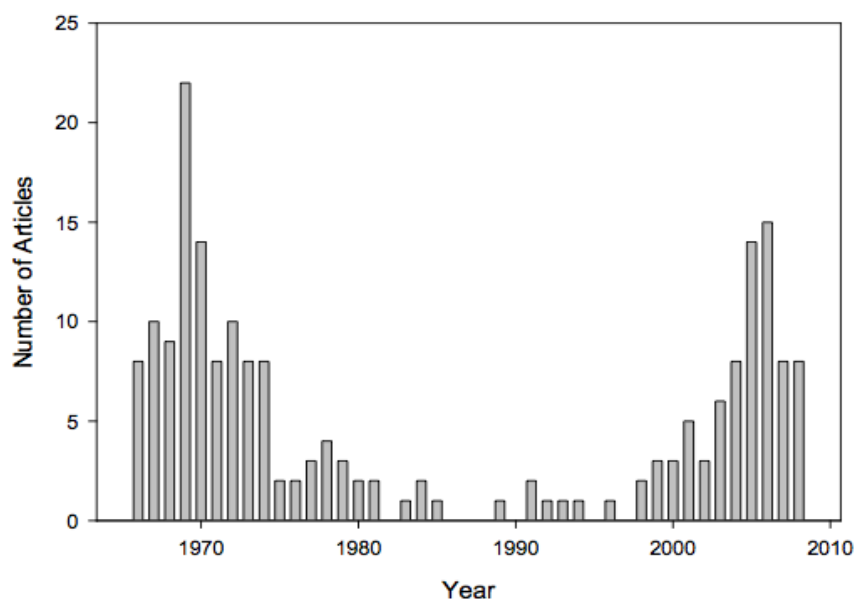


Figure 1. Number of scientific articles on *H. meleagridis* per year during the last four decades (van der Heijden, 2009)

treatments for histomoniasis (Duffy *et al.*, 2004). Focusing on immunizations against the disease, researchers have attempted vaccines with attenuated cultures of *H. meleagridis* *in vitro* and *in vivo*. The focus of this review is on prophylactic and therapeutic options for the control and treatment of histomoniasis in birds including chemotherapeutic drugs, plant substrate products, and vaccinations in the U.S. and the E.U.

Current treatment options

Chemotherapies

Arsenical compounds have been known to be very effective against histomonad infection by reducing mortality. However, the concern over arsenic toxicity resulted in the banning of these chemotherapies in the European Union in the period from the 1990s to 2003 (Byrne, 2001). Following the withdrawal of these compounds, scientists sought to find new drugs with antihistomonal properties and began the testing of various recognized antiprotozoal drugs.

In an *in vitro* experiment realized by Hu and McDougald (2004), nine antiprotozoal drugs were tested for activity against *H. meleagridis*, namely tinidazole, metronidazole, ornidazole, and diloxanide furoate, which are known as compounds active against amoebas and flagellates; albendazole and mebendazole, which are anthelmintics with antiprotozoal properties; and also some antibiotics including paromomycin sulfate, quinoline, and carbadox. Dimetridazole, an effective antihistomonal drug, which was previously banned in 2003 (Byrne, 2001) was used as a control in the study. In the *in vitro* experiment, a dose of 500,000 histomonads mL^{-1} was cultured in a modified Dwyer medium with different concentrations of the tested drugs. The results showed that growth of *H. meleagridis* was completely inhibited at $10 \mu\text{g mL}^{-1}$ or higher of dimetridazole, ornidazole, tinidazole, and metronidazole. Paromomycin sulfate only suppressed the growth of the parasite at $100 \mu\text{g mL}^{-1}$ or higher, while other drugs did not show any antihistomonal effects. These results led to an *in vivo* test where chickens, intracloacally inoculated with 200,000 *H. meleagridis*, were

subjected to the successfully tested drugs in the *in vitro* test. Dimetridazole, metronidazole, ornidazole, and tinidazole showed their high antihistomonal activity at 200-400 ppm, while paromomycin sulfate and carbadox neither improved weight gain nor reduced liver and caecal lesion scores (Hu & McDougald, 2004). These findings were quite persuasive since the authors used a strong study design where an *in vitro* experiment was followed by an *in vivo* experiment for confirmation. The sample size used in this study was sufficiently reliable ($n = 360$ birds). Moreover, the dose of *H. meleagridis* and the intracloacal inoculation has successfully been applied by previous researchers on various occasions (McDougald & Hu, 2001; Grabensteiner *et al.*, 2006; Zahoor *et al.*, 2011) which made the findings of this study more reliable.

In subsequent years, the prophylactic effect of paromomycin against histomoniasis was tested in turkeys (Hafez *et al.*, 2010). Similar to the methods of Hu and McDougald (2004), turkeys were challenged with *H. meleagridis* by intracloacal infection and then treated with different concentrations of paromomycin via a feed additive. These researchers found that the birds treated with paromomycin had significantly reduced mortality, reduced liver and caecal lesion scores, and an increased feed conversion rate compared to the control birds, indicating that paromomycin had exhibited its antihistomonal efficacy (Hafez *et al.*, 2010). This is in line with the results of a study conducted by Bleyen *et al.* (2009) where paromomycin, both in the commercial form of histoBloc and in drinking water, showed its preventative effect in 10,000 *H. meleagridis* intracloacally infected turkeys. However, this drug did not demonstrate a curative effect due to the aminoglycoside nature of paromomycin, which did not allow the drug to reach the liver stage (Bleyen *et al.*, 2009). Recently, van de Heijden *et al.* (2011) confirmed the effect of this antibiotic in the prevention of blackhead disease. Both *in vitro* and *in vivo* experiments were carried out with 3 different concentrations of paromomycin ranging from 100 to 400ppm. Although this antibiotic suppressed the growth of histomonads at all

concentrations under *in vitro* conditions, only the highest concentration provided full protection to challenged poult (Van de Heijden *et al.*, 2011). The high dose recommended in this study was not supported by the European Food Safety Authority due to the concern of bacterial resistance (EFSA, 2009). Kempf *et al.* (2013) proved the antimicrobial resistance of intestinal bacteria to paromomycin supplementation in feed additives in turkeys. *Escherichia coli*, *Enterococcus faecium*, and *Staphylococcus aureus* became resistant to paromomycin and other aminoglycosides in turkeys fed with paromomycin in feed additives. In addition, *E. coli* and *E. faecium* showed a co-selection for resistance to other antibiotics (Kempf *et al.*, 2013).

In essence, paromomycin has been identified as the most effective antihistomonal drug among various tested antibiotics despite the concern that a high dose of this drug could cause intestinal bacterial resistance in supplemented birds.

Tiamulin was another antibiotic of interest for scientists during the search for new drugs effective against blackhead disease. The curative effect of this drug was reported for the first time by Burch, who treated infected turkeys with tiamulin via drinking water at 25 mg kg⁻¹ body weight for five days (van der Heijden *et al.*, 2011). In this case, mortality was reduced significantly, and subsequently, the histomoniasis outbreak was controlled. According to this report, tiamulin had a therapeutic effect on blackhead disease, which shows promise that it could potentially be a new approach to the treatment of the disease. These findings, however, would be more convincing if the authors carried out a thorough microscopical diagnosis of histomoniasis or prepared a parasite culture to confirm the presence of *H. meleagridis* since caecal necrosis could be a result of coccidiosis (EFSA, 2009). In addition, it was likely that the authors ignored the co-effect of amoxicillin that birds had been previously supplied. Therefore, it would be beneficial to perform an *in vivo* experiment to confirm the antihistomonal effect of this antibiotic.

Hauck *et al.* (2010) carried out *in vitro* experiments in which the antihistomonal activity

of tiamulin was assessed. Tiamulin was tested at different concentrations ranging from 20 to 320 mg L⁻¹. In the experiments, different strains of *H. meleagridis* including strains 542/04, 551/04, and 1169/09 were used in different inoculation doses from 10,000 to 40,000 parasites mL⁻¹. In this study, tiamulin showed its inhibitory activity to histomonal growth at all tested concentrations. Furthermore, this antibiotic, at all concentrations, induced lethality to exposed histomonads of the low dose of inoculation while a high concentration (320 mg L⁻¹) of this antibiotic could completely destroy parasites at even a high dose (40,000 parasites mL⁻¹). This study demonstrated that tiamulin was effective against *H. meleagridis in vitro*. This finding, however, is not convincing since the doses of inoculation used in this study were relatively lower than those used in other studies. The highest dose of 40,000 histomonads mL⁻¹ was lower than 60,000 used in the study of van der Heijden (2009), and much lower than 500,000 in the experiment of Hu & McDougald (2004). Moreover, in this study, the authors pointed out that the effectiveness of the tested drug decreased when the dose of inoculation increased. Therefore, the effect of tiamulin would be questionable with a dose of inoculation as high as used in other studies.

A year later, van der Heijden *et al.* (2011) performed another *in vitro* experiment to evaluate the antihistomonal activity of tiamulin. Two-fold serial dilutions ranging from 2.5 to 400 µg mL⁻¹ of the antibiotic in PBS were added to cultures of the parasite. The starting concentration of histomonads used in the cultures was 550,000 parasites mL⁻¹. The results obtained from this experiment showed that the highest concentration of tiamulin (400 µg mL⁻¹) induced a slightly lower number of histomonads in the culture compared to the negative control cultures. This indicated that the antibiotic was unable to inhibit the growth of histomonads in the culture environment. The difference between the results of this study and Hauck's could be due to the variation in the doses of parasites used. In this study, paromomycin successfully showed its antihistomonal effect with the same doses of histomonads used in the tiamulin test. Therefore, the finding of this study was likely reliable.

In general, the effect of tiamulin in the prevention and treatment of histomoniasis is still a controversial issue. Its antihistomonal effect was reported in an *in vitro* experiment by Hauck (2010), while van der Heijden (2011) claimed the ineffectiveness of the drug in another test. The main difference between these two studies was the dose of the parasites, which probably explained the contradictory results obtained.

Arsenical Compounds

Nitarsonsone (4-nitrophenylarsonic acid), a pentavalent arsenical compound, was at one time the only available drug on the U.S. market for the purpose of preventing histomoniasis. Unfortunately, this drug was withdrawn by the U.S. Food and Drug Administration at the end of 2015 (Administration USFaD, 2015). There have been various studies assessing the effectiveness of this chemical and its commercial form, Histostat-50, on preventing blackhead disease.

In 2004, scientists from the research team led by Duffy (2004) compared the antihistomonal activity of dietary Natustat with Histostat, a commercial form of nitarsonsone. Six hundred one-day-old chickens were divided into four groups of 150 and then subjected to different treatments. The first group was the control in which birds were neither infected with histomonads nor supplemented with Natustat or Histostat. In the second group, the birds were given histomonads but not treatment drugs. Nitarsonsone at 0.1875 kg/Tone was supplemented to the birds in group 3, whereas Natustat at 1.925 kg/Tone was given to chickens in group 4. Then the birds in both groups were experimentally infected with histomonads. The infection of histomonads was induced by using litter with a confirmed presence of *Heterakis gallinarum* infected by *H. meleagridis*. The results of this study illustrated that the birds treated with nitarsonsone had significantly lower cecal and liver lesion scores compared to those of the infected-non-treated group, indicating the antihistomonal activity of this arsenic compound (Duffy *et al.*, 2004). Only one year later, the authors carried out a similar study, but this time in male turkeys. Once again, dietary Natustat for the control of *H. meleagridis* was assessed by comparing the effectiveness of

this plant substance product with that of nitarsonsone. The results obtained from this study showed that nitarsonsone substantially improved feed conversion ratios and reduced caecal and liver lesion scores in the nitarsonsone-supplemented turkeys compared with the infected non-supplemented groups. The average body weight at 42 days was greater in the nitarsonsone supplemented group than in the infected non-supplemented group (Duffy *et al.*, 2005). The findings of these two studies are not strongly supported by persuasive evidence, as the major limitation of these experiments was the unconvincing design. The authors applied a natural method of histomonad infection that had not been tested rigorously by previous researchers. The authors neither described this method in detail nor cited its referencing source. According to Lund *et al.* (1957) fewer than 0.5% of embryonated *Heterakis gallinae* eggs were found to harbor *H. meleagridis* infection, and more than 50% of the worms contained no eggs with *Histomonas* spp. Given that using litter containing *H. meleagridis*-infected *H. gallinarum* to introduce the infection is not a proven method, the findings of these studies require further confirmation.

Recently, Abraham *et al.* (2014) evaluated the sensitivity of *H. meleagridis* to nitarsonsone in culture media and turkeys. The three strains collected from different outbreaks were subjected to nitarsonsone in culture media. The results of the *in vitro* test showed that the strain of *H. meleagridis* obtained from the outbreak in Northern California had sustained resistance to nitarsonsone at 100ppm. In the *in vivo* experiments, no significant differences between the nitarsonsone-treated and the control group were found when comparing weight gain and caecal and liver lesions scores. This study illustrates that a strain of *H. meleagridis* has obtained partial resistance to nitarsonsone. The acceptable sample size and strong trial control design are supportive of the results of this study. These findings have raised concerns from scientists about the resistance of this parasite to the only permitted antihistomonal drug.

Following the ban of certain arsenic compounds, scientists began searching for an

alternative that could protect birds from histomoniasis. This search focused on the drugs available for poultry. Various drugs were tested *in vitro*, but not many of them successfully showed antihistomonal activity (**Table 2**). Several nitroimidazoles showed their effectiveness in the protection of birds from parasites (**Table 2**). However, they were banned in the E.U. and the U.S. due to the concern of carcinogenicity. Histostat-50, the commercial form of nitarosone, is the only arsenic compound that has shown potential effectiveness in preventing the disease. Recently, a strain of *H. meleagridis* was found to be partially resistant to this arsenic compound, which raised the concern of drug resistance to this unique drug. Among the various antibiotics tested, only paromomycin was capable of protecting turkeys from histomonad infection by reducing the mortality and the severity of pathological lesions, however, the use of this drug is tainted by the concern of antimicrobial resistance. The antihistomonal effect of tiamulin was still controversial since it showed a dose-reliant efficacy in *in vitro* experiments.

In brief, Histostat-50 and paromomycin are the two drugs that have been used in the U.S. for the prevention of histomoniasis in birds but there are certain concerns about the use of these drugs due to drug resistance and human safety. Histostat was already removed from the market

by the U.S. Food and Drug Administration at end of 2015 due to its carcinogenic property.

Plant extracts

As chemotherapeutics against *H. meleagridis* were banned in the European Union in 2003, farmers have been left without an effective treatment against blackhead disease. In the search for alternative treatments, researchers have investigated the use of natural plant-based products.

Natustat is a natural, plant-derived proprietary product containing yeast-derived mannanoligosaccharide, organic mineral nutrients, and plant extracts, and is used as an additive to feed for poultry (Duffy *et al.*, 2005). Various scientists have investigated the prophylactic effect of this herbal product against histomonad infections. The ability of Natustat to manipulate the composition of the intestinal microflora was proved by using PCR-DGGE techniques in the study of Waters *et al.* (2005). In the caecal contents of turkeys treated with Natustat, there were various probiotic species such as *Lactobacillus* spp. and *Bifidobacterium* spp., which may contribute to the success of this plant-derived product in the control of histomonad infection.

The antihistomonal effect of Natustat was assessed in the study of Duffy *et al.* (2004),

Table 2. Literature evaluating the antihistomonal effects of chemotherapeutic drugs

| Product | <i>In vitro</i> | <i>In vivo</i> (host species) | |
|---|-------------------------|-------------------------------|---|
| | | Chicken | Turkey |
| Diloxanide furoate, albendazole, mebendazole, quinolone, carbadox | Hu & McDougald (2004)* | | |
| Tinidazole, metronidazole, ornidazole, dimetridazole | Hu & McDougald (2004)** | Hu & McDougald (2004)** | |
| Paromomycin sulfate | Hu & McDougald (2004) | Hu & McDougald (2004)* | Hafez <i>et al.</i> (2010); Bleyen <i>et al.</i> (2010); van der Heijden (2011) |
| Histostat-50® (nitarosone) | | Duffy <i>et al.</i> (2004) | Duffy <i>et al.</i> (2005); Abraham <i>et al.</i> (2014) |

Note: * Proved ineffective for the protection of birds; ** Banned in the E.U. and the U.S.

which was evaluated in the section about arsenic compounds of this review. Among the different treatment groups, the chicks fed with Natustat had the heaviest average body weight, highest food conversion ratio, and lowest mortality. Further, the caecal lesion scores were significantly lower than in the nitarsone treated group. In a similar experiment performed in turkeys, Natustat fed birds had the same average body weights as the control (non-infected, non-supplemented) birds (Duffy *et al.*, 2005). Although the authors claimed that Natustat was effective for the prevention of histomoniasis in chickens and turkeys, the reliability of these papers could be questioned due to their unconvincing study designs.

Hauck *et al.* (2007) investigated the effect of Natustat in histomonad-infected turkeys. Three of eight birds in each cage were infected intracloacally. Heavy paper was used to cover the floor for one week in order to spread the infection to the other five birds. In this study, turkey poults received the Natustat at different rates (250g, 500g, or 1000g per ton of feed). The results revealed that treatments with Natustat at doses higher than 250g per ton yielded a significant reduction in caecal and liver lesion scores, and an increase in weight gain in indirectly exposed birds. The authors concluded that Natustat was effective in controlling the spread of histomoniasis within the flock. However, it was noticed that there was a significant difference in terms of mortality and weight gain between the directly infected and indirectly infected group, indicating unsuccessful indirect infections. These findings did not agree with the previous study conducted by McDougald & Fuller (2005) where the authors proved the direct transmission of histomoniasis among birds.

Zenner *et al.* (2003) carried out an *in vitro* experiment testing the antiparasitic activity of three essential oil extracts made from fresh cinnamon leaves, lemon pericarp, and cloves of garlic against *H. meleagridis*. A dose of 10^6 histomonads mL^{-1} was used for the anaerobic incubation, followed by the addition of different dilutions of the oils to determine the minimal lethal concentration (MLC). The synergistic action of the extracts was also analyzed. The

results of this experiment showed that the three tested oils had an *in vitro* antihistomonal activity despite the absence of synergy among them. A consistent experimental design, including an acceptable concentration of incubation (10^6 parasites mL^{-1}), the determination of the MLC, and the quantification of live parasites in culture made the findings of this study reliable.

Three years later, Hafez & Hauck (2006) continued their interest with these essential oils by conducting an *in vivo* study where the preventive efficacy of Protophyt, a herbal product containing essential oils of cinnamon, garlic, lemon and rosemary, against *H. meleagridis* infection was assessed. The authors divided 60 two-week-old turkey poults into three groups in a controlled trial. Group 1 was provided Protophyt as a feed additive 6 days prior to infection until the end of the experiment. Groups 2 and 3 remained untreated. At 3 weeks of age, the poults of Groups 1 and 2 were infected intracloacally with 147,500 *H. meleagridis* per bird. Lesion scores of the dead birds and euthanised birds were recorded. Real-time PCR was used to confirm the presence of histomonad DNA in the subjected birds. The results obtained in the study showed a 20% and 50% mortality rate in Groups 1 and 2, respectively, while no deaths occurred in Group 3. In addition, lesions were found only in caeca and liver, which is typical for histomonad infection. This study concluded that Protophyt was an effective alternative for the reduction of mortality in turkeys caused by histomoniasis. In this paper, the authors used a clear study design (control trial), an acceptable sample size (3 groups of 20 birds each), and a reliable statistic method (t-test). Also, a molecular technique with ensured specificity (real-time PCR) was applied to confirm the presence of the parasite in the subjected birds, which made these findings persuasive.

van der Heijden & Landman (2008a) performed a study to examine the *in vitro* effect of four herbal products against *H. meleagridis*, namely Aromabiotic, Enteroguard, Photophyt B (drinking water additive), and Photopyt SP (feed additive). A *H. meleagridis* strain was cultured in modified Dwyer medium prior to the

subculture. Dimetridazole and Histostat-50 were used as positive controls, while PBST was used as a negative control. Photophyt B at 0.24% caused a gradual decrease in the number of parasites over time. By using Enteroguard suspended at $400 \mu\text{g mL}^{-1}$, the decline in the number of histomonads was observed from 48h. A significant lower parasite count in comparison with the negative control was obtained only after 72h of incubation. Aromabiotic and Photopgyt SP showed no inhibitory effects on histomonad growth. In this *in vitro* experiment, the antihistomonal effects of the herbal products were not guaranteed since the growth of *H. meleagridis* can be inhibited only if the products were bactericidal to the caecal bacteria present in the culture of *H. meleagridis*. Using an antibiotic induced a complete inhibition of caecal bacterial growth that resulted in the inhibition of histomonads in this study. Moreover, the intestinal flora consisted of various species. A product may significantly destroy one or more bacterial species that are essential for histomonad growth. For these reasons, the results of this study are not reliable, and might need confirmation in an *in vivo* study.

Following the *in vitro* experiment, the authors performed an *in vivo* experiment with the same strain of *H. meleagridis* to confirm the antihistomonal activity of Enteroguard and Protophyt. Van der Heijden & Landman (2008b) conducted the experiment using 234 two-week-old turkeys divided into 13 groups ($n = 18$). The birds were either inoculated with 100, 3162, or 200,000 histomonads per bird. The negative control group was sham inoculated while the positive control was provided with no feed additives, Dimetridazole or Histostat-50. The study resulted in a mortality rate of 100% in the birds inoculated with 200,000 histomonads in all groups except the Dimetridazole-fed group. This study concluded that Protophyt and Enteroguard were ineffective at protecting birds from histomonad infection at a dose of 200,000 parasites/bird. The results obtained are convincing since the authors had designed a typical controlled trial with sufficient group sizes to justify statistical significance. Moreover, the authors used an adequate range of infection rates.

The method of intracloacal inoculation used in the experiment was likely more reliable in terms of avoiding re-infection as found in cases of using litter direct lateral transmission. However, it is necessary to revise the virulence of the strain and the dose used in this experiment. The dose of 200,000 histomonads per bird caused mortality in the group treated with Dimetridazole and 100% mortality in the group of Histostat-50 although these products had been reported as highly effective drugs against *Histomonas* spp. (Hu & McDougald, 2004).

Grabensteiner *et al.* (2008) performed a study to investigate the *in vitro* and *in vivo* antihistomonal activities of 43 plant substances from 18 organic wastes obtained from the food industry. During the *in vitro* experiment, only four ethanolic extracts completely inhibited the growth of *H. meleagridis* in the culture with an initial inoculation of $100,000 \text{ histomonads mL}^{-1}$, namely the ethanol extracts of saw palmetto, thyme, pumpkin fruit, and the water and ethanol extract of grape seed. These four extracts were selected for the subsequent *in vivo* experiment. The concentration of the extracts administered to the birds was increased from 0.1% to 1% during the experiment. The birds were experimentally infected with 10^3 histomonads/bird via cloaca. All four tested extracts showed only positive effects of delaying mortality for up to 18 days in comparison to the control group. The authors concluded that none of the tested substances were able to prevent *Histomonas* spp. infection in turkeys. These findings are strongly supported by an explicit control trial study with a reliable experimental method and valid *in vitro* and *in vivo* test system. In addition, all data were analyzed by reliable statistic software.

Responding to the demand of an antihistomonal alternative, various plant-derived products have been tested both *in vitro* and *in vivo*. Several plant products successfully inhibited the growth of *H. meleagridis* in culture media such as Enteroguard and several ethanolic extracts (**Table 3**). However, none of these demonstrated dependable protection for birds from histomoniasis in an *in vivo* environment. In three experiments, Natustat showed its effectiveness against histomoniasis in birds

(Table 3). The fact that these studies used infective methods made the results controversial. Therefore, it is recommended that further research should be conducted to evaluate the effects of Natustat using different modes of parasitic infection.

Similarly, controversies about the effects of Protophyt were found among the studies. This is one of a very few plant products that has shown an antihistomonal effect. It would be worthwhile for researchers to conduct further studies on this product.

Vaccinations

While chemotherapeutical products for the control of blackhead disease were used under high restrictions, and none of the plant-based substances demonstrated consistent protection for birds against the disease, vaccination became a compelling interest for scientists in the quest to discover an effective treatment and prevention for histomoniasis.

In 2008, Hess *et al.* (2008) investigated the efficacy of vaccines for the protection of turkeys from blackhead disease. The authors tested both

an inactivated vaccine and an attenuated vaccine. The former was obtained through a freezing and thawing process and the addition of formol to a *H. meleagridis* clonal culture, while the latter was achieved by passaging the cloned parasites for around 2 years in intervals of 2-3 days. In this experiment, 0.5mL of inactivated vaccine was intramuscularly injected, whereas the live vaccine was administered cloacally. The vaccinated birds were subjected to a challenge of 10,000 parasites/bird intracloacally. The turkeys that received the inactivated vaccine developed clinical signs and subsequently died, which indicated that the vaccine did not confer adequate protection. The birds that received the attenuated vaccine did not show any clinical signs specific for histomoniasis. In addition, no parasite DNA was recovered from the livers of the vaccinated birds, indicating that the vaccine successfully protected the birds.

An immunological study by Bleyen *et al.* (2009) was carried out to evaluate the effects of active and passive immunity in the protection of turkeys from histomoniasis. Active immunization was obtained by directly injecting

Table 3. Literature evaluating antihistomonal effect of plant substrate products

| Product | <i>In vitro</i> | <i>In vivo</i> (host species) | |
|---|---|-------------------------------|---|
| | | Chicken | Turkey |
| Aromabiotic | van der Heijden & Landman (2008a)* | | van der Heijden & Landman (2008b)* |
| Enteroguard | van der Heijden <i>et al.</i> (2008a) | | van der Heijden <i>et al.</i> (2008b)* |
| Natustat | | Duffy <i>et al.</i> (2004) | Duffy <i>et al.</i> (2005); Waters <i>et al.</i> (2005); Hauck <i>et al.</i> (2007) |
| Protophyt | Protophyt B van der Heijden <i>et al.</i> (2008a) | | van der Heijden <i>et al.</i> (2008b)* |
| (Essential oils of cinnamon, garlic, lemon, and rosemary) | Protophyt SP van der Heijden <i>et al.</i> (2008a)* Zenner <i>et al.</i> (2003) | | Hafez & Hauck (2006); Van der Heijden <i>et al.</i> (2008b)* |
| Ethanol extracts of saw palmetto | Grabensteiner <i>et al.</i> (2008) | | Grabensteiner <i>et al.</i> (2008)* |
| Ethanol extracts of thyme | Grabensteiner <i>et al.</i> (2008) | | Grabensteiner <i>et al.</i> (2008) |
| Water and ethanol extracts of grape seed | Grabensteiner <i>et al.</i> (2008) | | Grabensteiner <i>et al.</i> (2008) |
| Ethanol extracts of pumpkin fruit | Grabensteiner <i>et al.</i> (2008) | | Grabensteiner <i>et al.</i> (2008) |

Note: * Product was confirmed ineffective.

H. meleagridis antigens intramuscularly or by experimental infection via the cloaca, while passive immunization was implemented by intraperitoneal administration of antisera from immunized birds. These antisera were previously assessed for their capacity to destroy *H. meleagridis*. In addition, the transfer of antisera was assessed by measuring titers of anti-*H. meleagridis* antibodies. A strong antiparasitic serum antibody response was observed in both active immunization groups. On the contrary, the birds were not protected against a challenge with 3×10^5 histomonads in the passive immunized group despite the successful transfer of anti-parasite antibodies. This indicated that passive immunization by transferring serum antibodies did not have a protective effect in turkeys.

In the study of Liebhart *et al.* (2010), the efficacy of oral vaccination of turkeys using attenuated *H. meleagridis* was evaluated. The authors also assessed the negative effects of this vaccine prototype on the performance of the birds. Five different groups of birds were used for the experiments, namely cloacally challenged 2 weeks after being orally vaccinated, cloacally challenged 4 weeks after being orally vaccinated, non-vaccinated but inoculum challenged, vaccinated but not challenged, and non-vaccinated not-challenged. The results of this experiment showed that the birds that were challenged 4 weeks after vaccination, as well as the vaccinated but not-challenged and the non-vaccinated not-challenged groups, did not show any clinical signs related to histomoniasis. All the birds in the non-vaccinated group and 71.4% of the birds cloacally challenged 2 weeks after vaccination contracted blackhead disease. Moreover, none of the vaccinated but not-challenged birds showed any abnormal performance. These findings not only supported the claim of Hess *et al.* (2008) about the effectiveness of the attenuated vaccine against *H. meleagridis* but also proved that the vaccine could be administered orally (Liebhart *et al.*, 2010).

A study by Liebhart *et al.* (2010) evaluated the effects of virulent and attenuated *H. meleagridis* on turkeys and chickens. The virulent histomonads were obtained from clonal culture after 21 passages, while the attenuated

vaccine was achieved after 295 passages. The turkeys were subjected to either virulent histomonads or attenuated histomonads with a dose of 10^4 parasites administered orally, whereas the chickens were challenged with a similar infection regime and an additional cloacal dose. The results showed that liver and caecal lesions were found in both the chickens and turkeys receiving virulent infections, while no changes were observed in the birds infected with attenuated parasites. Moreover, the virulent parasites were found in several organs of the birds, whereas the attenuated isolates were present only in caecal samples in the PCR test. This indicated that attenuated *H. meleagridis* did not cause any adverse effects to infected chickens and turkeys. This was one of a very few studies focusing on vaccinations of this parasite in chickens. These findings could be useful for further investigations in the vaccine development for histomoniasis. However, the design of this study was not strongly convincing. The authors used a dose of only 10^4 histomonads per bird, which was significantly lower than the dose used by other scientists, ranging from 1.4×10^5 to 10^6 parasites per bird (Zenner *et al.*, 2003; Hu & McDougald, 2004; Hafez & Hauck, 2006; van Der Heijden *et al.*, 2008). It could explain why no changes were observed in the birds infected by attenuated parasites. Further studies are required to confirm the effectiveness of the immunization produced by these attenuated isolates.

A recent study conducted by Nguyen Pham *et al.* (2013) examined the protective effectiveness of the low-virulent *H. meleagridis* against blackhead disease in turkeys. The low-virulent *H. meleagridis* was produced by serial intracloacal passages, which are also known as back passages. The back passage comprised of an intracloacal inoculation of histomonad to 3-week-old birds. Thirteen days post-inoculation, these birds were euthanized to obtain the parasite for reinoculation of new birds. An examination of the virulence of the back passages showed that the last three intracloacal passages, did not cause any of the typical lesions found in turkeys. Subsequently, back passage 10 was used to test the protective capacity against *H. meleagridis*.

The birds vaccinated with this low virulent back passage yielded minor clinical signs, and lower liver and caecal lesion scores compared to the unvaccinated challenged group; and no mortality compared to 71% in the unvaccinated group in an experimental challenge with a virulent strain of the parasite. This result indicated that the low-virulent *H. meleagridis* attenuated by serial intracloacally passages was able to protect turkeys from a virulent strain of this parasite. A logical experiment design used in this study made its findings persuasive.

In general, most studies evaluating histomoniasis vaccination were conducted exclusively in turkeys. The transfer of serum antibodies from experimentally infected birds was unable to protect birds from histomonads infection, while active immunization created by injecting antigens showed protective activity. Several authors proved that a vaccination with *in vitro* attenuated parasites could provide protection to turkeys against *H. meleagridis* independently of administration (Table 4). Additionally, this type of vaccination did not alter the performance of the subjected birds. In a practical sense, the production of an effective vaccine would require not only sophisticated techniques but also a harmonization between laboratory experiments and field demand.

The future of histomoniasis treatments

Following the ban of nitroimidazoles, recognized as high potential antihistomonal drugs in the European Union and some other countries, due to the concern of ecological and human health threats, researchers were forced to search for viable alternatives in preventing histomoniasis. In general, three different approaches were investigated to deal with the situation. These were the application of

antibiotics showing antiprotozoal activity, testing plant substances, particularly some essential oils, and the attempt to develop effective vaccines.

Among the various drugs tested, Histostat-50 and Paromomycin are the two available drugs in some markets that possess antihistomonal activity despite recently raised concerns about deleterious side effects such as bacterial resistance and consumer safety. It is, therefore, crucial for policymakers to strike a balance between the benefits of the consumer and the sustainability of animal production.

A large number of plant substances were tested for their effectiveness against histomoniasis, none of which conferred reliable protection to birds in *in vivo* experiments. There was a lack of standardized methodology for infection among the studies that led to inconsistent results. It is therefore recommended to address this issue in further investigations.

Approaches to different types of immunizations showed that only vaccinations with attenuated parasites could induce reliable protection from histomoniasis in turkeys. However, the production of vaccines, particularly for this protist, would be challenging due to a high level of sophisticated techniques required.

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Table 4. Literature evaluating the effectiveness of vaccinations in the protection of birds from histomoniasis

| Passive vaccination | Active vaccination | | |
|------------------------------|-----------------------------|----------------------------|--|
| | Antigens | Inactivated parasite | Attenuated parasite |
| Bleyen <i>et al.</i> (2009)* | Bleyen <i>et al.</i> (2009) | Hess <i>et al.</i> (2008)* | Hess <i>et al.</i> (2008); Liebhart <i>et al.</i> (2010); Nguyen Pham <i>et al.</i> (2013) |

Note: * Proved to be ineffective to protect birds from blackhead disease.

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