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Short communication

# Detection of *Histomonas meleagridis* in turkeys cecal droppings by PCR amplification of the small subunit ribosomal DNA sequence

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**Abstract**

*Histomonas meleagridis* is a protozoan parasite that may cause histomoniasis, a disease of gallinaceous fowl characterized by necrotic typhlitis, hepatitis and high mortality. Diagnosis of this disease is based on direct identification or on cultivation of the parasite. With the aim of developing more sensitive, rapid and useful tools for parasite detection, PCR that amplified a DNA target of 209 pb of the 18S rRNA gene was designed to detect the genome of *H. meleagridis* and to differentiate it from the genome of *Tetratrichomonas gallinarum*, another common protozoan parasite of fowl. The sensitivity of the test was evaluated using serial diluted samples of cultured *H. meleagridis* and showed positive amplification for concentrations comprised between 10 and $10^{-1}$ parasites/ml of culture. The sensitivity for cecal droppings samples was assed using spiked material and was comprised between $3 \times 10^3$ and $3 \times 10^5$ parasites/ml of stool. The reliability of the PCR for the detection of *Histomonas* infection was also evaluated by experimental infection of turkeys. Results of the PCR appeared to be in agreement with the development of the clinical signs and of the cecal lesions. The PCR developed in this study may be a useful tool in the detection and identification of *H. meleagridis* for rapid, routine screening as a supplement to direct identification or cultivation of the parasite.


*Keywords:* *Histomonas meleagridis*; Diagnosis; Protozoa; PCR

## 1. Introduction

*Histomonas meleagridis* is a flagellated protozoan causing histomoniasis, a disease of gallinaceous fowl. This disease is characterized by necrotic typhlitis with tan-yellow sulfur feces, hepatitis and high mortality especially in turkeys. The progressive removal from the market of most of the active molecules against *Histomonas* (like Dimetridazol or Nifursol) by the European Council has lead to a dramatic increase in the prevalence of histomonasis in European countries generating important economic losses. In France, between May and December 2003 more than 50 outbreaks were recorded with a mortality reaching

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80% in some flocks (Comité Interprofessionnel de la Dinde Française, CIDEF).

Epidemiological data relating to this disease are still insufficient probably because tools for large scale studies are lacking. Diagnosis is mainly based on clinical signs, epidemiological informations and gross appearance of lesions. It may be confirmed by direct observation of organisms in scrapings of cecal mucosa or in fresh cecal droppings. Identification should be done by a poultry specialist because of the difficulties to differentiate it from other cecal parasites such as *Tetratrichomonas gallinarum* and *Blastocystis* sp. Cultivation of organisms in artificial media may also help in cases in which the parasites are absent in fresh smears but this should be carried out in a laboratory mastering the cell cultivation of the parasite (Zenner et al., 2002).

With the aim of developing more rapid and sensitive diagnostic alternatives, a polymerase chain reaction which amplified a 209 bp region from the small subunit ribosomal RNA gene of *H. meleagridis* was designed. This report describes the PCR assay and its first validation by testing cecal droppings from artificially infected turkeys.

## 2. Materials and methods

### 2.1. DNA extraction

Five microlitre of cultured parasites (*H. meleagridis* HmBR-a strain, Callait et al., 2002) or of a dilution of caecal droppings (3 μl of dropping diluted into 50 μl of $TE^{-1}$ buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0)) were dropped onto a FTA Indicating Card (Whatman, Middlesex, UK) and allowed to dry for 1 h at room temperature. A sample disc was taken from the sample spot using a 1.2 mm punch and placed in a PCR amplification tube. Samples were washed according to manufacturer's instructions, three times in 100 μl of FTA Purification Reagent and twice in 100 μl of $TE^{-1}$ buffer. Discs were dried at 56 °C for 10 min.

### 2.2. Primer designation

Primer designation was based on the partial sequence of *H. meleagridis* small subunit ribosomal RNA gene (GenBank™ accession number AF293056). Primers pairs designed HIS5F (5′-CCTTTAGATGCTCTGGGCTG-3′) and HIS5R (5′-CAGGGACGTATTCAACGTG-3′) defined a 209 bp fragment for *H. meleagridis* and a 181 bp fragment for *T. gallinarum*, another common fowl parasite protozoa.

BLAST search of the GeneBank™ sequences using the BLASTN search tool (www.ncbi.nlm.nih.gov/BLAST) revealed no identity with other fowl sequences or fowl parasites.

### 2.3. DNA amplification

Two microlitre of DNA or a 1.2 mm FTA sample disc were used as template in the polymerase chain reaction (PCR) which had a final volume of 12.5 μl and contained 1× buffer (Invitrogen, Carlsbad, CA), 1.5 mM $MgCl_2$, 60 μM of each dNTP (Amersham Biosciences, Buckinghamshire, UK), 5 pmol of each primer and 0.25 units of Taq polymerase (Invitrogen). They were performed in a TGradient thermocycler (Biometra, Goettingen, Germany). The reaction conditions were 5 min at 96 °C, 40 cycles each of 1 min at 95 °C, 1 min at 59 °C and 1 min at 72 °C, finished by the addition of a final extension period of 5 min at 72 °C. DNA fragments were separated on 3% (w/v) agarose gels and visualised by UV illumination of ethidium bromide stained DNA.

### 2.4. PCR controls

Five nanogram of total DNA extracted from cultured *H. meleagridis* and 3 ng of DNA from cultured *T. gallinarum* were used routinely as positive controls for PCR tests.

A negative extraction (a negative FTA sample disc treated as described below) control was included to check for cross-contamination during extraction and for quality control of extraction chemicals, and a negative PCR control was included in each run to quality control the PCR chemicals.

### 2.5. Evaluation of the sensitivity of the PCR

The sensitivity of the test was evaluated using serial diluted samples of cultured *H. meleagridis* HmBR-a strain (Callait et al., 2002) ranging from $10^6$ to

$10^{-2}$ parasites/ml. Five microlitre of each of these dilutions were dropped onto a FTA card.

In an attempt to assess the sensitivity of the technique for mixed infections with *H. meleagridis* and *T. gallinarum*, samples obtained by serial dilutions, containing various numbers of *H. meleagridis* parasites (1000, 500, 250, 100, 10 and 1) were mixed with a constant number (1000) of *T. gallinarum*, and vice versa, in a final volume of 5 μl dropped onto a FTA card.

The sensitivity of the PCR for dropping samples was assessed using spiked material cecal droppings of an uninfected turkey were inoculated with known numbers of *H. meleagridis* obtained by serial dilutions to obtain concentrations ranging from $3 \times 10^6$ to $3 \times 10^1$ parasites/ml of cecal droppings. Three microlitre of these dropping samples were diluted into 50 μl of $TE^{-1}$ buffer and were dropped onto a FTA card.

All these samples were subjected to DNA extraction as described below.

### 2.6. Experimental inoculations

Three turkeys (BUT9 strain) were obtained at 1 day of age from Eclosion (Roussay, France). They were battery raised in a quarantine geographically isolated from experimental areas until they were 4 weeks old and then placed separately in metabolism cages. Feed and water were provided ad libitum.

*Histomonas* used to prepare the inoculums was obtained from a strain isolated in 1998 from a farm breeding turkeys and chickens in the Dombes region of France. It was maintained in the laboratory by successive per cloacal infection of chickens; turkeys were used for the last infection before the experiment.

Each inocula was prepared by collecting the cecal contents and scraping the cecal mucosa of the infected turkeys. The collected material was suspended in a M 199 medium at 39 °C. The number of *Histomonas* per millilitre was determined using a Malassez cell; the inoculums contained $3 \times 10^6$ *Histomonas* per millilitre of the suspension liquid. The birds were then infected via the cloaca using a syringe fitted with a round-ended cannula.

Samples of cecal droppings were collected before the infection as a negative control and then on the morning of days 2, 5, 7, 9, 12, 14, 16 and 19 after infection and kept at −20 °C until analysis. Samples were analyzed by PCR as described below.

Thirty six other turkeys BUT9 were infected as described below. A group of four turkeys were sacrificed and autopsied at days 0, 2, 5, 7, 9, 12, 14, 16 and 19. At days 0, 6 and 13 control groups constituted by, respectively, 4, 2 and 3 uninfected turkeys were scarified and autopsied. Cecal lesion scores were used to measure severity of infection. A score was attributed to each cecum. Scoring (Zenner et al., 2004) were, score 0, absence of macroscopical lesions, fine cecal walls with characteristic longitudinal folds, homogenous cecal contents with a liquid to creamy consistency, dark coloured; no caseous exudates; 1, similar to score 0 but with either the presence of scattered petechiae on the cecal walls or a moderate thickening of the cecal walls or both; 2, moderate thickening of the cecal walls, modified cecal content with either caseous exudates or beginning of the formation of a caseous core or modification of the colour of the cecal contents or absence of cecal content; petechiae may be present; 3, greatly thickening of the cecal walls, either greatly modified cecal content (caseous core) or absence of cecal content; petechiae may be present; 4, greatly thickening of the cecal walls with fibrinonecrotic ulceration of the cecal mucosa, either presence of a caseous core or absence of cecal content; possibility of hemorrhagic ceca, possibility of a rupture of the cecum leading to peritonitis.

## 3. Results

### 3.1. Sensitivity of the assay

The sensitivity of the test was first evaluated on diluted samples of cultured *H. meleagridis*. This experiment was repeated four times. Positive amplifications were detected for concentrations comprised between 10 and $10^{-1}$ parasites/ml of culture (Fig. 1).

In the assay for determining the sensitivity of the technique for mixed infections, 100 *H. meleagridis* could be detected and differentiated in the presence of 1000 *T. gallinarum* and vice versa (Fig. 2). This experiment has been repeated twice giving the same results.

Finally, the sensitivity of the PCR for dropping samples was assessed. The experiment was repeated three times and positive amplifications were detected

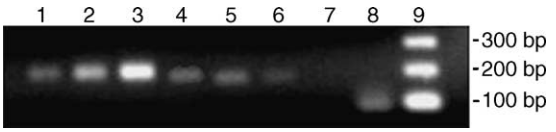


Fig. 1. Sensitivity of the PCR for the detection of *Histomonas meleagridis* HmBr-a strain. Products were separated on a 3% agarose gel, and a 100 bp DNA ladder was included at both extreme sides on every gel. Dilutions of cultured parasite strain were as follows: $3 \times 10^4$ parasites/ml in lane 1, $3 \times 10^3$ parasites/ml in lane 2, $3 \times 10^2$ parasites/ml in lane 3, $3 \times 10^1$ parasites/ml in lane 4, 3 parasites/ml in lane 5, $3 \times 10^{-1}$ parasite/ml in lane 6, control of culture media in lane 7, negative control in lane 8 and a 100 bp DNA ladder in lane 9.

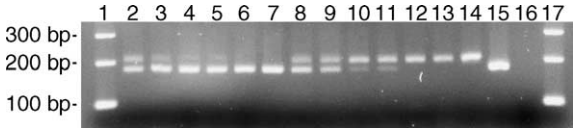


Fig. 2. Sensitivity of the PCR for the differentiation of *Histomonas meleagridis* and *Tetratrichomonas gallinarum* in mixed samples. To a sample containing 1000 *T. gallinarum* were added 1000 (lane 2), 500 (lane 3), 250 (lane 4), 100 (lane 5), 10 (lane 6), 1 (lane 7) *H. meleagridis*; to a sample containing 1000 *H. meleagridis* were added 1000 (lane 8), 500 (lane 9), 250 (lane 10), 100 (lane 11), 10 (lane 12), 1 (lane 13) *T. gallinarum*; in lane 14 positive control for *H. meleagridis*, in lane 15 positive control for *T. gallinarum*, negative control in lane 16 and a 100 bp DNA ladder in lanes 1 and 17.

for concentrations comprised between $3 \times 10^3$ and $3 \times 10^5$ parasites/ml of stool (Fig. 3).

### 3.2. Validation of the PCR on artificially infected turkeys

Before infection, all the three turkeys were negative by PCR and remained negative until day 2. Between D5 and D9, *H. melagridis* was detected by PCR amplification from all three turkeys. At D12 they are all negative again. At D14 parasites were detected in one turkey and in two turkeys at D16. At D19 no parasite was detected (Table 1).

All four autopsied turkeys presented a cecal lesion score of 0 at D0, before infection. At D2 only one turkey has a cecal score of 1. First notable cecal lesions appeared at D5. Mean cecal lesion scores increased from D5 to D12 followed by a progressive lowering of the intensity of the cecal lesions from D12 to D19 (Table 1). The negative controls reveal no lesions.

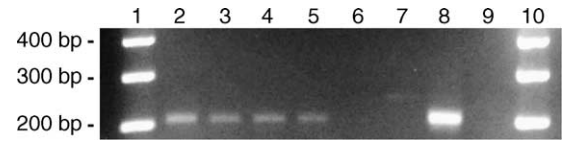


Fig. 3. Sensitivity of the PCR for droppings samples. A 3 μl sample of cecal dropping were spiked with $10^4$ (lane 2), $10^3$ (lane 3), $10^2$ (lane 4), 10 (lane 5), 1 (lane 6), $10^{-1}$ (lane 7); positive control for *H. meleagridis* in lane 8, negative feces control in lane 9 and a 100 bp DNA ladder in lanes 1 and 10.

## 4. Discussion

The protocol described provides a rapid diagnostic alternative for the detection of *H. meleagridis* directly in turkeys cecal droppings by PCR amplification.

Primers were designated on the partial sequence of *H. meleagridis* small subunit ribosomal RNA gene, an attractive target for PCR. Moreover, they are repeated genes and it has been shown that the detection limit of the PCR is lowered by the repetition of the target (Paugam, 1999).

Current PCR methods are greatly affected by droppings components (Denis et al., 2001). These inhibitors in feces are complex polysaccharides, possibly originating from vegetables material in the diet (Monteiro et al., 1997). To avoid these inhibitions problems, a filter-based DNA template preparation was chosen. It has been shown that the use of FTA filters appeared to limit or negate the effects of endogenous substances (Orlandi and Lampel, 2000). The impregnated filters sequester DNA within the matrix (Burgoyne, 1996) and factors that may interfere with PCR are effectively removed by washing the filters. Combined with a dilution of the fecal samples, this technique allows a PCR amplification of fecal droppings samples but lead to a reduced sensibility of the PCR compared to samples of cultured parasites.

The reliability of the PCR for the detection of *Histomonas* infection has been evaluated by experimental infection of turkeys. After infection, parasites were detected in cecal droppings by PCR from D5 for the three infected turkeys, and before the appearance of overt signs that occur most commonly 11 days post-infection (McDougald, 1997). Turkeys infected at the same time, in the same conditions and autopsied at the

Table 1
Artificially infected turkeys: cecal lesion scores and number of positive turkeys detected by PCR on cecal droppings

| Days after infection | Lesion scores ceca 1 | Lesion scores ceca 2 | Mean cecal lesion scores | Number of positive turkeys detected by PCR |
|---|---|---|---|---|
| D0 | C0 | C0 | 0 | 0 |
| | C0 | C0 | | |
| | C0 | C0 | | |
| | C0 | C0 | | |
| D2 | C0 | C0 | 0.25 | 0 |
| | C0 | C0 | | |
| | C0 | C0 | | |
| | C1 | C1 | | |
| D5 | C2 | C2 | 1.625 | 3 |
| | C0 | C1 | | |
| | C2 | C2 | | |
| | C2 | C2 | | |
| D7 | C3 | C3 | 2.875 | 3 |
| | C3 | C3 | | |
| | C3 | C4 | | |
| | C2 | C2 | | |
| D9 | C3 | C3 | 3.25 | 3 |
| | C3 | C4 | | |
| | C2 | C4 | | |
| | C3 | C4 | | |
| D12 | C3 | C4 | 3.625 | 0 |
| | C3 | C3 | | |
| | C4 | C4 | | |
| | C4 | C4 | | |
| D14 | C1 | C3 | 2.25 | 1 |
| | C3 | C3 | | |
| | C3 | C3 | | |
| | C1 | C1 | | |
| D16 | C1 | C2 | 2.25 | 2 |
| | C3 | C3 | | |
| | C2 | C3 | | |
| | C1 | C3 | | |
| D19 | C2 | C3 | 1.25 | 0 |
| | C2 | C2 | | |
| | C0 | C1 | | |
| | C0 | C1 | | |

same dates showed the first notable cecal lesions also at D5. The parasite is detected by PCR until D16. Complete clinical recovering of the turkeys is observed after D19 with a progressive lowering of the intensity of the cecal lesions from D12 to D19 on autopsied turkeys. Therefore, results of the PCR appeared to be in agreement with the evolution of the clinical signs and of the cecal lesions except at D12 where no positive droppings were detected. This could be explained by the elimination of caseous cores that has occurred for most of the turkeys between D7 and D14, probably leading to a kind of ''washing'' of the ceca and to the temporary negativation of the PCR.

The PCR developed in this study may be a potential tool in the detection and identification of *H. meleagridis* for rapid, routine screenings as an alternative to direct identification or cultivation of the parasite. Additional tests with experimentally

infected turkeys and surveys of animals in endemic areas would be necessary for a better evaluation of the diagnostic sensitivity of this tool. Some advantages of this PCR are that it does not require prior cultivation of the organisms and can be performed in less than 1 day. Samples of cecal droppings are easy to collect and the use of the FTA cards for DNA extraction also has an advantage in terms of safe handling and storage of the samples. In addition, large numbers of samples can be screened synchronously, as required for epidemiological studies.

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