Establishing mono-eukaryotic *Histomonas meleagridis* cultures from *in vivo* infection contaminated with *Tetratrichomonas gallinarum* and *Blastocystis* spp.

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(*Received 30 October 2012; revised 5 March, 5 April and 30 April 2013; accepted 1 May 2013; first published online 21 June 2013*)

SUMMARY

The necessity to easily establish *Histomonas meleagridis* cultures has been underlined extensively by many researchers in order to gain more insights in the biology of *H. meleagridis*. In addition the occurrence of different protozoa in the caeca of birds impedes, however, the isolation and propagation of *H. meleagridis* from field outbreaks. Therefore, in a kinetic study using transmission electron microscopy the deleterious effects of adventitious protozoa including *Tetratrichomonas gallinarum* and *Blastocystis* spp. on cultured *H. meleagridis* were examined. To overcome this issue, an easy and successful approach to establish the mono-eukaryotic *H. meleagridis* culture free of other host's protozoa is proposed. At 10 days post infection, liver lesions of *H. meleagridis*-infected birds were isolated and inoculated into culture media pre-incubated with caecal bacteria. After 48 h of incubation, presence of *H. meleagridis* in the cultures was confirmed through morphological evaluation. Additionally, TEM examination and analysis by PCR amplification of the small subunit rRNA gene could exclude the co-cultivation of *T. gallinarum* and *Blastocystis* spp. Furthermore, after successful propagation and maintenance of the cultured *H. meleagridis*, its pathogenicity was affirmed in an infection experiment in turkeys.

Key words: *Histomonas meleagridis*, *Tetratrichomonas gallinarum*, *Blastocystis* spp., liver, culture, pathogenicity, poultry, blackhead disease, histomonosis.

INTRODUCTION

Histomonas meleagridis is the causative agent of histomonosis (histomoniasis or blackhead disease or enterohepatitis) affecting gallinaceous birds, predominantly turkeys. Inflammation and ulceration of the caeca filled with sulphur-coloured exudates and necrotic liver lesions are considered to be pathognomonic (McDougald, 2005). Since the ban of all effective control measures (Anonymous, 1995, 2001, 2002), there are, at present, no therapeutic or prophylactic drugs available against *H. meleagridis*, resulting in an increasing number of fatal outbreaks in the poultry industry, particularly in turkey farms (McDougald, 2005; Callait-Cardinal *et al*. 2007). The current situation urges the need for basic knowledge on *H. meleagridis*. Although Tyzzer (1920) renamed the protozoon parasite observed by Smith (1895) as *H. meleagridis* and established its causal nature of this disease, many conflicting reports on the cause of enterohepatitis had been reported (Delappe, 1957). Based on frequent observations of other organisms, besides *H. meleagridis*, presumptions of coccidian protozoa, trichomonad species e.g. *Tetratrichomonas gallinarum* (Delappe, 1957), and moulds e.g. *Candida albicans* (Kemp and Reid, 1966*b*) as the etiological agent of blackhead disease had been made (Delappe, 1957). Further the co-infection of different protozoa in caeca, mainly *T. gallinarum* and *Blastocystis* spp., interferes and impedes the propagation of cultured *H. meleagridis* isolated from field outbreaks. In the past, many authors highlighted the necessity of cultural investigations. Many reports dealt with *in vitro* isolation of *H. meleagridis* from embryonated eggs of the *Heterakis gallinarum* nematode (Ruff *et al*. 1970), from caecal contents (DeVolt, 1943; Dwyer, 1970; McDougald and Galloway, 1973), from caecal discharges (Delappe, 1952, 1953; Delappe and Pierce, 1953), from feces (Drbohlav, 1924; Bishop, 1938) and from liver lesions (Bayon and Bishop, 1937; Delappe, 1957). However, few data on growth behaviour and life-time of the cultured parasites were reported. Therefore, it is not clear whether other

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doi:10.1017/S0031182013000723

host-derived pathogens were present in the *in vitro* isolates of *H. meleagridis*. Exclusion of interfering organisms such as *Blastocystis* spp. and *T. gallinarum* is essential for the maintenance of *H. meleagridis* cultures. So far, a clonal *H. meleagridis* culture from caecal content was established through micromanipulation (Hess *et al*. 2006*b*). This technique depends on precise microscopic recognition and selection of a single *H. meleagridis* parasite complicated by the morphological similarity to other host organisms such as *T. gallinarum* (Allen, 1936; Harrison *et al*. 1954; Delappe, 1957) and *Blastocystis* spp. (Delappe, 1952; Harrison *et al*. 1954) commonly present in caecal content of birds. The development of the mono-eukaryotic *H. meleagridis* culture from a number of cells mimics the field isolate, as opposed to cloning, and offers a key tool for future in-depth studies of *H. meleagridis*. With this perspective, the objective of this study was to develop an efficient technique to isolate and set up *H. meleagridis* cultures free of other avian protozoa starting from mixed infections of *H. meleagridis, T. gallinarum* and *Blastocystis* spp. Following the successful *in vitro* propagation of the isolated *H. meleagridis* parasites, their sustained pathogenicity was verified *in vivo* in the turkey model.

MATERIALS AND METHODS

Histomonas meleagridis *mixed strain: origin and storage of the stock*

The virulent field strain *H. meleagridis*/Turkey/France/HNA.C2.L2/06 was isolated from the caeca and liver of diseased birds from a clinical outbreak at a French commercial breeder in June 2006. The *H. meleagridis*-infected liver and caeca were collected at autopsy and macerated in warm PBS. For long-term storage in liquid nitrogen 8% heat-inactivated horse serum (Gibco TM, Invitrogen) and 8% dimethylsulfoxide (Sigma Aldrich, Germany) were added to the suspension and frozen to −80 °C under controlled conditions (±1 °C/min). The presence of *H. meleagridis* and other host protozoa in the suspension was verified with an inverted light microscope at 200–400× magnification and confirmed by PCR amplication of the small subunit rRNA gene of *H. meleagridis* (Bleyen *et al*. 2007) of *T. gallinarum* and of *Blastocystis* spp. (Grabensteiner and Hess, 2006). For further ease of reference this suspension will be referred to as 'mixed strain'.

Comparison of the growth kinetics of H. meleagridis *with* T. gallinarum *and* Blastocystis *spp. in cultures of the mixed strain*

The mixed strain was resuscitated in culture. Hereby, the cryostabilate stored in the liquid nitrogen was defrosted as fast as possible in 37 °C warm water. The mixed strain was carefully transferred into a culture flask containing culture medium. This culture medium consisted of 90% M199 medium supplemented with Hanks salts, L-glutamine, 25 mM HEPES and L-amino acids (Gibco TM, Invitrogen), 10% heat-inactivated horse serum (Gibco TM, Invitrogen) and 12 mg of rice starch (Gibco TM, Invitrogen). The medium was inoculated with one loop of the turkey's caecal bacterial culture grown on a Columbia agar plate with 5% sheep blood (Biotrading, Belgium). The culture was incubated at 40 °C under anaerobic conditions sealing the culture flasks tightly. Parasite growth was examined with an inverted light microscope at 200–400× magnification. For the relative kinetic growth studies of different cultured protozoa, the cryostabilate of the mixed strain and culture samples from different incubation times (3, 4, 5, 6, 12 and 24 h) were processed for transmission electron microscopic (TEM) analysis. The cell suspensions were centrifuged and the cell pellets were treated for TEM analysis of ultra-fine sections as described by Mast *et al*. (2005). Briefly, cells were fixated in a cacodylate buffer containing 2·5% glutaraldehyde and 2% paraformaldehyde as active compounds and subsequently in 1% (wt/vol) osmium tetroxide. Thereafter the samples were fixated with 2% (wt/vol) uranylacetate in distilled water and dehydrated in ethanol. The cells were embedded in Epon-Spurr (1 : 1) medium. Ultrafine sections were cut with a Leica Ultracut ultramicrotome (Leica) and were stained with Reynolds lead citrate and uranyl acetate. Twenty to 100 sections of culture sample of each incubation time were analysed with a Technai Spirit transmission electron microscope, thereby taking digital pictures with a Eagle 4*4 camera (FEI).

Birds/poults

Commercial male B.U.T. 9 turkeys (British United Turkeys; Claeys, Kruishoutem, Belgium) and broiler chickens (Ross; Belgabroed NV, Merksplas, Belgium) were housed in a disinfected stable. At arrival, the one-day-old birds were kept together in a floor pen. Water and feed were supplied *ad libitum*. The experiments were approved by the Ethical Committee for Animal Experiments of the KU Leuven with licence number P029/2011 according to international regulations.

The mono-eukaryotic H. meleagridis *culture*

To establish a *H. meleagridis* culture free of other host intestinal protozoa, 5-week-old B.U.T. 9 turkeys and 3-week-old chickens were cloacally inoculated with a cryostabilate of *H. meleagridis*/Turkey/France/HNA.C2.L2/06. Ten days after inoculation the birds were euthanized by cranial dislocation. When the liver

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showed very small off-white foci, these starting-necrotic liver lesions were collected while still warm. They were chopped into small pieces and put into 9 mL culture medium. The culture medium was prepared and inoculated with caecal bacteria one day before and incubated at 40 °C under anaerobic conditions. Cultures were microscopically examined daily, and were maintained according to Hauck *et al*. (2010*a*). Every second or third day, 1 mL of the culture was transferred into a new culture flask containing 9 mL culture medium. The parasites were identified as *H. meleagridis* by the diagnostic Hime-PCR test for the detection of the *H. meleagridis* 18S rRNA gene (Bleyen *et al*. 2007). Furthermore, to exclude the presence of other protozoa, PCR-amplification of the small subunit rRNA gene of *T. gallinarum* and of *Blastocystis* spp. was performed (Grabensteiner and Hess, 2006). TEM analysis was performed to ascertain the identity of the cultured protozoa, as histomonads show morphological similarity to other intestinal protozoa under light microscopic conditions (Kemp and Reid, 1966*a*).

Confirmation of the sustained pathogenicity of the mono-eukaryotic H. meleagridis *culture in turkeys*

Forty birds were randomly allocated to the infected group (IG) and the uninfected control group (UC) at a 1 : 1 ratio. At the age of 17 days, the birds of the IG were intracloacally inoculated with $1{\cdot}7 \times 10^5$ *H. meleagridis* per animal (culture passage 8). The amount of cultured *H. meleagridis* was determined using a Neubauer counting chamber and inverted light microscopy at 200–400× magnification. Subsequently, the suspensions were diluted in culture medium to obtain the required concentration for inoculation. During the study all birds were examined daily and mortality was recorded. At 14 days post infection (dpi), all surviving birds were euthanized by cranial dislocation for investigation of the macroscopic histomonosis caecal and liver lesions applying the adapted 0–4 lesion score system of McDougald and Hu (2001). Normal caeca with dark-coloured content were scored as 0, whereas caecal lesions were scored as follows: 1 = caecal content is yellow, slimy and foamy to fluid and little or no thickening of the caecal wall with some small petechiae or few scattered, small lesions on the mucosa; 2 = thickening of the caecal wall with some bleeding or inflammation of mucosa, caecal content is yellow, foamy to fluid with some fibrinous exudates; 3 = caeca enlarged, thickening of caecal wall, bleeding and/or necrotic confluent lesions on mucosa, beginning of ulcers visible, caeca empty or yellow, foamy to fluid content with blood and/or caeseous core; 4 = death from histomonosis, severe thickening of caecal wall, distended caeca with severe necrotic lesions or ulcers on the mucosa, caeca empty or filled with yellow caseous material. Macroscopic liver lesions were scored as follows: 0 = normal; 1 = a few very small foci; 2 = lesions covering up to 50% of liver surface, lesions are off-white and variable in appearance; 3 = more than 50% of the liver surface covered with necrotic lesions; 4 = death from histomonosis, at least 70% of the liver show coalescing huge necrotic lesions with craters or huge amount of smaller necrotic spots.

RESULTS

Comparison of the growth kinetics of H. meleagridis *with* T. gallinarum *and* Blastocystis *spp. in cultures of the mixed strain*

The culture of the mixed strain was not pure. Besides *H. meleagridis*, presence of at least two other protozoa was detected by microscopic examination and PCR analysis. Based on their characteristic morphology, *T. gallinarum* and *Blastocystis* spp. were identified. The kinetic TEM study showed the relative amounts of *H. meleagridis, T. gallinarum* and *Blastocystis* spp. (Fig. 1). In the cryostabilate of the mixed strain as well as in the 3-h incubated culture predominantly *T. gallinarum* were observed (Fig. 1A). After the incubation time of 4 and 5 h equal amounts of *Blastocystis* spp. (Fig. 1D) and *T. gallinarum* (Fig. 1B) were present in the culture samples. After 6 h of incubation *T. gallinarum* were still observed (Fig. 1C), however most of the cultured protozoa were identified as *Blastocystis* spp. (Fig. 1E) and after 12 and 24 h the culture consisted of only *Blastocystis* spp. (Fig. 1F), no other protozoa were observed. Throughout the study, the relative amount of *H. meleagridis* remained relatively low: only one *H. meleagridis* could be demonstrated on all examined sections of the TEM analysis. Obviously, the culture of the mixed strain contained several bacterial species originating from the host's caeca. The bacterial population shifted from Gram-negative to Gram-positive bacteria and, interestingly, these Gram-positive bacteria could be consumed by the only remaining *Blastocystis* spp. (Fig. 1F).

The mono-eukaryotic H. meleagridis *culture*

At post-mortem examination of the euthanized birds (10 dpi), liver and caeca of the infected turkeys and chickens displayed typical histomonosis lesions. The caecal lesions were scored as 2 to 3. Score 2 was assigned to the turkey liver lesions and score 1 for the chicken liver lesions. Overall, the liver lesions were still small and were off-white coloured. Twenty-four hours after inoculation of the cultures with turkey liver material no *H. meleagridis* could be detected. However, after 48 h *H. meleagridis* protozoa were numerous so 10 mL fresh culture medium was added. In order to maintain the parasite's proliferation *in vitro* for more than 1 year, every 2 or 3 days at

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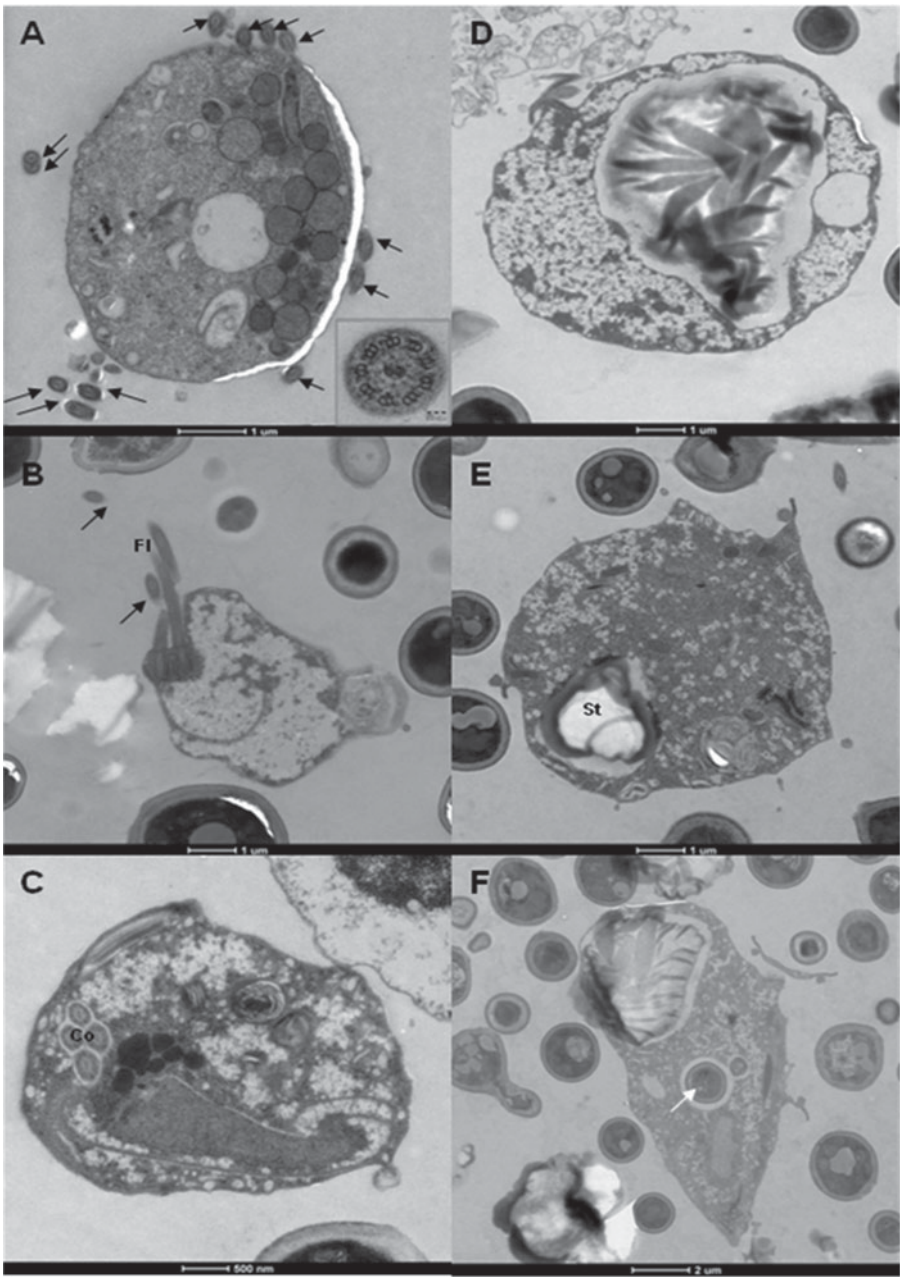

Fig. 1. TEM analysis at several incubation time points to study the relative amount of *Histomonas meleagridis* to *Tetratrichomonas gallinarum* and *Blastocystis* spp. in a culture of caecal content originated from turkeys suffering from histomonosis. *T. gallinarum* were identified based on the presence of 4 flagella (black arrow) outside the cell or 4 costas (Co) in the cell at incubation time of 0 (**A**), 4 (**B**), and 6 (**C**) hours. *Blastocystis* spp. were identified at incubation time of 4 (**D**), 6 (**E**), 24 (**F**) hours. The black arrows show transversal sections of the flagellum (Fl). On the insert of the picture (**A**) the cross-section of the axenoma with the characteristic 9*2 plus 2 (9 fused pairs of microtubule doublets surrounding 2 central ones) organization of microtubules is shown. Gram-positive bacteria with the characteristic thick peptidoglycan layer were observed in the environment of the protozoal cells but also inside *Blastocystis* spp. (white arrow in **F**).

growth peak ($\pm 10^6$ *H. meleagridis* mL^{-1}) the culture needs to be passaged (1/10 dilution). No other protozoa could be detected by light microscopic examination (Fig. 2) and specific PCR analysis as shown in Fig. 3. Irrespective of the passage number of the culture originating from liver lesions of *H. meleagridis* infected turkeys, only the protozoon *H. meleagridis* was identified in the culture (Fig. 3A). No other protozoon such as *Blastocystis* spp. (Fig. 3B) and *T. gallinarum* (Fig. 3C) were detected in the culture, whereas the PCR controls with genomic DNA from protozoa frequently occurring in caeca of poultry were positive. Through electron microscopic examination the cultured parasites were unequivocally identified as *H. meleagridis* (Fig. 4A). Remarkably, a high number of Gram-negative bacteria surrounding *H. meleagridis* was observed (Fig. 4B). Similar observations resulted from

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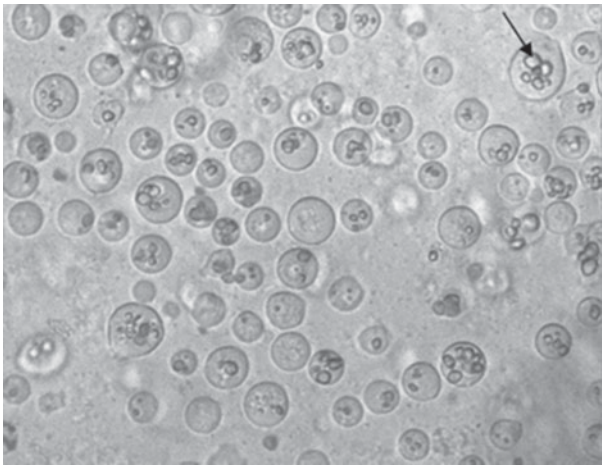

Fig. 2. Light microscopic examination (400×) of the cultured protozoa confirmed the presence of *Histomonas meleagridis* and the absence of any other protozoa in the mono-eukaryotic culture originating from turkey liver lesions. The parasites showed phagocytosis of rice starch granules (black arrow).

examination of the mono-eukaryotic *H. meleagridis* culture originating from liver lesions of chickens after infection with the *H. meleagridis* mixed strain (data not shown).

Confirmation of the sustained pathogenicity of the mono-eukaryotic H. meleagridis *culture in turkeys*

Experimental infection of turkeys with the isolated *H. meleagridis* culture resulted in the cumulative mortality rate and mean lesion scores of liver and caeca of birds presented in Table 1. One bird of the IG group was excluded from the data as it was decapitated by other birds. Since 18 of 19 birds of the group IG deceased due to histomonosis, from 7 dpi to 14 dpi, score 4 was assigned to the caecal and liver lesions. The caecal wall was thickened and showed necrotic to caseous exudates. Strong necrotic lesions with ulceration were noticed on the mucosa of the caeca and on the liver. The one remaining bird was killed at termination of the study and its caecal and liver lesion scores were 1 and 0 respectively. The caecal lesion score was 1 based on the observation of the aspecific yellow and foamy content. The mean caecal and liver lesion scores for the group IG resulted in 3·84 and 3·79, respectively. Meanwhile, for the group UC lesion scores of 0·2 and 0·0 were assigned to the caeca and the liver, respectively. Seventeen non-infected birds had normal caecal content whereas in 3 birds of the UC group the content was yellow and foamy. During the experiment none of the non-infected birds died due to histomonosis.

DISCUSSION

Since the first *in vitro* isolation of *H. meleagridis* by Drbohlav from feces (Drbohlav, 1924), a wide range of culture media and conditions have been studied to optimize growth of *H. meleagridis* (DeVolt, 1943; Dwyer, 1970; van der Heijden *et al*. 2005; Hauck *et al*. 2010*a*). In the current investigation and in accordance with Tyzzer's observations (Tyzzer, 1920), besides *H. meleagridis* other protozoa such as *T. gallinarum* and *Blastocystis* spp. were identified in cultures inoculated with caecal contents of birds from field outbreaks of histomonosis. Moreover, the frequent contamination of field isolates with other protozoa such as *T. gallinarum* and *Blastocystis* spp. hampers the propagation and maintenance of cultured *H. meleagridis*. The deleterious effect of *T. gallinarum* (Delappe, 1957) and *Blastocystis* spp. on *H. meleagridis* has been mentioned without specific details (Delappe, 1952; Tyzzer, 1934). Taking into account these perspectives, an efficient approach to isolate and propagate *H. meleagridis* free of other host's protozoa was described in the present study. Thereby, the contamination of *H. meleagridis* with *T. gallinarum* and *Blastocystis* spp. was analysed by TEM at different culture incubation times. The parasite population shifted from mainly *T. gallinarum* to only *Blastocystis* spp. Concomitantly, the main bacterial populations also shifted from Gram-negative to Gram-positive bacteria. Possibly, the inhibitory power of *T. gallinarum* and *Blastocystis* spp. in contaminated cultures could be attributed to their rapid growth driven by their selective bacterial preference as a food source, in agreement with Delappe's findings (Delappe, 1952). Conversely, in our pure *H. meleagridis* culture pre-inoculated with caecal bacteria, predominantly Gram-negative bacteria were present. Unfortunately, the underlying explanation for the shift from Gram-negative to Gram-positive bacteria when cultures were overgrown by *Blastocystis* spp. needs further investigation.

Furthermore, the successful establishment of mono-eukaryotic *H. meleagridis* cultures from liver was possible by pre-incubation of the culture medium with caecal bacteria one day before, highlighting the indispensable addition of bacteria. Previously, many attempts to culture *H. meleagridis* in an axenical manner failed (Bishop, 1938; Delappe, 1952; Lesser, 1961; Hauck *et al*. 2010*a*). Although underlying reasons remain unsolved, it is generally assumed that the co-cultured caecal bacteria are able to produce an anaerobic environment (Delappe and Pierce, 1953) suitable for *H. meleagridis*, a facultative anaerobic flagellate (McDougald, 2005), and serve as food for the cultured protozoa as observed in vacuoles (Munsch *et al*. 2009). Possibly, the inclusion of host's caecal bacteria promotes the parasite to switch from its amoebic tissue form to the flagellated stage increasing the success rate of establishing mono-eukaryotic *H. meleagridis* cultures from bird liver lesions. As previously described by Lee *et al*. (1969), the flagellated form in the caecal lumen consumes

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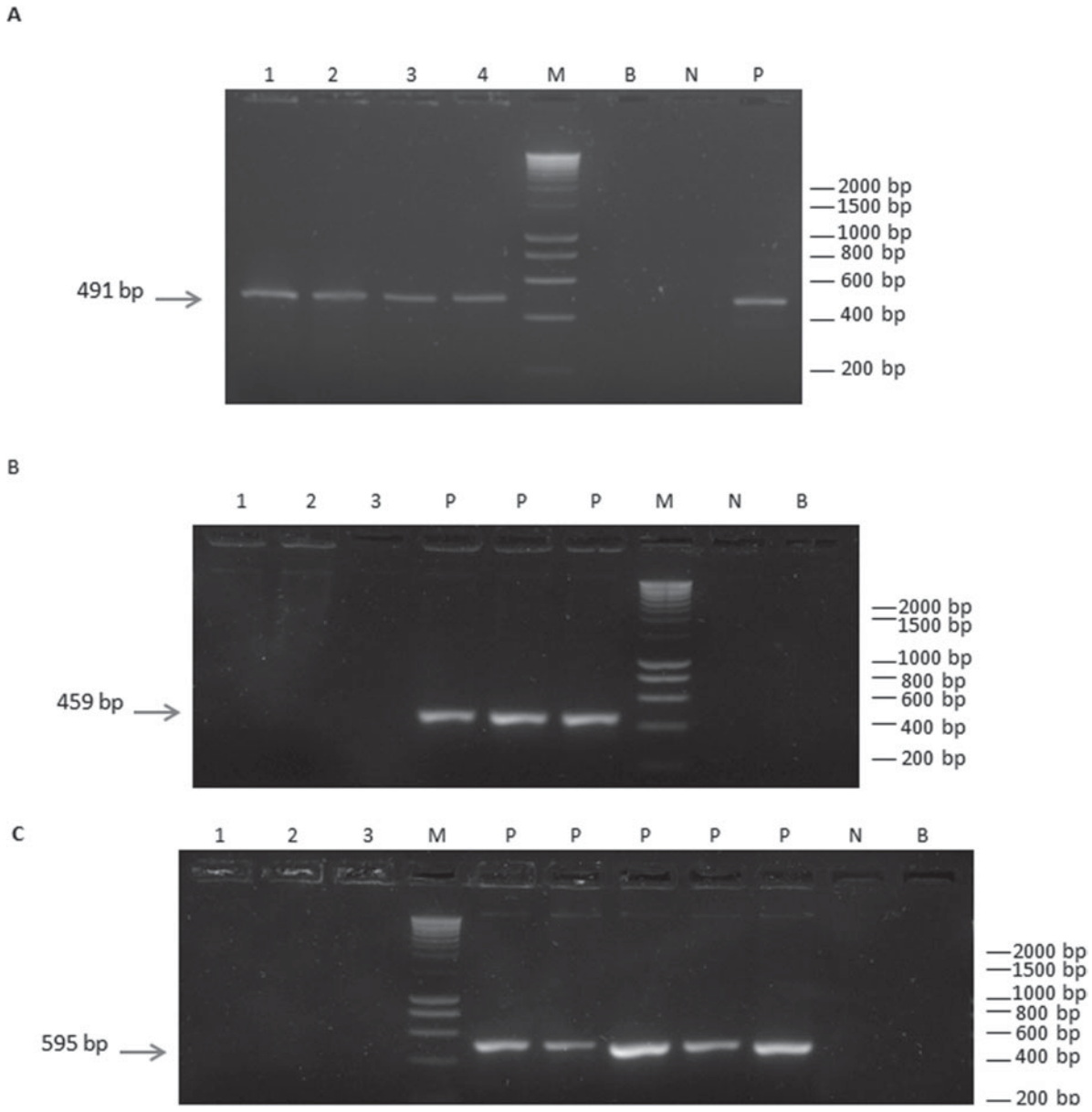

Fig. 3. Diagnostic PCR analysis detected *Histomonas meleagridis* but no other protozoa were detected in the mono-eukaryotic culture originating from turkey liver lesions. To amplify the small subunit rRNA gene of each protozoon species, specific primers for *Histomonas meleagridis* (**A**), *Blastocystis* spp. (**B**) and *Tetratrichomonas gallinarum* (**C**) were used in the mono-eukaryotic culture at passage 6 (lane 1), passage 34 (lane 2), passage 49 (lane 3) and passage 52 (lane 4). M: molecular size marker (Smartladder SF, Eurogentec); B: blanco PCR control without DNA template; N: negative PCR control; P: positive PCR controls, respectively for *Histomonas meleagridis* (**A**), *Blastocystis* spp. (**B**) and *Tetratrichomonas gallinarum* (**C**).

bacteria by phagocytosis while the non-flagellated tissue form ingests dissolved host liver by pinocytosis. In our study, flagellated *H. meleagridis* could be detected microscopically 48 h after inoculation of the cultures. However, flagellated parasites were already observed 24 h after establishment of *H. meleagridis* cultures from liver lesions of hen or turkey (Bayon and Bishop, 1937; Goedbloed and Bool, 1962). Unfortunately, specific identification of cultured organisms, growth behaviour and effective propagation of the cultures in those previous studies were not documented in detail, impeding interpretation of their findings. The only in-depth defined *H. meleagridis* culture was established through micromanipulation by picking up one single cell (Hess *et al*. 2006*b*) rather than from a population of cells as performed in our study. The propagation of the latter cultures allows maintaining the characteristic nature of a field isolate which enables their use for *in vitro* screening of candidate antihistomonal compounds. Long-term cultivation of *H. meleagridis* however, might narrow the genetic variation between cells.

In the present study, the ultra-structural characteristics of the parasites from our pure cultures

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Table 1. Confirmation of the sustained pathogenicity of the cultured *Histomonas meleagridis* originated from turkey liver lesions in turkeys intracloacally infected with the mono-eukaryotic *H. meleagridis* culture passage 8

Group[a]	Total[b]	Infection dose[c] (*H. meleagridis*/bird)	Mortality (%)	Mean lesion scores[d]	
				Caeca	Liver
UC	20	0	0	0·2	0·0
IG	19	$1{\cdot}7 \times 10^5$	94·74	3·84	3·79

[a] UC: uninfected group; IG: infected group.
[b] Total number of birds in each group.
[c] Except for UC group, all birds of IG were intracloacally infected at 17 days of age.
[d] The mean lesion scores of the caeca and the liver of each group, assigned at day of autopsy (14 dpi).
Birds that died from histomonosis during the study were given a lesion score of 4 per organ.

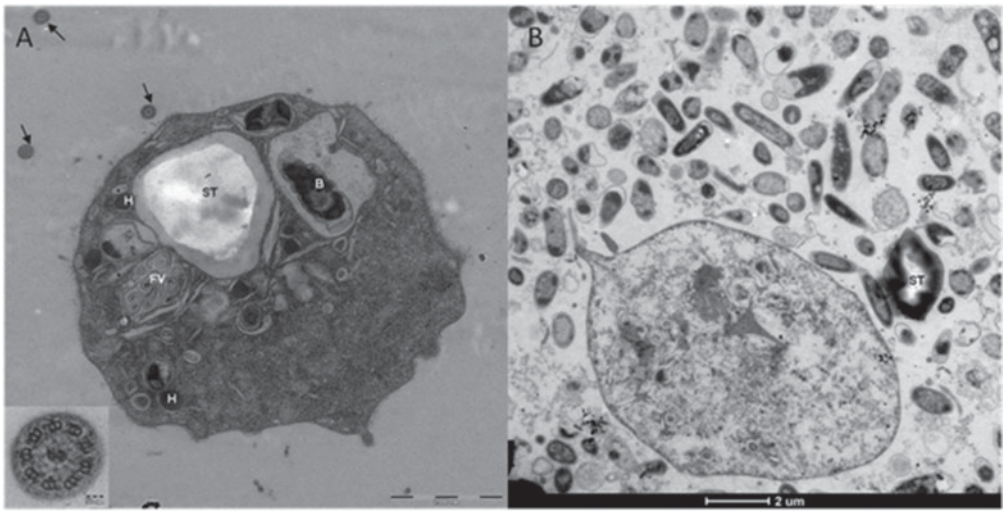

Fig. 4. TEM examination identified unequivocally the cultured protozoon to be the flagellated *Histomonas meleagridis*. Large part of the cytoplasma consists of organelles surrounded by membranes as hydrogenosomes (H), food vesicles containing bacterial debris (FV) or phagocytosed bacteria (B) or rice starch granules (ST). Black arrows show transversal sections of the flagellum with the typical microtubule organization presented in the inserted picture (**A**). Very high number of Gram-negative bacteria and rice starch granules (ST) were observed in the mono-eukaryotic *H. meleagridis* culture (passage 3) (**B**).

matched the descriptions and observations of *H. meleagridis* in the literature (Schuster, 1968; Rybicka *et al*. 1972; Mielewczik *et al*. 2008). In our study, only *H. meleagridis* could be isolated from the liver of birds which had been inoculated with a contaminated stabilate, whereas in other studies liver of *H. meleagridis*-infected birds appeared to be contaminated with other organisms including bacteria and protozoa (Harrison *et al*. 1954; Delappe, 1957; Goedbloed and Bool, 1962; Grabensteiner and Hess, 2006; Hauck *et al*. 2010*b*).

Investigations using PCR amplification of the SSrRNA-gene (Grabensteiner and Hess, 2006; Hauck *et al*. 2010*b*) ascertained the microscopic identification of *Blastocystis* spp. (Delappe, 1952) and *T. gallinarum* (Delappe, 1957) in the necrotic liver lesions of birds, besides *H. meleagridis*. Although they are regarded as apathogenic possibly due to subtype differences, contradictions remain about the pathogenic potential of *Blastocystis* spp. (Stensvold *et al*. 2009) and *T. gallinarum* (Amin *et al*. 2010). In addition, the amount of detected bacteria in liver samples of turkeys killed during the course of the disease was much lower than in birds which died from histomonosis (Harrison *et al*. 1954). In this regard, *H. meleagridis* causes extensive damage of the caecal tissue allowing dissemination of other host's organisms as postulated by Delappe (1957). So, it is worth mentioning to precisely determine the moment of isolating liver lesions for obtaining the mono-eukaryotic *H. meleagridis* culture as *H. meleagridis* might play the first invader role causing liver lesions in birds subjected to a mixed infection. Clearly, development of *H. meleagridis* cultures free of growth-impeding protozoa such as *T. gallinarum* and *Blastocystis* spp. is an important tool to characterize this protozoon parasite.

Next to the successfully acquired mono-eukaryotic *H. meleagridis* culture, an infection study in turkeys

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was performed to confirm the sustained pathogenicity of this *H. meleagridis* culture. In accordance with reports about mortality and development of histomonosis regardless of the several variable factors (Hess *et al*. 2006*a*), the mortality due to histomonosis reached 95% by 14 dpi. At autopsy severe inflammation with necrosis in the caeca and liver displaying the maximum lesion score 4 were seen, while in the IC control group no clinical signs or death due to histomonosis were observed. So, the mono-eukaryotic *H. meleagridis* cultures were still considered as very pathogenic.

In conclusion, it was possible to establish mono-eukaryotic *H. meleagridis* cultures in an efficient and successful way from liver lesions of birds infected with a contaminated *H. meleagridis* strain. Hereby, the co-cultivation of caecal bacterial flora is essential for the *in vitro* multiplication of *H. meleagridis*. As the pathogenicity of the parasite was not altered by propagation *in vitro*, this method of producing pure *H. meleagridis* cultures provides an important tool to gain more in-depth information about the protozoon *H. meleagridis* itself. Moreover, the mono-eukaryotic culture could reveal questions about the interactions between *H. meleagridis* and its bacterial environment in the culture or in the host.

ACKNOWLEDGEMENTS

We would like to thank Marcel Samain, Nele Steen and Sharon Beernaert for their technical assistance. Special thanks to Ward Heggermont MD for his careful reading of the manuscript.

FINANCIAL SUPPORT

This work was financially supported by the 'Agency for Innovation by Science and Technology' of Belgium (IWT grant number SB-71506).

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