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In vitro and *in vivo* Sensitivity of a Flagellated Protozoan, *Histomonas meleagridis*, to Metronidazole and Nitarsone

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ABSTRACT

Histomoniasis or blackhead disease is a life-threatening disease of turkeys that is caused by a flagellated protozoan, *Histomonas meleagridis*. Currently metronidazole and nitarsone are used commonly against blackhead disease in Pakistan. Initially we tested the sensitivity of *H. meleagridis* collected from field outbreak in turkey to metronidazole (Flagyl®) and nitarsone (Histostat-50TM®) using *in vitro* culture conditions. *H. meleagridis* at 200 and 500 ppm of nitarsone showed reduced growth in comparison to respective untreated culture. However, there was no inhibition of growth *H. meleagridis* treated with metronidazole at 200 ppm, while reduced growth was seen at 500 ppm. To test the sensitivity of *H. meleagridis* strain to nitarsone and metronidazole *in vivo*, poults offered Flagyl® and Histostat® premix diet or a control diet were inoculated cloacally with *H. meleagridis*. The Histostat® treated group of birds showed significant difference while metronidazole-treated group showed non-significant difference compared to that of infected control group especially at 200 ppm when measuring weight gain and liver and cecal lesions scores. *Histomonas meleagridis* were re-isolated from the Flagyl® fed chicks and subjected to the *in vitro* assay. Isolated *H. meleagridis* maintain their resistance to metronidazole at 200 ppm. This study demonstrates that field isolate of *H. meleagridis* has acquired partial resistance to metronidazole. *H. meleagridis* strains currently present in Pakistan seems to be more sensitive to nitarsone than metronidazole; hence metronidazole should be replaced with nitarsone for better prevention and control of black head disease in poultry.

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Authors' Contributions:
MIK, SA, MY and SA maintained turkeys. SU collected clinical lesion data and wrote the article. MU and FS performed *in vitro* sensitivity tests. QN took care of protozoan culture.

Key words:
***Histomonas meleagridis*, blackhead disease, nitarsone, metronidazole, turkeys.**

INTRODUCTION

Histomoniasis is a disease of gallinaceous fowl, caused by a flagellated protozoan, *Histomonas meleagridis* (Hauck and Hafez, 2009). It is transmitted horizontally through a cecal nematode, *Hetarakis gallinarum*, or through direct contact (only in turkeys) between infected and uninfected birds through reverse peristalsis of infected feces through the cloaca to the ceca. This form of transmission occurs when sick turkeys huddle together and allows for rapid spread of blackhead disease in the absence of *H. gallinarrum* (McDougald, 2005). Due to the absence of the huddling behavior in chickens, this mode of transmission is not observed in chicken flocks, and therefore, is not shown to be an important factor in the transmission of blackhead disease in chickens (Hu *et al*., 2004). Affected birds show reduced body weight, morbidity, yellowish diarrhoea and sometimes high mortality (especially in turkeys) with necrotic foci in the liver and ulcers in ceca. Lesions may also be seen in other tissues e.g kidney, lungs and bursa of fabricius (Zaragatzki *et al.*, 2010; Sentı-es-Cue *et al.*, 2009; McDougald *et al.*, 2012). The economic significance of histomoniasis is difficult to ascertain, but annual losses from mortality in turkeys has been estimated to exceed two million dollars in the United States (McDougald, 1997). Histomoniasis is often less severe in chickens, causing moderately bloody discharge in cecal droppings, decreased production, poor uniformity of layers, and increased culling. Sometimes clinical signs may be unnoticed, thus they are potential reservoirs of infection for turkeys. In general, economic losses in chickens are estimated to be greater than in turkeys because of the frequency of occurrence and the numbers of birds involved (AAAP Committee on Disease Reporting, 1986). Turkey farming is flourishing in

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Pakistan. Turkeys, which were previously kept in most households as pets are now being reared in farms.

Until 1950, arsenicals were the only compounds that were used to control histomoniasis in the field (Joyner *et al.,* 1963). Then nitroimidazoles, and dimetridazole in particular, became available. Historically, blackhead disease could be readily treated or prevented by the use of nitroimidazole drugs (Lucas, 1962; van der Heijden and Landman, 2007; Reynolds *et al*., 2009). They were used for many years in feed or water for the treatment and prevention of the disease (McDougald 1997). However, the use of these products has been disallowed in the United States and Europe, resulting in an upsurge of blackhead disease cases (McDougald *et al.,* 2012; Lotfi *et al.*, 2012; Faita *et al.,* 2013; Nawaz *et al*., 2010). The use of nitroimidazole drugs (Flagyl®) is still in use for the treatment of blackhead disease of poultry in Pakistan. There is no approved vaccine or treatment for blackhead disease, although an organoarsenic compound, nitarsone (4-nitrophenylarsonic acid), may be used for prevention (McDougald, 2005; Grabensteiner *et al*., 2007; Abraham *et al*., 2014). There is no previous report of reduced sensitivity of *H. meleagridis* under lab conditions to metronidazole. Recent field observations regarding blackhead disease outbreak in Pakistan and United States have shown that there is a possibility of drug resistance of *H. meleagridis* to metronidazole and nitarsone (data not published). This raises concerns for the future of blackhead prevention and management in commercial poultry. In the present work we tested strain of *H. meleagridis* obtained from outbreaks of blackhead disease for sensitivity to nitarsone and metronidazole.

MATERIALS AND METHODS

Nitarsone and metronidazole

Purified nitarsone and metronidazole was obtained from Sigma-Aldrich (St. Louis, MO) for use *in vitro*. The compound were suspended in dimethylformamide and diluted to working concentrations of 200 and 500 ppm in Hank's balanced salt solution. For *in vivo* studies Histostat-50TM® and Flagyl® (Pfizer Animal Health, Fort Washington, NJ) was used in turkey starter ration at 0.0187% (187 ppm).

Parasites and culture

H. meleagridis was obtained from blackhead outbreaks in turkey showing no response to medication from Faisalabad, Pakistan in 2013. The parental isolates of *H. meleagridis* generated from the *in vitro* culturing of the ceca of affected birds at 40°C in modified Dwyer medium consisting of 0.08% (w/v) rice powder and 5% horse serum in Medium199 with Hank's salts (van der Heijden and Landman, 2007; Grehold *et al*., 2010).

In vitro *testing of the sensitivity of* H. meleagridis *to drugs*

After 48 h of growth in media, cultures were counted using a hemocytometer (Hausser Scientific, Horsham, PA) and diluted to 50,000 cells/ml in fresh culture medium and transferred to 50 ml culture flasks (10 ml/flask). For *H. meleagridis* isolate, nitarsone and metronidazole were tested at 0, 200, 500 ppm in three replicate cultures. *H. meleagridis* in each flask were counted after 12, 24, 36, and 48 h of growth and an average count of each time were calculated.

In vivo *testing of the sensitivity of* H. meleagridis *to drugs*

Experimental birds

The study was conducted on 40 turkey poults (2 week old) procured from South Asia breeding company Khairpur Mirs, Sindh Pakistan. The birds were kept in isolators under standard management conditions with water and feed *ad libitum*. Experimental study protocol was approved by the Animal Care and Research Committee and experimentation was carried out according to the guidelines of committee.

Experiment design

Turkey poults were individually weighed, marked for identification, and distributed into four groups of 10 poults each. One group was infected with *H. meleagridis* by means of direct cloacal inoculation (HMI), second and third groups were infected in a similar manner but were fed a diet with nitarsone (HMN) and metronidazole (HMM) respectively (feed mixed with 187.5 ppm of Histostat®-50 and 250 ppm Flagyl®) from the first day of life. The 4th group remained uninfected (UC) and served negative control. Turkey poults were inoculated intracloacal using a blunt-tipped pipette inserted about 3 cm into the cloaca. A dose of 45,000 cells/bird was given in 1 ml of culture medium. Ten days post-infection (dpi) birds were weighed and necropsied to determine cecal and liver lesions scores (Hu *et al.,* 2004).

Lesion scoring

Thickening or reddening of cecal wall was scored as 1. Scores of 2 and 3 indicated increasing severity of the lesion like thickening of the cecal wall, formation of a cheesy cecal core, and inflammation of the mesenteries. Extreme lesions like complete involvement of the ceca and the engorgement of lumen with cecal cores were scored as 4. The liver lesions were scored 1–2 with only a few discrete surface lesions (score 1) or a 3 with

increasing severity. A complete involvement of the liver with lesions was scored 4 (Umar *et al*., 2016; Abraham *et al*., 2014; Hu and McDougald, 2004b).

In vitro *testing of the sensitivity of re-isolated* H. meleagridis *to drugs*

Ceca of birds in the infected group (HM-M200 and HM-M500) of the *in vivo* study showing highest lesions of histomoniasis were transferred to culture media, and *H. meleagridis* was grown for 24 h followed by transfer of 1 ml of the culture to 10 ml of fresh culture media. After attaining optimum growth, re-isolated *H. meleagridis* were transferred to fresh culture media and allowed to grow for 48 h. These 48 h cultures were counted using a hemocytometer (Hausser Scientific, Horsham, PA). Cultures were diluted to 50,000 cells/ml in fresh culture medium and tested for nitarsone and metronidazole sensitivity as described above.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software Inc. La Jolla, CA, USA). Results of the *in vitro* study were analysed statistically by a two-way ANOVA (time and concentrations) and three replicates for each treatment. In the live-bird study, the average weight gain and average lesion scores of liver and ceca were analyzed by one-way ANOVA followed by a Tukey multiple comparison test. Statistical significance was set at $p<0.05$ unless otherwise stated.

RESULTS AND DISCUSSION

In vitro *sensitivity of* H. meleagridis *to drugs*

To test whether *H. meleagridis* was sensitive to nitarsone and metronidazole, *in vitro* cell cultures were performed using low (200 ppm) and high (500 ppm) concentrations of the drugs. Untreated culture of *H. meleagridis* (UHM) grew rapidly for 24 h to an average count of 148,657 cells/ml, while cultures treated with nitarsone (HM-N200 and HM-N500) showed diminished growth within 12 h post-inoculation with decreased counts observed for each time point for HM-N200 or HM-N500. UHM showed a significant difference ($P<0.05$) from nitarsone-treated groups (HM-N200 or HMN500) during the entire period of growth (Figs. 1, 2). On the other hand, culture treated with metronidazole (HM-M500) showed diminished growth within 12 h post-inoculation while metronidazole (HM-M200) had no significant difference on cell count from UHM for the first 12 h, and their growth patterns for the remaining time points were similar. There was no statistically significant difference between the average histomonal count of UHM and HM-M200 ($p>0.05$). M 500 had only a small increase in the number of *H. meleagridis* at 12 h, with a decline thereafter. UHM and HM-M200 showed a significant difference form of HM-M500 during the entire period of growth ($P <0.05$).

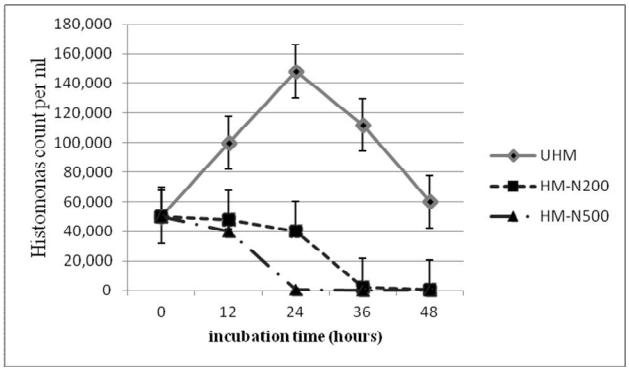

Fig. 1. Growth of *H. meleagridis in vitro*, as affected by addition of 200 or 500 ppm of nitarsone. Cell counts were done 12, 24, 36, and 48 h post-inoculation. UHM, untreated *H. meleagridis* culture; HM-N200, *H. meleagridis* culture treated with 200 ppm nitarsone; HM-N500; *H. meleagridis* culture treated with 500 ppm nitarsone)

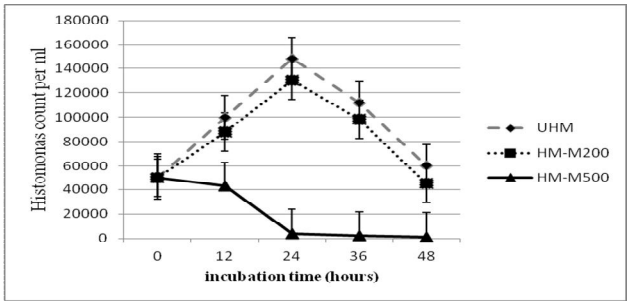

Fig. 2. Growth of *H. meleagridis in vitro*, as affected by addition of 200 or 500 ppm of metronidazole. Cell counts were done 12, 24, 36, and 48 h post-inoculation. UHM, untreated *H. meleagridis* culture; HM-M200, *H. meleagridis* culture treated with 200 ppm metronidazole; HM-M500, *H. meleagridis* culture treated with 500 ppm metronidazole.

In vivo *sensitivity of* H. meleagridis *to drugs*

An *in vivo* study was conducted with *H. meleagridis* to validate the results of the *in vitro* study in infected turkey poults treated with nitarsone and metronidazole. There was no mortality in UC and HMN but a cumulative mortality of 42.2% and 36.67% was recorded in HMI and HMM, respectively at 8 dpi. Average weight gains of the UC, HMI, HMN and HMM

were 188.45, 97.71, 139.46 and 108.31 g, respectively (Fig. 3a). There was no significant difference in average weight gain of the HMM birds compared to that of HMI birds (P >0.05) while birds in HMN showed significant difference from birds of HMI (p<0.05). Average liver lesion scores of HMI, HMM and HMN were 3.00, 2.38 and 0.5 respectively (Fig.3b) and average cecal lesion scores were 3.94, 3.32 and 1.6 (Fig. 3c). There was significant difference (p<0.05) in liver and cecal lesions of the HMN birds compared to that of HMI. Although HMI birds had numerically reduced average weight gain and higher average lesion scores than HMM birds, there were no significant differences in the liver lesion and cecal lesion scores (p>0.05).

In vitro *sensitivity of H. meleagridis isolated from the* in vivo *study to nitarsone and metronidazole*

To test whether nitarsone and metronidazole sensitivity was maintained in *H. meleagridis* after passage in turkey poults, the re-isolated *H. meleagridis* strain was again tested *in vitro*. Compared to the original strain of *H. meleagridis*, the isolated strain grew more robustly reaching a count of 251, 254 cells/ml compared to 148,657 cells/ml at 24 h post-incubation (Fig. 4). Isolated *H. meleagridis* treated with nitarsone at 200 ppm and 500ppm showed significant decrease in cell count (p<0.05) while metronidazole at 200 ppm showed average cell count almost equal to untreated control group at all measured times. At 500 ppm the isolated strain again showed diminished growth. Isolated HM-M200 had a significant increased cell count compared to both regenerated UHM and isolated HM-M500 (P<0.05).

Although metronidazole has been banned in Europe and USA but is still very common in use for prevention and treatment of blackhead disease in Pakistan, suggesting high possibility of drug resistance for metronidazole than other drugs. In the present study, we demonstrated that *H. meleagridis* isolated from field outbreak has diminished responsiveness to metronidazole *in vitro*, while the growth of *H. meleagridis* was inhibited by nitarsone. This reduced responsiveness of *H. meleagridis* strain to metronidazole *in vitro* correlated with inability of metronidazole to prevent disease caused by *H. meleagridis* strain in poultry. Based on the *in vivo* and *in vitro* studies, we believe that the culture method can be used as a rapid test for assessing the metronidazole and nitarsone sensitivity of field isolates. The data suggest that *H. meleagridis* that are resistant to metronidazole at 200 ppm in culture will be resistant to the recommended dose of metronidazole (200 ppm) used in the feed. This needs to be evaluated with other metronidazole-resistant strains. Even though the feed contained metronidazole at a higher level (250 ppm) than

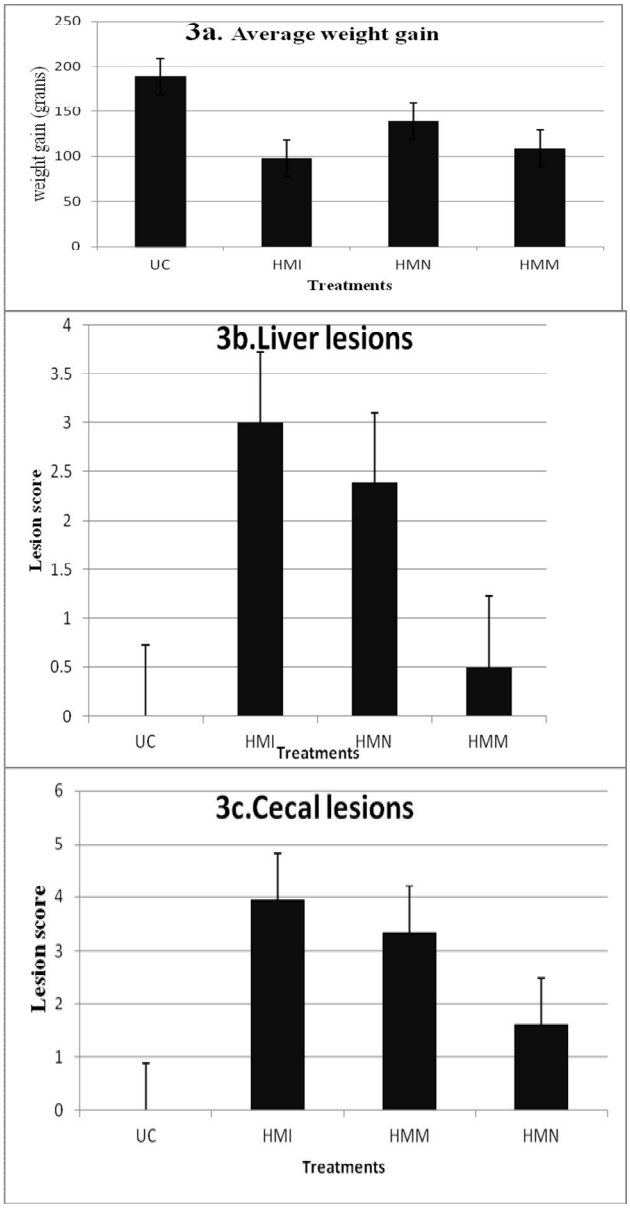

Fig. 3. Effect of nitarsone (187.5 ppm in feed) and metronidazole (250ppm in feed) on weight gain (g) and lesion scores in the liver and ceca at necropsy 10 dpi with 45,000 *H. meleagridis*/bird in turkey poults. Scores within a graph with no common lowercase letters differ significantly: P <0.05. **3a**, average weight gain; **3b**, liver lesion; **3c,** cecal lesion. UC (uninfected control group), HMI (*H. meleagridis* infected group), HMN (*H. meleagridis* infected + nitarsone treated group*)*, HMM *(H. meleagridis* infected +metronidazole treated group*).*

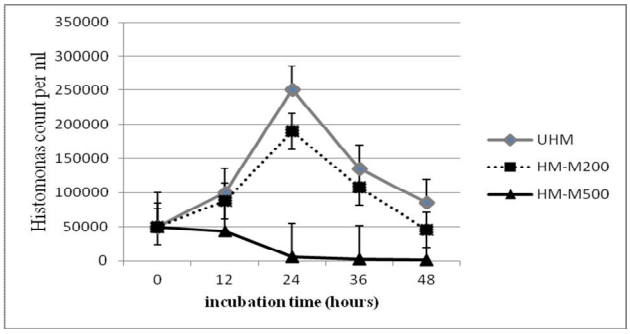

Fig. 4. Growth of re-isolated *H. meleagridis* (isolated from *in vivo* study) *in vitro*, as affected by addition of 200 or 500 ppm of metronidazole. Cell counts were done 12, 24, 36, and 48 h post-inoculation. UHM, untreated *H. meleagridis* culture; HM-M200, *H. meleagridis* culture treated with 200 ppm metronidazole; HM-M500, *H. meleagridis* culture treated with 500 ppm metronidazole.

the culture conditions (200 ppm), dilution in the gut with ingested water and digestive juices most likely reduced the effective concentration to a level where *H. meleagridis* strain showed resistance *in vitro*. Although increased levels of metronidazole inhibited growth of *H. meleagridis* strain in culture, increasing levels of metronidazole in the feed to prevent this strain from causing disease would be toxic to poultry (Plumb, 2008). Treatment of *H. meleagridis in vitro* with 500 ppm of metronidazole resulted in reduced growth, suggesting that this strain was responsive to the higher doses of drug but had developed a mechanism to deal with metronidazole at lower concentrations. The observation that the drug resistance was maintained after passage through turkey poults suggests that a genetic modification to the organism is most likely responsible for this phenotype and makes this *H. meleagridis* isolate a valuable resource to study drug resistance in protozoal organisms. Although metronidazole has been used as an antiparasitic drug in poultry rations for decades, but its efficacy against present *H. meleagridis* isolate of field is a question mark. Organic compounds such as 5-nitroimidazole, may affect *H. meleagridis* by disrupting DNA formation process (CVMP, 1997; van der Heijden and Landman, 2008a,b). This makes it likely that mutation resulting in the change of a target protein may contribute to metronidazole resistance. It has been reported that *H. meleagridis* has several transporters which can play a key role in drug resistance development by reducing import and export of chemicals. (Leprohon *et al.*, 2011; Kodnicki *et al.*, 2012; Abraham *et al*., 2014).Characterizations of these transporters may provide insights into the mode of metronidazole resistance. The difference susceptibility to metronidazole that we observed in *H. meleagridis* tested strains is similar to variation seen in the response of difference monocultures of *H. meleagridis* to natural organic compounds (Grabensteiner *et al.*, 2007; Abraham *et al.*, 2014). Together, these results suggest inefficacy of metronidazole at recommended dose level to current field isolates of *H. meleagridis* and should be considered when designing experiments and testing compounds to inhibit blackhead disease. In addition, our studies show high efficacy of nitarsone than metronidazole against field isolate of *H. meleagridis*. Reduced efficacy of metronidazole may be due to development of partial drug resistance by *H. meleagridis*. Hence, the use of nitarsone is recommended for better prevention and control of black head disease.

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